Riboflavin-Induced Photochemistry

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Riboflavin (RF), which is also known as vitamin B2, is a water-soluble vitamin. RF is a nontoxic and biocompatible natural substance. It absorbs light (at wavelengths of 380 and 450 nm) in the presence of oxygen to form reactive singlet oxygen ($^{1}O_{2}$). The generated singlet oxygen acts as a photoinitiator to induce the oxidation of biomolecules, such as amino acids, proteins, and nucleotides, or to initiate chemical reactions, such as the thiol-ene reaction and crosslinking of tyramine and furfuryl groups.

Keywords: riboflavin ; riboflavin phosphate ; visible light ; photochemistry ; thiol-ene reaction ; furfuryl group

1. Introduction

In the biomedical field, biopolymers are applied in wound healing, tissue engineering, drug delivery, and cosmetics [1][2]. Natural biopolymers have suitable biological properties for application in the biomedical field; however, they have limitations in their original state. The poor mechanical properties of biopolymers, such as low strength and rapid decomposition, must be improved. The crosslinking of biopolymers is a method for improving their mechanical properties. Crosslinking increases the molecular weight of biopolymers or forms an intermolecular network ^[3].

Biopolymers can be crosslinked via enzymatic reactions, light irradiation, and temperature and pH variations ^[4]. Although various stimuli can induce crosslinking, the light-mediated photochemistry-based crosslinking of biopolymers have recently gained significant research attention ^[5]. Crosslinking using light is more beneficial for spatiotemporal control than that using other stimuli ^[6], as it is challenging to swiftly control localized temperature changes, pH, and enzyme concentration ^[5]. However, light has a unique property: it can be triggered at a precise time and location. In addition, temperature, pH, and enzymes are internal parameters that maintain homeostasis in the body; however, light is an external trigger that is not controlled in the body. Other external triggers include ultrasound, magnetic fields, electric fields, and exogenous chemicals; however, light can induce rapid changes in local areas ^[5].

The light-induced chemical reactions require a photoinitiator. The following discussion covers photochemical reactions that use riboflavin (RF) or riboflavin phosphate (RFP) as photoinitiators. The natural substances RF and RFP, which act as photoinitiators by absorbing light in the UV and blue light spectrums, are promising because of their excellent biocompatibility.

2. Riboflavin-Induced Photochemistry

2.1. Oxidation

The RF-mediated oxidation process transforms the substrate into an oxidation product that uses oxygen as a mediator $^{[Z]}$. An oxidation product is formed when the energy of RF, excited to the triplet state, is transferred to oxygen. Bonds are then formed between the biopolymers by oxidation, and crosslinking is induced. Depending on the object to which the RF energy is transferred, two types of pathways exist for oxidation product formation. In Type 1, the RF transfers the excited energy to the substrate to form RF-free radicals. Subsequently, the free radicals interact with ground-state oxygen molecules to generate oxidation products. In Type 2, the energy of the excited RF is transferred to the oxygen molecules to form singlet molecular oxygen, which then reacts with the substrate to produce an oxidation product. Type 1 is the preferred pathway when the oxygen concentration is low, whereas the singlet molecular oxygen generated in the Type 2 pathway is more reactive than the RF radicals [8].

RF-induced oxidation reactions are involved in various biological redox processes. Reactive functional groups on biomolecules react with one another to form crosslinks when reactive oxygen species (ROS), OH, $O_2^{2^-}$, and O_2^- , are produced from Type 2 reactions ^[9]. Protein crosslinking can be achieved by inducing the bonding of amino acids that constitute proteins. Histidine converts the imidazole moiety to electrophilic imidazolone by inducing ROS and reacts with

nucleophilic amino acids, such as lysine, to form covalent crosslinks ^{[10][11]}. In addition to histidine–lysine, crosslink formation between the amino acids to form tyrosine–tyrosine, tyrosine–tryptophan, and tyrosine–lysine is possible under oxidative conditions ^[12].

RF-induced oxidation is used to crosslink proteins rich in amino acids, such as histidine, lysine, tyrosine, and tryptophan. Certain amino acids, including cysteine, methionine, tyrosine, tryptophan, and arginine, undergo oxidation in the presence of RF. In addition, several biomolecules, such as ascorbate and nicotinamide adenine dinucleotide hydrogen, in tissue can serve as electron donors to the PS triplet excited states without oxygen mediation ^[13]. Collagen crosslinking is a widely employed crosslinking method for RF and RFP. It is specifically used in medicine as a healing technique for keratoconus corneas. The collagen crosslinking of the corneal stromal layer is induced by light irradiation after RFP is applied to the layer ^{[10][14]}. In clinical treatments, UV light with a wavelength of 365 nm, which has a lower transmittance than visible light in the blue wavelength region, is employed to induce crosslinking at depths <200–300 μ m. UV irradiation with a weak intensity of 3 mW cm⁻² is applied for a period of approximately 30 min. Kang et al. demonstrated that RFP-induced crosslinking increased the Young's modulus and tensile strength of skin tissues by approximately three times by applying the same procedure to skin collagen as that for corneal collagen ^[15]. Blue light is safer than UV light when applied to the skin; therefore, visible light with a wavelength of 450 nm was employed. In addition, the irradiation time was decreased to 5 min using a high intensity of 100 mW cm⁻².

Kim et al. performed the RFP-induced crosslinking of keratin proteins ^[16]. The thiol group of the cysteine residue, which is abundant in keratin, participated in crosslinking using photoexcited RFP, and the crosslinking efficiency was increased using citric acid as a crosslinker. The resulting product could be used as an environmentally friendly personal care product, demonstrating significant effectiveness in the setting and strengthening of the hair by inducing crosslinking.

Because RF-mediated protein crosslinking, which is achieved by inducing oxidation, is typically performed by binding amino acid residues, it has the advantages of crosslinking, which can be performed without protein modification. However, the crosslinking density differs owing to the different amino acid compositions of various proteins. Additionally, its application to biopolymers is challenging, except for proteins such as polysaccharides. Another drawback is that the degree of crosslinking cannot be precisely controlled. However, if the appropriate conditions are determined and applied to obtain the required degree of crosslinking, it can be employed for crosslinking proteins in various tissues constituting the human body.

2.2. Thiol-Ene Reaction

The thiol-ene reaction, which is frequently referred to as photo-click chemistry, is a versatile reaction owing to the high selectivity between thiol and vinyl groups. The thiol group loses a proton after the initiator is activated, forming a thiyl radical [1Z]. This radical then reacts with the vinyl bond, resulting in the formation of a thioester bond.

The type of alkene used in the thiol-ene reaction affects the reaction rate and properties, such as alkene conversion. Norbornenes are common alkenes used in thiol-ene reactions. Norbornene forms a thioester bond with a thiol group through a low-energy reaction, resulting in a rapid reaction with a high conversion rate [17]. However, the light stability is low, and the selectivity for progressing the reaction by irradiating light at a particular time decreases in terms of the light-responsive reaction.

Thiol-ene reactions are frequently employed after modifying the target biomaterials with thiol or alkene groups because they can promote crosslinking when paired with thiols and alkenes. Therefore, synthetic polymers, which are more straightforward to modify than natural biopolymers, are frequently used. For example, the synthetic polymer, poly(ethylene glycol) (PEG), has been crosslinked via the thiol-ene reaction. Stenzel's group created a crosslinked hydrogel by reacting 4-arm PEG-norbornene with dithiothreitol (DTT) ^{[18][19]}. When RFP is exposed to light, the excited RFP removes hydrogen from the thiol group of DTT to form a thiyl radical. This radical then reacts with the alkene of norbornene to form a thioether linkage.

Methacrylate is another type of alkene used in thiol-ene reactions. It is more dependent on the light energy because it requires more energy for thiol-ene reactions than norbornene. Therefore, the reaction can proceed selectively when blue light is irradiated using RFP as the photoinitiator. Previous studies have demonstrated that the reaction progress varies depending on the RFP concentration ^[20]. RFP concentrations between 0.001% and 0.1% were investigated, and the highest acceleration of gelation was achieved using 0.01% RFP. An RFP concentration of 0.001% was insufficient to initiate treatment. At 0.1%, the dark yellow color of RFP prevented the penetration of blue light, leading to insufficient gelation. Therefore, the key to regulating the gelation rate is to use an appropriate amount of RFP.

Methacrylated biopolymers often have been crosslinked by photoinitiation of RF without a thiol group. Due to the lack of photoinitiation energy of RFP, methacrylated biopolymers typically require RFP as well as an extra co-initiator for vinyl crosslinking. Goto et al. crosslinked methacrylated gelatin (GelMA) using RF with triethanolamine as a co-initiator ^[21]. In terms of GelMA hydrogel properties, the combination of RF and visible light-induced crosslinking was comparable to that of Irgacure2959 and UV.

Hyaluronic acid (HA) is a crosslinked biopolymer created by the reaction of thiol groups with methacrylate $\frac{[20][22]}{2}$. HA is a biocompatible polymer that promotes cell migration and proliferation, and it reduces water evaporation owing to its attraction to water. HA interacts with CD44, which is involved in autoimmune diseases and cancer $\frac{[23]}{2}$. In addition, HA is occasionally used in combination with medications because of its therapeutic effects $\frac{[22]}{2}$. After introducing methacrylate and thiol groups to HA, the two HA components were mixed with RFP and exposed to blue light to produce an HA hydrogel via the thiol-ene reaction $\frac{[20]}{2}$. Because the thiol-ene reaction between the methacrylate and thiol groups is slower than that between the norbornene and thiol groups, a higher intensity or longer irradiation time is required. Abdul-Monem et al. utilized RFP to crosslink methacrylated HA without the thiolated HA part, and dimethylaminoethyl methacrylate was added as a co-initiator $\frac{[24]}{2}$.

The thiol-ene reaction is a light-responsive crosslinking reaction that is more selective than the oxidation reaction; therefore, it is an appropriate method for inducing crosslinking between specific biomolecules. The oxidation reaction requires an irradiation time of several minutes to several tens of minutes, whereas the thiol-ene reaction can induce sufficient crosslinking within several tens of seconds to several minutes. Although the reactivity varies depending on the type of alkene, the reaction rate between the thiol and alkene groups can be significantly increased by the photoinitiation of RFP. In addition, it does not produce any reaction by-products and is not sensitive to O_2 , rendering it suitable for application in living organisms.

2.3. Tyramine and Tyrosine Groups

Tyramine or tyrosine groups create crosslinks by forming dityramine or dityrosine via excited RF. Tyrosyl radicals are created by the tyrosine group when RF is excited, and these radicals then form carbon–carbon or carbon–oxygen bonds to create dityrosine bonds ^[13]. Both tyramine and tyrosine possess a phenyl group, which acts as an electron donor and bond-forming site; therefore, the same mechanism can be applied. Tyrosine, which is an amino acid building block, is assumed to contribute to the oxidation mechanism that causes protein crosslinking. Tyramine is simple to conjugate through various chemical processes because it contains a primary amine group at its terminal. Therefore, tyramine can be used to form a pendant phenyl group in a biopolymer, and RF photosensitization initiates the crosslinking.

Kim et al. synthesized tyramine-modified alginate by conjugating tyramine to alginate using 1-ethyl-3-(3dimethylaminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysulfosuccinimide (NHS) chemistry ^[25]. Dityramine was produced after 3 min of exposure to visible light at 440 nm and 2500 mW cm⁻². It has been demonstrated that dual crosslinking can be employed to regulate the mechanical properties of alginate hydrogel because alginate possesses ionic crosslinking capabilities. Hong et al. synthesized tyramine-modified HA using EDC/NHS chemistry to prepare HA hydrogels ^{[26][27]}. RFP and sodium persulfate (SPS) were employed as the photoinitiator and co-initiator, respectively, to induce photocrosslinking, which was achieved by exposing the material to visible light at 2500 mW cm⁻² and 440 nm for 30 s. The resulting mechanical strength of the hydrogel was comparable to that of the commercial filler, Restylane, and an in vitro cytocompatibility assay demonstrated that the dityrosine crosslinking caused by RFP was biocompatible.

Silk fibroin is a biocompatible and biodegradable biopolymer and is used as an optical element owing to its high transparency ^[28]. Applegate et al. demonstrated that photoexcited RF formed a dityrosine bond between the tyrosine residues of silk fibroin, and a high-resolution pattern of approximately 50 μ m was fabricated via photolithography ^[29]. Piluso et al. manufactured a silk fibroin hydrogel that could be used in tissue engineering and cell delivery applications through dityrosine crosslinking ^[30]. Tyrosine residues, which constitute approximately 9% of the hydrophobic blocks in silk fibroin, were used without any additional chemical modifications. RF and SPS were used as the photoinitiator and co-initiator, respectively, to induce the reaction. Visible light in the 400–700 nm range was used as the light source for 10 min. The silk fibroin hydrogel exhibited a storage modulus > 10^3 Pa and led to a cell viability of >80% even after one week of cell encapsulation in the hydrogel. Silk fibroin has the advantage of forming dityrosine crosslinks without additional chemical modifications.

The formation of tyrosyl radicals from the tyrosine group using excited RF is a type of amino-acid oxidation. RF-mediated oxidative crosslinking results from a random combination of oxidation products of amino acid residues contained in proteins. Although all amino acid residues cannot create links ^[31], a higher crosslinking efficiency can be achieved because tyrosine creates a dityrosine between tyrosine residues to form a specific link.

2.4. Furfuryl Group

The furfuryl group is a chemical structure comprising a furan ring and has a visible-light-reactive functional group. Furfuryl groups are crosslinked by forming furan endoperoxides through a photo-oxidation crosslinking mechanism in the presence of singlet oxygen ^{[32][33]}. Because the furfuryl group is not a functional group found in natural biopolymers, the chemical modification is essential. Therefore, selective reactivity to light can be imparted by introducing a furfuryl group into the biopolymer.

Gelatin, after functionalization with a furfuryl group, is widely employed as a biopolymer. As a partially hydrolyzed collagen, gelatin is a preferred biopolymer in the biomedical industry because of its biocompatibility and biodegradability. Kong et al. created an injectable gelatin hydrogel by adding a furfuryl group to gelatin, which can be employed as a controlled drug delivery system ^[33]. Selective bonding was used to create the drug delivery system by combining the furfuryl and maleimide groups, representing a type of Diels–Alder click chemistry. The epidermal growth factor (EGF) was conjugated to a maleimide group and then linked to furfuryl-modified gelatin through a Diels–Alder reaction. Hydrogels were then created using the remaining furfuryl groups by crosslinking the gelatin. Chemically immobilized EGF in the gelatin matrix was sustainably released from the hydrogel.

Joddar et al. demonstrated the applicability of furfuryl-modified gelatin as a tissue engineering scaffold ^{[34][35]}. The cell viability, network formation, and proliferation were excellent after the cells were enclosed in a 3D gelatin scaffold with 400 nm light irradiation ^[34]. In addition, a nanofiber scaffold was formed using furfuryl-modified gelatin by electrospinning and exhibited mechanical properties similar to those of the extracellular matrix found in cardiac tissues ^[35]. The utility of photocrosslinked gelatin electrospun fibers for tissue engineering was demonstrated by the adhesion and growth of cardiomyocytes and induced pluripotent stem cells.

Heo et al. introduced a furfuryl group into alginate to produce visible-light-curable alginate and employed it as a controlled release system ^{[36][37]}. RF-mediated photocrosslinked alginate hydrogel exhibited mechanical and release properties similar to those of alginate hydrogels physically crosslinked by calcium ions, demonstrating that it is appropriate for application in drug delivery systems ^[36]. In addition, alginate has been employed as a visible-light-curable anti-adhesion agent because it exhibits non-adhesive properties in tissues ^[37].

However, a limitation of the aforementioned approach is that the furfuryl group can only be used when it is introduced into a biopolymer for the RF-mediated photocrosslinking reaction. However, because it has been introduced into various biopolymers, as mentioned previously, the furfuryl group is a promising candidate for application in the biomedical industry. The storage stability of the thiol group participating in the thiol-ene reaction, which also requires functional group modification, is inferior to that of the furfuryl group. However, because the thiol-ene reaction is better in terms of photoreactivity, the RF-mediated mechanism can be selected based on the required purpose.

The RF-induced photochemistry with applied biomaterials and light irradiation conditions are summarized in Table 1.

Photochemistry	Biopolymer	Photoinitiator	Light Source Wavelength (nm)	Light Intensity (mW cm ⁻²)	Irradiation Time	Refs.
Oxidation	Collagen (Cornea)	RF	405	3	30 min	[<u>10]</u> [<u>14]</u>
	Collagen (Skin)	RFP	450	100	5 min	[<u>15</u>]
	Keratin (Hair)	RFP	450	3	5 min	[<u>16</u>]
Thiol-ene reaction	НА	RFP	450	100	40 s	[<u>20]</u> [<u>22]</u>
Tyramine group	Alginate	RF	440	2500	3 min	[25]
	НА	RFP	440	2500	30 s	[<u>26]</u> [27]
	НА	RF	365	10	20 min	[38]
	Silk fibroin	RF	450	18.7	20 min	[29]
	Silk fibroin	RF	400-700	-	10 min	[30]

Table 1. Types of RF-induced photochemistry and crosslinked biomaterials.

Photochemistry	Biopolymer	Photoinitiator	Light Source Wavelength (nm)	Light Intensity (mW cm ⁻²)	Irradiation Time	Refs.
Furfuryl group	Gelatin	RFP	458	100	10 min	[<u>33</u>]
	Gelatin	RF	400	-	2.5 min	[<u>34]</u> [<u>35]</u>
	Alginate	RF	445	200	10 min	[<u>36]</u> [<u>37]</u>
	НА	RF	-	-	10 min	<u>[39]</u>

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