REVIEW PAPER

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Fluorescent spectroscopy of collagen as a diagnostic tool in medicine

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ABSTRACT

Medicine continuously needs to improve the existing diagnostic solutions or introduce new ones. Despite the fact that collagen is a well described protein, collagen-related diseases represent the disorders requiring improvement particularly in terms of research tools. These diseases include connective tissue diseases, keratoconus, where a change in the structure of collagen is observed causing a deformation of the cornea, as well as neoplasms, in which the amount of collagen is multiplied in cells.

Fluorescence spectroscopy constitutes a highly sensitive, non-invasive research method, thus, its use in medicine can contribute to the development of excellent diagnostic methods. This method allows to determine the changing amount of the tested fluorophores, as well as the change of the pH of the environment in which these fluorophores are located. Until now, numerous studies on collagen have been performed using fluorescence spectroscopy. However, a detailed analysis of the literature revealed some discrepancies which have been summarized in this paper.

The collected experimental results allowed to conclude that the discrepancies in the obtained fluorescence excitation and emission spectra of collagen may result from the structural richness of collagen. Another reason for the variability of the results is the different experimental conditions, i.e. the excitation and detection of collagen fluorescence at different wavelengths. Therefore, it should be emphasized that collagen spectroscopy constitutes an extremely promising method, although the determination of the exact conditions of the experiment and their standardization are required in the research on the diagnostic use of this technique.

Introduction

Collagen is the most abundant protein in the human body. Thus, a detailed description of all its properties, including the spectral characteristics, is necessary. This need results undeniably from the fact that many connective tissue diseases are associated with the changes in the functioning and structure of collagen. Other diseases can also be caused at least partly by the functional changes in collagen. An example of this type of disease is an eye condition referred to as keratoconus, where a reduction in the amount of collagen causes the cornea to reshape. Therefore,

a detailed understanding of the spectroscopic properties of collagen may contribute to the development of new diagnostic methods.

Currently, there are no studies with regard to connective tissue diseases using fluorescence spectroscopy. Nevertheless, connective tissue diseases are often associated with collagen impairment. Moreover, changes in tissue collagen fluorescence during the neoplastic process have been demonstrated in many studies. In fact, the experimental results of some experiments indicate that low collagen and high NAD(P)H intensity of fluorescence characterizes high-grade dysplastic lesions, compared to non-dysplastic tissues [1]. These experiments indicate that once the method is standardized, fluorescence spectroscopy can become an indispensable tool in the diagnosis and/or treatment of certain neoplasms as measurements are performed quickly and can be performed *in vivo* in some cases. Therefore, the idea of using fluorescence spectroscopy in the diagnosis of connective tissue diseases seems to be justified and promising.

Over the last few years, a number of spectroscopic studies of collagen have been carried out. However, the issue of collagen spectroscopy has not been sufficiently described in the literature, and reports are inconsistent. Therefore, the aim of this study is to compile and verify the current knowledge related to the stationary fluorescence spectroscopy of collagen.

Literature Search

All available literature databases were searched. The literature was reviewed at PubMed, Medline (Webhost EBSCO) and Web of Science from February 2021 to June 2021. The initial database searches were made against the following keywords: collagen and fluorescence. Other keywords were added subsequently, allowing to specify the research method being sought: spectroscopy, excitation and emission spectra. The analysis was limited to the English language. Papers which do not provide information on collagen excitation and emission spectra were excluded.

As shown in **Figure 1**, the review uses information from 36 papers, 9 of which contain the complete spectra of excitation and / or emission of collagen fluorescence, whereas 20 papers provide additional information on the fluorescent properties of collagen. The remaining 8 articles contain information on the structure of collagen and fluorescence spectroscopy.

Steady State Fluorescence Spectroscopy

The use of fluorescence in life sciences has gained importance over the past thirty years. Fluorescence spectroscopy is regarded as a significant research tool in biotechnology and biophysics [2], with fluorescence detection as an extremely sensitive and specific method. It uses molecules called fluorophores/fluorochromes. Excitation of fluorophores by a light beam of a lower wavelength elevates the electrons to an excited state, which then emit energy as light of a longer wavelength during their return to the ground state [3]. Autofluorescence uses the neutrally occurring chromophores in the tested cells/tissues. Fluorescence spectroscopy allows for the observation of a minimal change: the amount of fluorophore, the environment of the fluorophore and even the conformation of proteins. Fluorescence measurement devices include fluorimeters and spectrofluorimeters. Depending on the method of analysis, measurements can be divided into stationary and time-resolved measurements [2, 3].

The method of fluorescence analysis can be a very useful alternative in the diagnosis of diseases accompanied by changes in the structure, environment or the number of endogenous fluorophores. Most frequently, individual fluorophores differ from each other in the wavelength at which they give the maximum excitation and/or maximum emission of fluorescence [4]. The excitation maximum of the fluorescence is the wavelength at which the absorption of light is the highest, whereas the emission maximum of the fluorescence is the wavelength at which the highest intensity fluorescence is observed. The difference between the position of the maximum of the absorption band in relation to the emission band is known as the Stokes shift [2, 4]. The abovementioned differences often allow for the identification of specific fluorophores. Nonetheless, in the case of stationary measurements, the spectra of

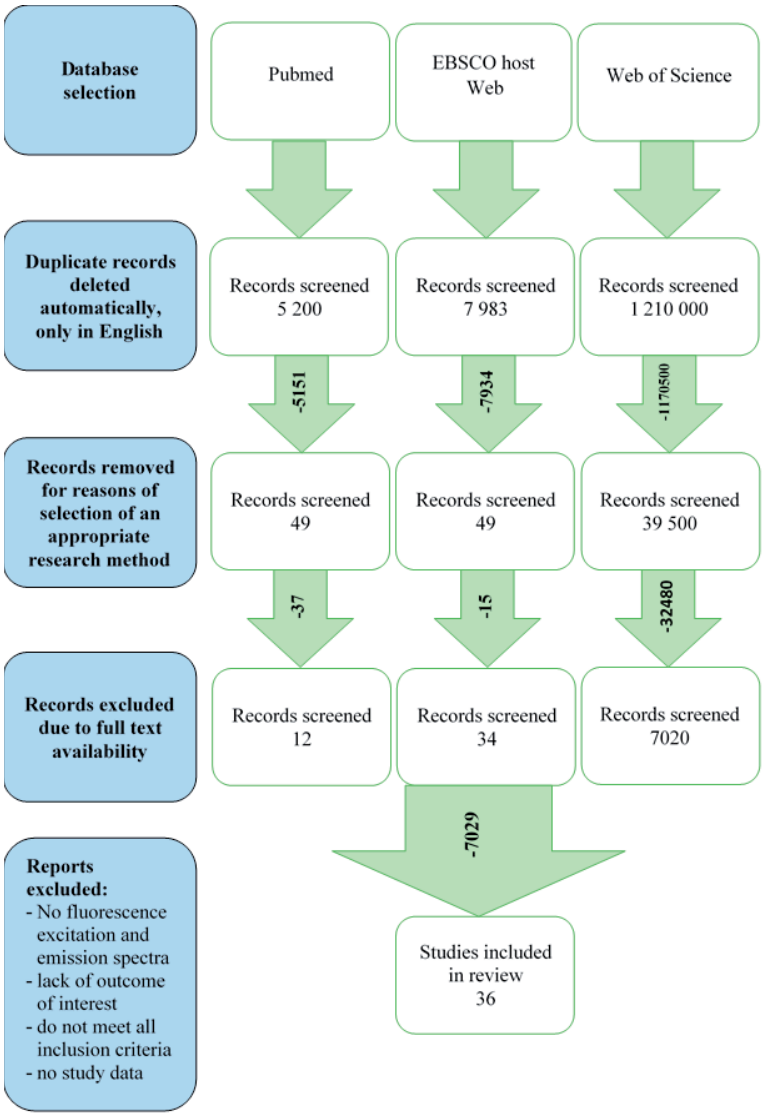

Figure 1. Literature search strategy. As indicated by the search algorithm, information from 36 papers was used

some chromophores may coincide. Therefore, in some cases, much more sensitive measurements of time-resolved spectroscopy may provide a useful extension to the research [3]. However, samples which possess the same autofluorophores in their structure can be distinguished by the amount of chromophores or by the influence of the environment in which these chromophores are located, e.g. a change in the hydrophobicity of the environment may change the spectral properties of the tested molecules. These factors may occur in the course of formation of pathological tissue lesions, as in the case of a neoplasm [4, 5]. Well-known endogenous fluorophores capable of emitting autofluorescence include aromatic amino acids (phenylalanine, tyrosine and tryptophan), molecules of cellular energy metabolism: nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), vitamins, lipids, elastin and collagen, the main component of the extracellular matrix and the most commonly found protein in the human body [2, 5].

An indisputable advantage of the optical methods is their minimal invasiveness, as measurements can be performed *in vivo*. Therefore, there is no cell procurement, or possibly only a small piece of the tissue is removed during surgery. Additionally, blood or urine samples can be also used as research material, whereas real-time research allows to obtain the results immediately. Thus, by using spectrophotometry, the experiment can be performed in a relatively short time.

Collagen is used as a marker of pathological changes in *in vivo* experiments [1, 4, 6, 7]. Due to their availability, the most common experiments involve epithelial tissues, such as the skin [1, 6, 7]. However, with ever greater miniaturization of equipment and more sophisticated research tools, it is possible to examine previously inaccessible locations, such as the colon [4]. The examination involves (1) the insertion of the fiber optic probe as an endoscope, (2) the excitation of the tissue fragment with the light of a certain wavelength / wavelengths, (3) collecting the fluorescence from the tissue into the detector and (4) its further analysis [4]. Hence, as the research on epithelial cells shows, fluorescence spectroscopy is a very promising method of examining pathological changes such as cancer [1, 6, 7]. However, to date it has been limited to locations where fiber optic courts can be introduced. Nevertheless, the increasing miniaturization allows the device for reaching the previously inaccessible sites in the human body.

Collagen structure

Although the understanding of the pathogenesis of many collagen-related diseases remains insufficient, the structure of collagen has been well described. So far, 28 types of collagen have been identified. Some amino acid residues found in the structure of collagen are capable of autofluorescence, and this type of amino acids possesses an aromatic ring in their structure, namely tryptophan, tyrosine and phenylalanine. Furthermore, proline-hydroxyproline-glycine is the most common amino acid sequence observed in the collagen chain. This triplet constitutes about 10.5% of the collagen sequence. Hydrogen bonds formed between proline and glycine residues of adjacent chains link them with each other. The hydrogen bonds help hold three helices together, thus creating a triplet superhelix. Procolagen fibers formed in this way undergo further post-translational processing, the product of which is tropocollagen. Divalent cross-links are formed between tropocollagen fibers with the use of the enzyme lysyl oxidase, and the cross-links may be further modified [8].

In addition to aromatic amino acid residues, the cross-links are also capable of autofluorescence in the collagen structure (**Figure 2**). Since aromatic amino acids can be found in various proteins, the cross-links present in this protein comprise the structural elements determining the importance of spectroscopic studies on collagen [8]. As the human body ages, immature divalent cross-links are converted into mature trivalent cross-links [9]. Moreover, the divalent cross-link may undergo modifications, e.g. glycosylation. Depending on the period of development and external factors to which the examined tissue has been exposed, various modified cross-links are formed. They may be detected due to the differences in the fluorescence spectra of the sample [9–11].

Collagen in its natural form occurs as a solid molecule, insoluble in water and very resistant

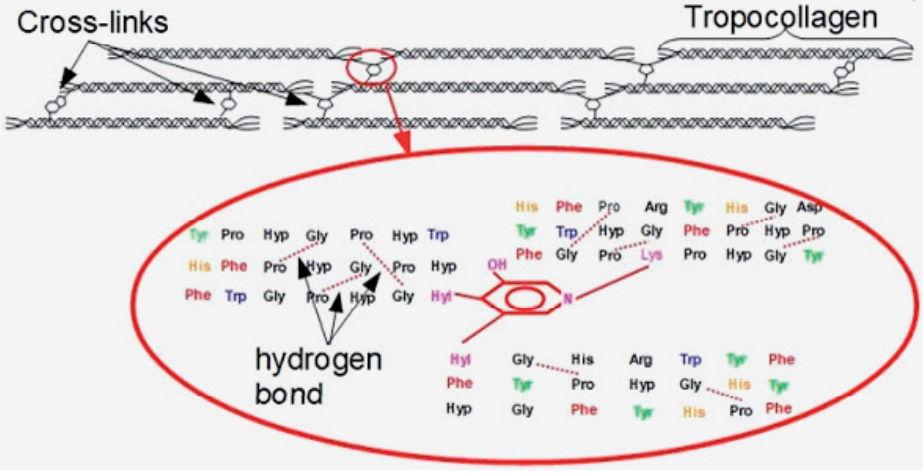

Figure 2. Structure of collagen in which hydrogen bonds (dotted lines) and cross-links between tropocollagen fibrils (center of the Figure) can be distinguished

to degradation [8, 12]. Due to its resistance during isolation, collagen can be divided into acetic acid-soluble and insoluble collagen, the latter of which can be isolated with the use of enzymes, such as pepsin or collagenases [6, 13].

Fluorescence Spectroscopy of Collagen

In the literature, varied information on spectroscopy of collagen can be found. According to the majority of literature data, the absorption maximum of collagen ranges between 340–370 nm [11, 13–15]. For the collagen emission maximum, more diverse data are observed, which indicate that it is 380 nm [14], around 400–410 nm [1], or around 440–450 nm [1, 6, 7, 11, 13]. In various studies, only one peak in the fluorescence emission spectra of collagen has been indicated [1, 6, 7, 11, 13]. However, several other studies have found collagen spectra more complex with several fluorescence peaks [7, 16].

Robert Gillies et al. [6] divided the emission spectra of collagen according to the enzyme used for isolation. Pepsin-soluble collagen was determined to have the excitation maximum at 335 nm and the emission maximum at about 390 nm. On the other hand, collagenase-soluble collagen had the excitation maximum of 370 nm and the emission maximum of 440–460 nm. Smirnova et al. [7] determined the fluorescence spectrum of type IV collagen suspended in water with the emission maximum at 440 nm, and several local peaks at 380, 420, 465 and 495 nm following excitation with the light of approximately 370 nm. Julian Ionita et al. [16] obtained emission spectra of pure hydrolyzed collagen powder with maxima at wavelengths of 430 nm and 460 nm with the local maximum at about 390 nm. Calfskin acid-soluble collagen isolated by Deyl et al. [15] showed the fluorescence maximum at 440 nm and 495 nm and an additional peak at 410 nm, as well as excitation spectra with a maximum at 345, 370 and 410 nm. Researchers from the Massachusetts Institute of Technology determined four excitation/emission wavelength pairs of powder Bovin collagen: (280 / 310 nm), (265 / 385 nm), (330 / 390 nm), and (450 / 530 nm), using a contour map representation of the fluorescence (EEMs) [17]. The summary of these data is presented in **Table 1**.

In order to obtain a consistent representation of collagen fluorescence spectrum data, several publications with excitation / emission spectra of collagen fluorescence were selected [for a complete list of publications see **Figures 3–4**]. Subsequently, on the basis of the figures found in these publications, the excitation as well as emission bands were digitized and compiled together in the collective figures of collagen excitation spectra (**Figure 3**) and collagen emission spectra (**Figure 4**).

Based on the literature data, the absorption/fluorescence spectrum of collagen can be divided into two fractions. The former fraction includes the amino acid residues present in collagen, while the latter belongs to the cross-links. The amino acid fraction can be excited in the range between 250–290 nm, and fluorescence emission spectra can be related to the range from 250 nm to 440 nm [18]. Furthermore, the aromatic amino acids responsible for collagen fluorescence in this wavelength range are phenylalanine [Phe], tyrosine [Tyr] and tryptophan [Trp] [19]. Their ratio of the fluorescence excitation maximum to the fluorescence emission maximum is 258/282 nm, 275/303 nm, and 275/348 nm, respectively. Based on this information, it can be concluded that the fluorescence excitation bands of collagen (marked as No. 2 and 5 line in **Figure 3**) and the fluorescence emission bands of collagen (marked as 4 and 6 line in **Figure 4**) are related to these amino acids [18]. The cross-links form the latter fraction responsible for collagen absorption / fluorescence which includes trivalent cross-links and advanced glycation end-

Table 1. Summary of literature data with regard to the excitation maximum and emission maximum of collagen fluorescence

Reference	Excitation maximum	Emission maximum
[15]	345 nm, 370 nm, 410 nm	440 nm, 495 m
[7]	370 nm	380 nm, 420 nm, 440 nm, 465 nm i 495 nm
[16]	360 nm	430 nm, 460 nm
[17]	280 nm, 265 nm, 330 nm, 450 nm	310 nm, 385 nm, 390 nm, 530 nm

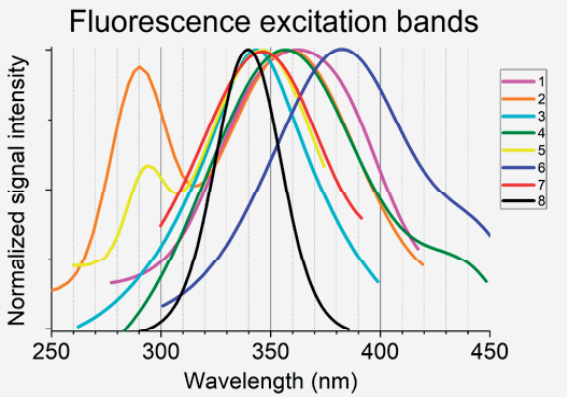

Figure 3. The differences in the excitation bands of collagen prepared by various methods. For this purpose, fluorescence spectra presented in different publications were digitized point by point in order to obtain minimal differences compared to the original bands

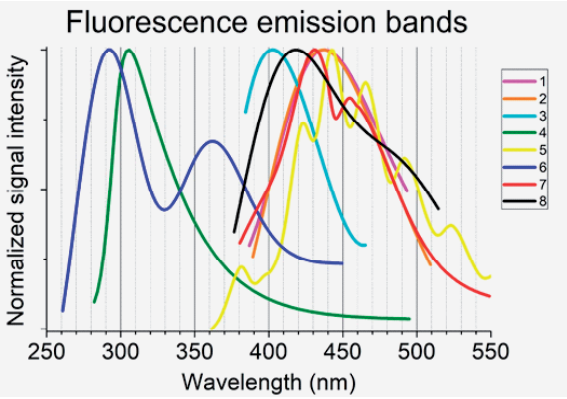

Figure 4. The differences in the emission bands of collagen prepared by various methods. For this purpose, fluorescence spectra presented in different publications were digitized point by point in order to obtain minimal differences compared to the original bands

products (AGEs), varying in terms of their spectrophotometric properties [9].

Collagen cross-links spectroscopy

Until now, several types of cross-links have been identified using HPLC [9]. Nonreducible cross-links, such as pyridinoline (Pyr) or deoxypyridinoline (Dpyr) have been identified using their natural ability of autofluorescence. They are characterized by the maximum wavelength in excitation spectrum at 295 nm, 325 nm and 340 nm (depending on the pH of the environment) with the light emission maximum of 395 nm [20]. Moreover, pentosidine (Pen) is also naturally capable of fluorescence and its excitation emission maximum amounts to 335/385 nm [9, 20, 21]. Another cartilage-specific cross-link, 2,6-dimethyldifuro-8-pyrone (DDP) has been identified and isolated. It is capable of autofluorescence with a maximum ratio of 306/395 nm (excitation/emission) [22]. Additionally, other divalent reducible cross-links, including dihydroxylysinonorleucine (DHLNL), hydroxylysinonorleucine (HLNL), lysinonorleucine (LNL) and trivalent nonreducible cross-link histidinohydroxylysinonorleucine (HHL), have been detected by generating O-phthalaldehyde derivatization because they do not have the ability to naturally fluorescent [9].

According to the literature data, AGEs can constitute the main fraction of cross-links associated with collagen fluorescence [10, 23, 24]. They are responsible for a fluorescent excitation-emission maximum at 360/460 nm [10, 23, 24]. The initial amount of this type of bonds is small, but it increases with age, resulting in the collagen molecule aging. However, in the case of diabetic patients, AGEs are multiplied [9, 25–29]. Pen, as mentioned above, belongs to the well-described AGEs. This cross-link has a ratio of excitation-emission maximum which is significantly different from other AGEs [9, 21]. Other collagen cross-link AGEs capable of autofluorescence include vesperlysine A and B, vesperlysine C, lysyl-pyrropyridine, FFI, Argpyrimidine, Crossline, Fluorolin, and threosidine [21, 27, 29–31]. The wavelengths at which the excitation-emission maxima were measured are summarized in **Table 2**. Among AGEs identified in collagen, cross-links which do not possess a benzene ring in their structure have been described, thus, these cross-links are incapable of autofluorescence, e.g. N- (carboxyethyl) lysine (CEL), N- (carboxymethyl) lysine (CML), methylglyoxal-lysine dimer (MOLD) and glyoxal-lysine dimer (GOLD), and formyl threosyl pyrrole (FTP) [10, 28, 29, 32].

During the development of the organism, divalent immature cross-links are converted into non-reducible mature or glycation cross-links. Due to the very long half-life of collagen in various tissues, e.g. cartilage or skin, mature cross-links and AGEs accumulate with age [9, 27–29, 33]. As reported in the literature, numerous additional AGEs are formed during the incubation of cartilage with threose [10]. Furthermore, AGEs

Table 2. Summary of the identified cross-links capable of fluorescence.

Name of cross-link	Excitation /Emission maximum [nm]	References
Trivalent cross-links		
Pyridinoline (Pyr), Deoxypyridinoline (Dpyr)	295 nm (acid), 325 nm (neutral), 340 nm (alkaline)/395 nm	[20]
Hydroxylysyl pyridinoline (HP), Lysyl pyridinoline (LP)	295 nm / 395 nm	[32]
2,6-dimethyldifuro-8-pyrone (DDP)	306/395 nm	[15,32]
Advanced glycation endproducts (AGEs)		
Pentosidine (Pen)	335 nm/385 nm	[9,21,32]
Vesperlysine A i B	366 nm/ 442 nm	[21]
Vesperlysine C	345 nm/ 405 nm	[21]
Lysyl-pyrropyridine	370 nm/ 448 nm	[21]
FFI	380 nm/ 440 nm	[21]
Argpyrimidine	335 nm/400 nm	[21]
Crossline	379 nm/ 463 nm	[21]
Fluorolink	380 nm/ 460 nm	[21]
threosidine	328 nm/ 402 nm	[31]

contribute to the aging of tissues (cartilage, skin or bones) and changes in their properties, e.g. their ability to deform [10, 28, 29]. Additionally, the results of various experiments indicate that cross-links, such as CML, CEL and Pen, constitute only about 4.7% of the newly formed AGEs [10].

Conclusion

The discrepancy in the literature data on collagen spectroscopy does not entirely imply that they are incorrect. In fact, different results of spectrophotometric tests of collagen may stem from collagen structural diversity and different spectrophotometric parameters of the experiment. Therefore, the result of the experiment is closely related to the type of collagen tested, or the physical parameters of the experiment. Thus, it seems essential to define the exact test parameters and identify the collagen type when presenting the experiment, or to provide an accurate description of the investigated tissue, since this may help other researchers relate to the experiment. Moreover, the unification of parameters with regard to the spectrophotometric tests may contribute to a faster progress in research on collagen.

Acknowledgements

List of abbreviations
NADH - nicotinamide adenine dinucleotide
FAD - flavin adenine dinucleotide
AGEs - advanced glycation end-products
Pyr - pyridinoline
Dpyr - deoxypyridinoline
Pen - pentosidine
DDP - 2,6-dimethyldifuro-8-pyrone
DHLNL - dihydroxylysinonorleucine
HLNL - hydroxylysinonorleucine
LNL - lysinonorleucine
HHL - histidinohydroxylysinonorleucine
CEL - N-(carboxyethyl) lysine
CML - N-(carboxymethyl) lysine
MOLD - methylglyoxal-lysine dimer
GOLD - glyoxal-lysine dimer
FTP - formyl threosyl pyrrole
HP - Hydroxylysyl pyridinoline
LP - Lysyl pyridinoline

Conflict of interest statement
The authors declare no conflict of interest.

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