Progress in the detection and quantification of collagens: a review

A H M Gameil¹, F Yusof¹², A S Azmi¹ and N I Mohamad Puad¹

¹Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, P.O. Box 50728 Kuala Lumpur, Malaysia

²Corresponding author’s email: yfaridah@iium.edu.my

Abstract Collagens are an important and ubiquitous family of proteins. They have many functions in the human body and similarly have found numerous, potent applications in various industries including the manufacture of biomaterials. The ever-increasing demand for collagen has made necessary the exploration of alternative sources such as bacterial collagen-like proteins which have a triple-helical domain of Gly-X-Y amino acid repeats. Detection and quantification of native collagens have been well-established. However, collagen-like proteins differ in their composition and do not have the unique abundance of hydroxyproline and hydroxylysine found in vertebrate collagens. Thus, this poses a problem in the detection and quantification of collagen-like proteins. This paper evaluates reports on the detection and quantification of collagens and collagen-like proteins. A systematic search of the PubMed database was conducted in May 2021, to which five additional papers were added. The 310 unique search results were then subjected to a screening and elimination process, at the end of which 22 papers were included in the study. The findings were summarized and presented in a table that highlights progress in this field. While novel methods have been developed for the detection and quantitation of collagens in general, mainly using enzyme digestion, hybridization, and fluorescence, there is a need for a rapid, one-step method that selectively and sensitively detects and quantitates collagen and collagen-like protein samples with ease.

1. Introduction

Collagens represent a diverse and ubiquitous family of proteins in the animal kingdom, forming the skin, cartilage, bones, and organs of vertebrates as well as the exoskeleton of invertebrates such as crustaceans and nematodes. The collagen family serves various functions such as tensile strength and elasticity, protection, tissue architecture, cell signaling, and some collagens carry out complex functions related to cell metabolism [1-3]. As we begin to understand and appreciate these functions, collagen finds more utility and a newer purpose in enhancing our quality of living. Collagen supplements serve various pharmaceutical and cosmeceutical functions, and collagen has innumerable uses in the surgical and wound care aspects of various medical departments. Collagen scaffolds and novel biomaterials are also being developed to regenerate tissues [4, 5]. The scientific field also experienced developments in order to draw level with these developments and enable progress in these fields. Testing and assays must be more robust to cater to small-scale experiments as well as large-scale productions. Concurrently, a relatively recent development in the area is the discovery of bacterial and viral collagens, known collectively as collagen-like proteins [6, 7]. These collagen-like proteins have collagen-like, triple-helical domains of glycine-X-Y repeats that show similar thermal and structural stabilities to animal
collagens [4-7]. This development is very promising for the biomaterial, medical and biomedical applications of collagen, among others, in the effort to provide a sustainable supply of collagens as well as in making “designer collagens” for various applications [4]. However, new questions emerged to be dealt with, for instance, scientists need to easily ensure and confirm that the protein that has been produced is indeed the recombinant or collagen-like protein of interest. For instance, mammalian collagen is composed of large amounts of hydroxyproline and hydroxyllysine, whereas prokaryotic collagens do not have these unique amino acids – what techniques, if any, are available to detect and quantify these different types of collagens? This study sought to answer these questions and to determine the gap(s) in the field.

2. Methodology
This review was carried out by using a systematic search guided by the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines [8]. The keywords were determined using the PICO (Population, Interventions, Comparisons, and Outcomes of study) system, as shown in Table 1. The keywords and synonyms presented were considered and utilized to formulate the search string. The information source used was the PubMed database (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/pubmed/advanced). The systematic search was performed in the database as shown in Table 1, in May 2021.

Table 1. Keyword summary used to develop the search terms.

| Characteristics | Keywords | Synonyms |
|-----------------|----------|----------|
| **Population**  | Collagen* | Collagen-like proteins, bacterial, prokaryotic, collagen mimetic peptide, triple-helix |
| **Intervention**| method   | technique, assay, fluorescent*, colorimetric, hybridization, enzymatic, derivatization, absorbance, spectrophotometric |
| **Comparison**  | native and recombinant mammalian collagens | human, bovine, mammalian, type |
| **Outcome**     | detection, quantification | detect*, quantif*, determination |
| **Search string** | ((collagen*[Title]) OR ("collagen-like protein"[Title]) OR ("bacterial collagen"[Title])) AND ((method[Title/Abstract]) OR (assay[Title/Abstract]) OR (technique[Title/Abstract]) OR (detect*[Title]) OR (quantif*[Title]) OR (hybridiz*[Title/Abstract]) OR (fluorescen*[Title/Abstract])) NOT (role[Title]) Filters: Journal Article, in the last 10 years |

These papers were then imported into the online Parsifal tool, using TexMed tool and Zotero software (Version 5.0.88). All titles and abstracts retrieved from the comprehensive search were screened and evaluated based on the inclusion and exclusion criteria. Only English-language, journal articles are included in this study, and review articles, conference papers, and book sections were excluded. Publications on the detection and/or quantification method of any type of collagen were included, except those considered as not assays. The data extracted from each study were its basis, principal reagent type, advantages and limitations, detection limit, and the name and type of collagen studied.

3. Results
The systematic search returned a total of 305 unique citation results via the PubMed database. An additional five articles were identified via citation-checking techniques. Imported studies were made up of all the 310 records. Of these, 310 abstracts were screened by title and/or abstract screening and 287 articles were excluded as they did not fit the inclusion and/or exclusion criteria. In vivo and ex vivo staining and imaging of collagenous tissue or fibers and other histochemical staining techniques in the study of numerous diseases formed the major portion of the excluded literature found on collagen detection and quantitation. The remaining 23 records [9-31] went through full-text screening. The full...
A total of 22 papers were identified and included in the data extraction process. The findings of each study are summarized in Table 2.

**Table 2. Summary of findings.**

| Ref | Method Keywords                                      | Collagen type                        | Application                                          | Advantages                                      | Limitations                      |
|-----|------------------------------------------------------|--------------------------------------|-----------------------------------------------------|-------------------------------------------------|----------------------------------|
| 9   | Thermal nanoparticles, silver nanoparticles          | Porcine, human type I, rat tail collagen | Denatured mammalian collagen                         | Rapid (30 minutes) Reproducible Highly sensitive Inexpensive Specific (high affinity) | Not Available                   |
| 10  | enzyme-linked immunosorbent assay (ELISA), antibody, matrix-metalloproteinase (MMP) | human type III                        | Digested fragment of human type III collagen         | Specific (high affinity) For each specific biomarker, an antibody is required. | Not Available                   |
| 11  | Hybridization, FRET (fluorescence resonance energy transfer) graphene oxide | Collagen peptide                     | Collagen triple-helical domains                      | Rapid Sensitive Multiple samples specific       | Not Available                   |
| 12  | Alkaline hydrolysis                                 | Mammalian collagen (porcine)          | Hydroxyproline-containing collagen                   | Rapid Sensitive                                | Complex                          |
| 13  | Alkaline hydrolysis, Woessner method                | Hoki skin collagen                    | Hydroxyproline-containing collagen                   | Accurate Reproducible High throughput          | Requires strict adherence to protocol. Complex Specific biomarker required   |
| 14  | chromatography, mass spectrometry                   | Animal collagen, type I collagen       | Thermally denatured, trypsin digested animal collagens | Semi-destructive Sensitive Small sample quantity Reduced steps Versatile | Not Available                   |
| 15  | Hybridization, phototriggered caged CMP, fluorescence, near infrared tags, (carboxyfluorescein and IR680) | Collagen mimetic peptides             | Any triple-helical collagen peptide                  | Versatile                                       | Not Available                   |
| 16  | Hybridization, fluorescence, carboxyfluorescein bound collagen hybridizing peptides | Collagen mimetic peptides             | Denatured mammalian collagen peptides                | Rapid                                           | Not Available                   |
| 17  | Polyclonal antibodies, gold nanoparticles           | Rat tissue                            | Any triple-helical collagen domain                   | Rapid sensitive                                | Not Available                   |
| 18  | chromatography and mass spectrometry                | Raw and cooked pork                   | Hydroxyproline-containing collagens                 | Rapid sensitive multiple samples               | Costly                           |
4. Discussion
Several assay methods including colorimetric [9, 19, 20], chromatographic [14, 18, 24, 27, 31], antibody-based [10, 17], nanoparticles-based [9, 17] and radio-isotope labelling [24] approaches have been offered for the quantification of collagens. First, the picrosirius red method is mentioned in one included paper [19]. This colorimetric assay of collagens, using the Sirius red dye in picric acid solution, provides a simpler and more rapid quantification of collagens when compared to older techniques. However, drawbacks such as low selectivity of the colorimetric method have been a problem, in addition to its

|   | Methodology                                                                 | Materials                                                                 | Advantages                                                                 | Drawbacks                                                                 |
|---|------------------------------------------------------------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|
| 19 | Colorimetric, Sirius red                                                     | Rat tail collagen, porcine skin gelatin                                   | Low-cost, simple, rapid, versatile, small sample volume                    | Interference from Bovine Serum Albumin and elastins.                      |
| 20 | Hybridization, colorimetric, fluorescence, dye-labeled collagen-like peptide | Collagen mimetic peptides                                                 | Any triple-helical collagen peptides                                       | Not available                                                            |
| 21 | Hybridization, fluorescence, graphene oxide, FAM                             | Collagen mimetic peptides                                                 | Hydroxyproline-containing collagen peptides                                | Not available                                                            |
| 22 | Hybridization, fluorescence, graphene oxide, FAM (5-Carboxyfluorescein)     | Collagen mimetic peptides                                                 | Hydroxyproline-containing collagen peptides                                | Not available                                                            |
| 23 | Hybridization                                                                | Collagen mimetic peptides                                                 | Hydroxyproline-containing collagen peptides                                | Not available                                                            |
| 24 | Isotope-labeling, LCMS, chromatography, mass spectrometry                    | Collagen mimetic peptides                                                 | Hydroxyproline-containing collagen peptides                                | Complex, costly                                                          |
| 26 | Hybridization, fluorescence, fluorescein, cyclic collagen mimetic peptides   | Collagen mimetic peptides                                                 | Hydroxyproline-containing collagen peptides                                | Not available                                                            |
| 27 | Hybridization                                                                | Collagen mimetic peptides                                                 | Hydroxyproline-containing collagen peptides                                | Not available                                                            |
| 28 | Acid hydrolysis, modified HPLC, chromatography and mass spectrometry        | Animal collagens                                                           | Hydroxyproline-containing collagen peptides                                | Costly                                                                   |
| 29 | Enzymatic digestion, fluorescence, 3,4-dihydroxyphenylacetic acid            | Animal collagens                                                           | Hydroxyproline-containing collagen peptides                                | Costly                                                                   |
| 30 | Fluorescence 4-chlorobenzene-1,2-diol                                        | Animal collagens                                                           | Hydroxyproline-containing collagen peptides                                | DNA-based                                                                |
| 31 | Hydroxyproline-containing collagens                                          | Hydroxyproline-containing collagen peptides                                | Hydroxyproline-containing collagen peptides                                | Not available                                                            |
limitation for collagen-like proteins which do not have hydroxyproline. Hence, several modifications of the assay have been proposed.

Another approach to collagen determination involves the use of enzymatic or chemical degradation of collagens followed by high-performance liquid chromatography (HPLC) and spectrophotometry or mass spectrometry (MS). Detection of native eukaryotic collagens has often utilized the unique abundance of hydroxyproline residues that is characteristic of collagen proteins. Aside from collagen, only a handful of other mammalian proteins have hydroxyproline-containing collagen-like (triple-helical) domains, notably the elastins and argonaute-2. Thus, the detection of the presence of hydroxyproline is used in the detection of collagen, where hydroxyproline is derivatized to enable its colorimetric detection. Hydroxyproline, released by acid or alkaline hydrolysis of collagens, can be quantified by full amino acid analysis using chromatography followed by post-column detection. However, the most widely used method is a colorimetric assay based on the reaction of oxidized hydroxyproline with Ehrlich’s reagent (p-dimethylaminobenzaldehyde). For example, hydroxyproline can be oxidized to pyrrole-2-carboxylic acid using hydrogen peroxide as an oxidizing agent in the presence of alkaline copper sulfate. The excess oxidizing agent is then removed by heating, and the oxidation product is reacted with Ehrlich’s reagent in the presence of dilute sulfuric acid [13].

Finally, the quantification of collagens by enzyme-labeled immunosorbent assay (ELISA) using specific antibodies to each type of collagen or a specific collagen degradation product is another popular approach especially for the detection of collagen peptide biomarkers of diseases in biological samples. Even though it is quite specific, it is an expensive, complex, time-consuming approach. One included article [15] utilized a unique, novel approach that would enable quantitation with some modifications, as well as simultaneous imaging of collagen.

Newer approaches for the detection of collagens make use of another unique aspect of collagen proteins, which is its triple-helical structure. Inspired by the hybridization of denatured DNA strands with a probe, several articles have been published where denatured collagens have been detected by using triple-helical hybridization with single-stranded, fluorescence-tagged, and cyclic collagen-mimetic peptides. As mentioned previously, one included article involves a photo-triggered hybridizations step that can then be used to image the collagen in vivo and ex vivo as well [15]. During the data extraction, a method keywords column was created to analyze the data, and the most common method keyword that appears is fluorescence together with hybridization seven times [11, 15-16, 20-22, 26]. Hybridization methods offer higher affinity towards collagen peptides than antibodies, and thus can be useful as less expensive methods for biomarker studies.

Finally, a relatively recent assay proposed that the collagen can be derivatized via amine-labeling of its glycine residues by using chromophores or fluorophores, since the collagen triple helix is made up of consistent repeats of glycine residues. For this to be used, the collagen must first be digested to expose its free N-terminal glycine, by using bacterial collagenases such as Clostridium histolyticum. After the derivatization, the dyes fluoresce upon reaction with the amine residues in a linear manner of increasing fluorescence with protein concentration that can be compared against other collagen standards of known concentrations [29, 30]. This unique approach solves the crucial problem of detecting recombinant and prokaryotic collagen-like proteins which lack hydroxyproline and otherwise cannot be detected or quantified by the picrosirius method. In addition, it offers a sensitive alternative to assay collagen peptides. However, the method has its own limitations, chiefly, the need for a spectrofluorometer and the sample preparation time of a few hours. As the collagen triple helix is resistant to protease digestion by pepsin and trypsin, one way to enhance the selectivity of this approach would be to first digest a sample with trypsin, hence eliminating other non-collagenous proteins.

5. Conclusion

Collagens represent a vast family of useful biomaterials whose uses span several industries including the pharmaceutical and food industries. Collagen peptides are also important in the biomedical diagnostics field for understanding and diagnosing diseases using products of collagen degradation, or collagen biomarkers. In addition, collagen-like domains of proteins were found to be common in bacteria.
including pathogenic ones. This means that there will be more reasons to understand and utilize these unique triple-helical proteins. Hence, there is a need for novel, one-step methods to rapidly and selectively detect and quantitate the various types of collagens and collagen-like samples with ease. Most of the recent existing approaches, however, address the detection and quantification of hydroxyproline-containing biological samples. Here, the picrosirius red assay remains ideal for most applications of mammalian collagens where speed, low cost, and sensitivity are required. While relatively recent fluorometric techniques currently exist, the preparation steps are still laborious and assume the availability of the fluorophore, assay reagents, and costly spectrofluorometric instrumentation. The same can be said of liquid chromatography mass spectrometry, and thus, there exists room for an improved collagen detection and quantitation technique. As for the case of collagen-like proteins, only two studies offer a lucrative approach [29, 30]. Furthermore, this review provides a preliminary step towards systematic mapping of collagen detection and quantitation literature. By modifying the systematic search to be more inclusive, a more comprehensive search of databases can be used to map the literature and identify gaps in those areas of collagen detection and quantitation that were not relevant to this paper, such as in imaging of collagenous tissue samples.

Acknowledgments
This study is funded by the Ministry of Higher Education, Malaysia via the Transdisciplinary Research Grant Scheme (TRGS/1/2018/UIAM/01/1/3). The authors report no conflict of interest.

References
[1] Silvipriya K, Kumar K, Bhat A, Kumar B, John A and Lakshmanan P 2015 *J. Appl. Pharm. Sci.* 5 pp 123-7
[2] Sandhu S V, Gupta S, Bansal H, Singla K and Yadav N S 2012 *J. Orofac. Res.* 2 pp 153-9
[3] Gelse K, Poschel E and Aigner T 2003 *Adv. Drug. Deliv. Rev.* 55 pp 1531-46
[4] An B, Kaplan D L and Brodsky B 2014 *Front. Chem.* 2 40
[5] Ramshaw J A, Werkmeister J A and Dumsday G J 2014 *Bioengineered* 5 pp 227-33
[6] Xu C, Yu Z, Inouye M, Brodsky B and Mirochnitchenko O 2010 *Biomacromolecules* 11 pp 348-56
[7] Yu Z, An B, Ramshaw J A and Brodsky B 2014 *186* pp 451-61
[8] Moher D, Liberati A, Tetzlaff J, Altman D G and PRISMA Group 2009 *PLoS Med.* 6
[9] Ahumada M, McLaughlin S, Pacioni N L and Alarcon E I 2016 *Anal. Bioanal. Chem.* 408 pp 1993-6
[10] Barascuk N, Vassiliadis E, Larsen L, Wang J, Zheng Q, Xing R, Cao Y, Crespo C, Lapret I, Sabatini M *et al.* 2011 *Clin Biochem.* 44 pp 900-6
[11] Cai X, Yang Q, Ding J, Ye W, Li X and Xiao J 2016 *J. Mater. Chem. B.* 4 pp 7009-13
[12] da Silva C M, Spinelli E and Rodrigues S V 2015 *Food Chem.* 173 pp 619-23
[13] Hofman K, Hall B, Cleaver H and Marshall S 2011 *Anal Biochem.* 417 pp 289-91
[14] Kumazawa Y, Hattori S and Taga Y 2019 *Anal. Chem.* 91 pp 1796-800
[15] Li Y, Foss C A, Pomper M G and Yu S M 2014 *J Vis Exp.*
[16] Lin A H, Zitnay J L, Li Y, Yu S M, and Weiss J A 2019 *J. Orthop. Res.* 37 pp 431-8
[17] Masiri J, Benoit L, Barrios-Lopez B, Thienes C, Mesghii M, Agapov A, Dobritsa A, Nadala C and Samadpour M 2016 *Meat Sci.* 121 pp 397-402
[18] Qiu B, Wei F, Sun X, Wang X, Duan B, Shi C, Zhang J, Zhang J, Qiu W and Mu W 2014 *Mol. Med. Rep.* 10 pp 1157-63
[19] Rodriguez-Rodriguez P, Arribas S M, de Pablo A L, Gonzalez M C, Abderrahim F and Condezo-Hoyos L 2013 *Anal. Bioanal. Chem.* 405 pp 6863-71
[20] Sun X, Fan J, Li X, Zhang S, Liu X and Xiao J 2016 *Chem. Commun. (Camb).* 52 3107-10
[21] Sun X, Fan J, Ye W, Zhang H, Cong Y and Xiao J 2016 A highly specific graphene platform for sensing collagen triple helix. *J. Mater. Chem. B.* 4 pp 1064-9
[22] Sun X, Qiao Y, Li W, Sui Y, Ruan Y and Xiao J 2020 *J. Mater. Chem. B.* 8 pp 6027-33
[23] Sun X, Yao L, Fu C, Luo L, Wang J and Xiao J 2019 *J. Mater. Chem. B.* 7 pp 7676-82
[24] Taga Y, Kusubata M, Ogawa-Goto K and Hattori S 2014 J. Agric. Food Chem. 62 pp 12096-102
[25] Takita K K, Fujii K K, Ishii K and Koide T 2019 Org. Biomol. Chem. 17 pp 7380-7
[26] Takita K K, Fujii K K, Kadonosono T, Masuda R and Koide T 2018 Chem. Biochem. 19 pp 1613-7
[27] Vatansever B, Senal M O, Akgoz M and Goren A C 2015 Anal. Bioanal. Chem. 407 pp 1981-7
[28] Wang Y, Yi L, Pan X, Zhang J and Duan R 2018 Food Chem. 256 pp 40-4
[29] Yasmin H, Kabashima T, Rahman M S, Shibata T and Kai M 2014 Sci. Rep. 4 4950
[30] Yasmin H, Rahman M S, Shibata T, Kabashima T and Kai M 2015 Chemical Papers 69
[31] Yuswan M H, NH A J, Mohamad H, Keso S, Mohamad N A, Tengku Md Yusoff T S, Ismail N F, Abdul Manaf Y N, Mohd Hashim A, Mohd Desa M N, et al. 2021 Food Chem. 337 127762