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Chemical Tools for Monitoring and Targeting Collagen Cross-linking

Matthew C. Deen, Linus B. Boll, and Helma Wennemers*

Abstract: The formation of collagen, the most abundant protein in mammals, is vital for the integrity of skin, tendons, and tissue in essentially any organ. Excessive collagen formation is, however, characteristic of fibrotic and malignant diseases, which include major global health issues. The diagnosis of abnormal collagen production and deposition is, therefore, critical for disease prognosis and helps guide treatment decisions. Here, we summarize our research on the development of tailored tools for monitoring and targeting excessive collagen cross-linking. We anticipate these tools will provide a deep understanding at the molecular level of collagen formation in normal and disease conditions with applications in imaging and disease treatment.

Keywords: Chemical biology · Collagen · Fluorescent sensors · Lysyl oxidase · Peptide

Linus Boll studied Chemistry (MSc) at ETH Zurich. For his Master thesis, he joined the group of Ronald T. Raines (MIT, USA), where he investigated and developed chemistry for the chemoselective modification of proteins. In 2022, he returned to ETH Zurich to start his PhD with Prof. Helma Wennemers. In the Wennemers group, Linus is developing chemical tools for the diagnosis and targeted therapy of fibrotic diseases.

Matthew Deen completed his PhD degree under the supervision of Prof. David Vocadlo (Simon Fraser University, Vancouver, Canada). During his doctoral studies, he developed fluorogenic chemical tools to quantitatively image the activity of glycoside hydrolases. In 2023, he joined the group of Prof. Helma Wennemers at ETH Zurich where he is focused on developing new selective tools to study lysyl oxidases and their role in fibrotic pathologies.

Helma Wennemers received her PhD from Columbia University for research with Prof. W. Clark Still. She carried out postdoctoral studies with Prof. Hisashi Yamamoto at Nagoya University before joining Basel University as the Bachem-endowed Assistant Professor in 1999. In the fall of 2011, Helma moved to ETH Zurich, where she is a Professor of Organic Chemistry. Her research uses peptides and the power of organic chemistry to address questions in asymmetric catalysis, chemical biology, and supramolecular chemistry.

1. Introduction

Collagens, the most abundant proteins in mammals, are the main constituents of the extracellular matrix (ECM).[1] The fibrous structure of collagens is key for the strength and stability of connective tissue.[1–3] Collagen formation is, therefore, vital for the integrity of skin, tendons, and tissue in essentially any organ. Excessive collagen formation is, however, characteristic of fibrotic and malignant diseases.[4–6] Estimates associate 45% of all deaths in the industrialized world with diseases possessing fibrotic pathologies.[7] Methods to visualize and target excessive and abnormal collagen production and deposition are therefore important. Effective tools can help disease prognosis and guide decisions such as whether or not to perform surgery.

Collagen fibrils consist of triple-helical peptide strands with amino acids in Xaa-Yaa-Gly repeating units where Xaa and Yaa are most commonly proline (Pro, P) and (2*S*,4*R*)-4-hydroxyproline (Hyp, O), respectively (Fig. 1a).[1] The cyclic nature of these amino acids enforces Φ torsion angles that are ideal for polyproline II (PPII) helices, the conformation of the single strands within the triple helices. Aside from Pro and Hyp residues, many other amino acids, including lysine (Lys), occur in collagens.[1,8]

During tissue formation, lysyl oxidases (LOX) play a key role in the collagen maturation process.[9] This family of enzymes oxidizes the ε-amino group of Lys residues to aldehyde groups (allysine) that then undergo spontaneous aldol and related reactions with other lysine, hydroxylysine, or lysyl-aldehyde (allysine) residues to form covalent bonds between collagen fibrils as well as within elastin (Fig. 1b).[9–12] This LOX-initiated cross-linking process is essential for normal tissue homeostasis, but prolonged and excessive LOX activity leads to excessive collagen cross-linking and impaired tissue function as observed in fibrotic and malignant diseases. Tools that allow for visualizing and targeting excessive cross-linking within the ECM, thus, present an opportunity for detecting diseased tissues.

Lysyl oxidases and their aldehyde products in the ECM offer unique opportunities for precise detection through chemical sensing. Classic LOX activity assays monitor the hydrogen peroxide generated by LOX through the horseradish peroxidase-catalyzed conversion of Amplex red to fluorescent resorufin.[13] While this Amplex red assay was a major advancement over early assays using tritiated lysine it is susceptible to fluorescence quenching or artifacts from other endogenous sources of peroxide. This coupled-enzyme assay is, therefore, incompatible with complex biological environments and *in vivo* use. Fluorogenic probes that report directly on the enzymatic activity of LOXs avoid some of these drawbacks.[14–16] Yet, these probes diffuse freely and,

*Correspondence: Prof. H. Wennemers,
E-mail: helma.wennemers@org.chem.ethz.ch
Laboratorium für Organische Chemie, ETH Zurich, D-CHAB, Vladimir-Prelog-Weg 3
CH-8093 Zurich

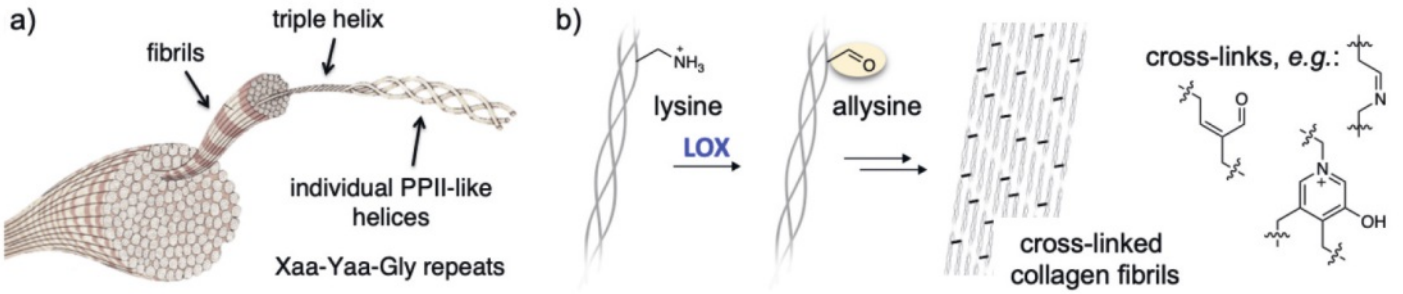

Fig. 1. a) Cartoon of a collagen fibre. b) LOX-initiated collagen cross-linking.

therefore, can not offer spatial control over the location where LOX activity and cross-linking of collagen occur.

Herein, we present recently developed chemical tools and their application for the simultaneous imaging and targeting of LOX-generated cross-linking of collagen in the ECM.

2. A Chemical Probe for Monitoring and Targeting LOX-initiated Collagen Cross-linking

Our laboratory has long-standing expertise in using collagen model peptides (CMPs) to understand the stability of collagen at the molecular level and to establish functional synthetic collagen triple helices.[17–25] These include pH-responsive synthetic collagen,[20,26–28] hyperstable triple helices,[22–24] and heterotrimeric collagen.[25] Our studies established protocols that allow for the functionalization of CMPs with essentially any desired moiety at the Xaa or Yaa position.

Building on this knowledge, we developed a chemical probe (**1**) for the simultaneous imaging and targeting of LOX-generated cross-linking of collagen in the ECM (Fig. 2).[29] The system consists of three components: a) a turn-on fluorescent LOX sensor, b) a collagen mimetic peptide, and c) an aminooxy group (Fig. 2a). The coumarin-based turn-on sensor uses a LOX-reactive aminopropyl group to mask the fluorescence. Bright fluorescence emerges upon LOX-mediated oxidation of the amino group to an aldehyde that triggers rapid β-elimination of acrolein (Fig. 2b). This turn-on sensor is dark in the masked state (λ_{ex} < 360 nm, Φ = 0.09) and brightly fluorescent (Φ = 0.89) upon activation by LOX, with an excitation window between 360–405 nm. This sensor is biocompatible, reacts quickly with LOX in a dose-dependent manner, and is so sensitive that less than picogram quantities of enzyme can be monitored.[29] The aminooxy group ensures chemoselective ligation to the endogenous aldehydes generated by LOX in the extracellular space. The collagen peptide provides selectivity for sites of collagen remodeling; in the absence of the CMP (probe 2), non-specific staining was observed (Fig. 2e).[29]

In contrast to previously developed CMPs for *in vivo* applications[30–32] that rely on non-covalent interactions and are therefore prone to wash-out, the combination of the CMP with the aminooxy group for chemoselective reaction ensures covalent tethering. All three components of the synthetic reporter work in concert to visualize LOX-created cross-linking sites during extracellular collagen maturation. *In vivo* studies with mice and *ex vivo* studies with histological sections from tumors revealed an unprecedented level of specificity and spatial resolution in tissues (Fig. 2c,d).[29]

3. Illuminating Collagen Remodeling During Wound Healing

During wound healing, the structural integrity and functionality of tissue is restored after injury. A key aspect of this complex process is the dynamic remodeling of the ECM, which includes

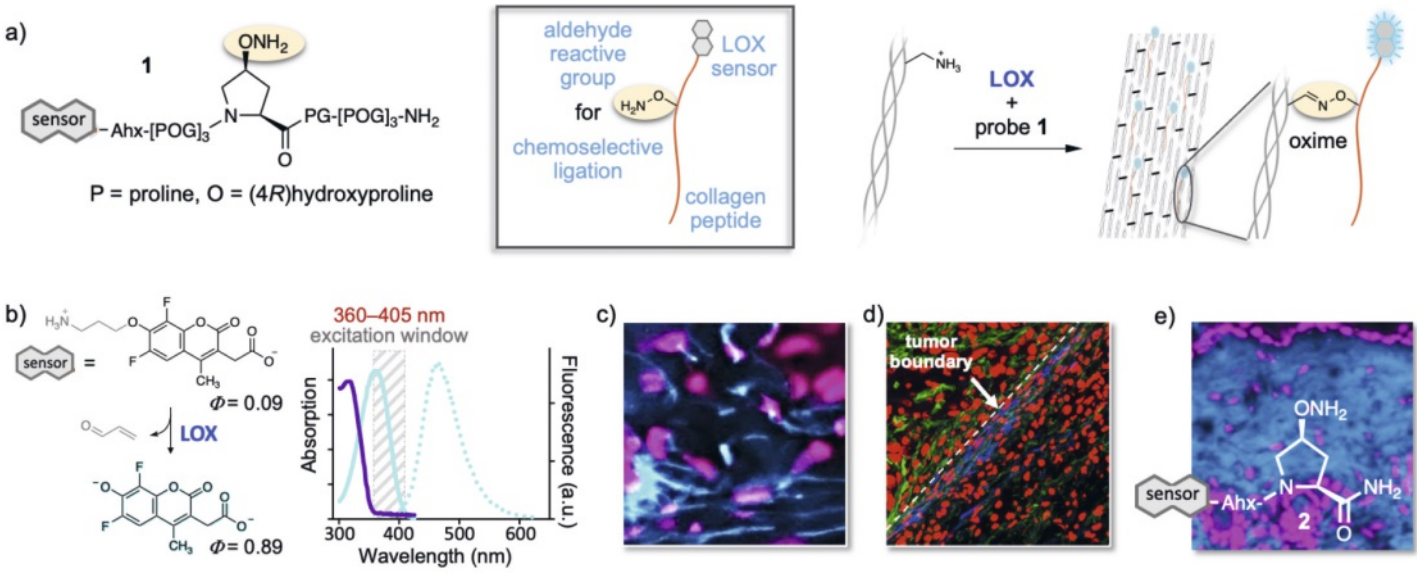

Fig. 2. Monitoring and targeting of LOX-initiated collagen cross-linking. a) Probe **1**. b) LOX sensor with masked fluorophore (absorption, purple) and unmasked fluorophore (absorption, turquoise solid line; emission, turquoise dotted line). c) Fluorescence staining of a skin section 5 days after intradermal injection of **1** into the back skin of mice (pink, nuclear stain). d) Fluorescence staining of a cutaneous squamous cell carcinoma ear skin xenograft by probe **1** (blue), (red, nuclei; green, immunofluorescence stain for collagen I). e) Fluorescence staining of a skin section 5 days after intradermal injection of control compound **2** into the back skin of mice (pink, nuclear stain). Ahx = 6-amino hexanoic acid. Reproduced and modified with permission from *Nature Chemical Biology*.

the deposition and maturation of fibrillar collagens. Insufficient or excessive collagen deposition and maturation are associated with abnormal LOX activity and can lead to pathological conditions, such as chronic non-healing wounds or hypertrophic scars, respectively.[33–35] Traditional methods for evaluating collagen remodeling during tissue repair rely on indirect measurements, often based on assessments of tissue histology, collagen concentration, and protease activity.[36-38] These methods lack precision in identifying specific sites and stages of collagen remodeling, and many of them cannot be used in routine diagnostics. We, therefore, envisioned that our probe could fulfil an unmet need and serve as a powerful and nuanced tool for monitoring the wound healing process.

In collaboration with Prof. Sabine Werner (ETH Zurich), we demonstrated that the collagen peptide sensor probe **1** selectively binds to and illuminates areas of active collagen production and remodeling during murine and human wound healing.[39] In studies with tissue sections of wounds, probe **1** localized at the wound margins and wound interior, the areas where newly formed collagen and active collagen remodeling take place (Fig. 3a). A comparison of wound sections on days 5 and 14 showed a clear progression of the healing progress toward the center of the wound (Fig. 3b).

The ability of probe **1** to report selectively on the localization of newly formed and remodeling collagen was further corroborated by combining confocal microscopy imaging with second harmonic generation (SHG), a technique that visualizes mature, fibrillar collagen (Fig. 3c).[40] Fluorescence arising from probe **1** is in close proximity, but not overlaid with mature fibrillar collagen, as visualized by SHG. This result implies that probe **1** binds and anchors to emerging collagen still in formation and not sufficiently mature to emit SHG signals. The staining of sections from hemizygous transgenic mice with wound healing alterations revealed that probe **1** discerns variations in collagen formation and remodeling during the wound healing process, even allowing for quantification of such differences. *In vivo* studies showed that probe **1** illuminates newly forming and remodeling collagen in wounds of live mice. The probe migrated from the subcutaneous injection site to the wound, highlighting its ability to penetrate tissue and bind only in specific locations.

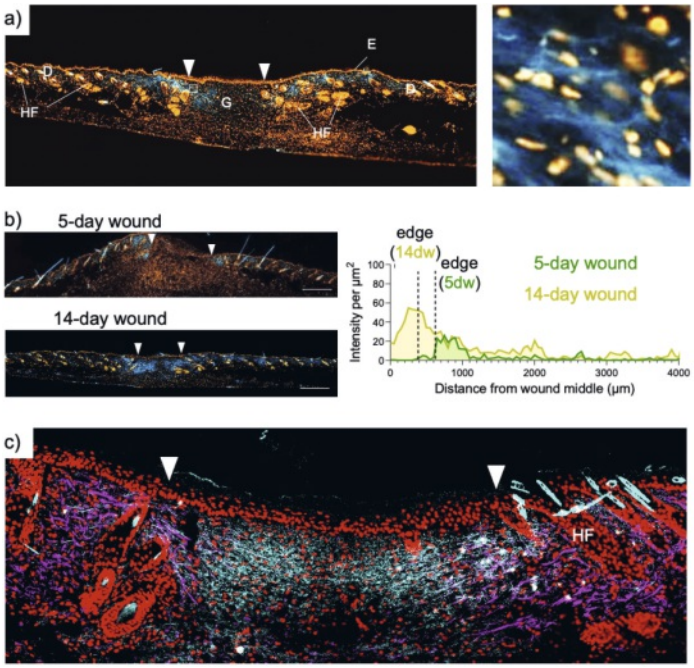

Fig. 3. Monitoring of LOX-initiated collagen cross-linking in wounds: a) Representative images of frozen sections from 14-day murine excisional wounds stained with probe **1** (blue; nuclei, red). White arrowheads indicate the wound margins, D: Dermis; E: Epidermis: G: Granulation tissue; HF: Hair follicle. b) Representative images of 5-day and 14-day murine excisional wounds stained with probe **1** (blue; nuclei, red). Comparative quantification of the spatial distribution of probe **1** staining in 5- *vs*. 14-day wounds. N = 4-5 wounds per group c) Representative image of a 14-day murine excisional skin wound stained with probe **1** collected using confocal microscopy coupled with multi-photon microscopy and second harmonic generation (SHG, magenta) to visualize fibrillar collagen (nuclei, red). Hair autofluorescence in blue. Reproduced and modified with permission from *Matrix Biology*.

The ensemble of *in situ* studies, *in vivo* animal models, and human wound samples underscored the selectivity and precision of **1** in identifying areas of active collagen formation/remodeling within and around the wound during the healing process. The results illustrate the utility of **1** as a tool to monitor, in real-time, collagen remodeling and progression of collagen formation during wound healing *in vivo*.

4. Conclusions and Outlook

The ability of probe **1** to map areas of active collagen formation and remodeling opens up exciting avenues for the use of **1** as a diagnostic tool to probe collagen remodeling dynamics in various physiological and pathological scenarios. We are currently expanding the tool kit of chemoselective collagen peptide LOX sensors, *e.g.* to sensors that operate in the near-infrared, and develop functional probes towards therapeutic applications.

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