Title: Targeting Collagen for Diagnostic Imaging and Therapeutic Delivery

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Keywords: Drug delivery, collagen binding molecules, collagen mimetic peptide, collagen hybridizing peptide, theranostics, collagen remodeling, denatured collagen, ECM remodeling

Abstract

As the most abundant protein in mammals and a major structural component in extracellular matrix, collagen holds a pivotal role in tissue development and maintaining the homeostasis of our body. Persistent disruption to the balance between collagen production and degradation can cause a variety of diseases, some of which can be fatal. Collagen remodeling can lead to either an overproduction of collagen which can cause excessive collagen accumulation in organs, common to fibrosis, or uncontrolled degradation of collagen seen in degenerative diseases such as arthritis. Therefore, the ability to monitor the state of collagen is crucial for determining the presence and progression of numerous diseases. This review discusses the implications of collagen remodeling and its detection methods with specific focus on targeting native collagens as well as denatured collagens. It aims to help researchers understand the pathobiology of collagen-related diseases and create novel collagen targeting therapeutics and imaging modalities for biomedical applications.

Introduction

Collagen is the most abundant protein in mammals and is a major component of extracellular matrix (ECM) providing a vital structural matrix for tissue development, maintenance, and regeneration [1]. The three main types of collagen, type I, II, and III, account for nearly 90% of the collagen found in the body; all of these types are fibrous and share a triple-helical molecular structure. Normal tissue development involves a dynamic collagen remodeling process, which includes both collagen production and degradation. An imbalance in these processes causes abnormalities in the structure and metabolism of collagen, leading to various pathological conditions, including fibrosis, arthritis, and cancer [2]. Thus, the ability to detect structural and metabolic anomalies in collagen would be significant in both diagnosing and treating related pathological conditions.

In this review, we highlight the importance of recognizing two different states of collagen—triple helical, intact collagen, and unfolded, denatured collagen (dn-collagen). Methods of detecting and targeting each of these collagens are also reviewed in order to show new approaches in utilizing collagen for delivery of drug molecules and imaging modalities.

Targeting and Monitoring Native Collagen

As the most abundant protein in humans, collagen constitutes approximately 25% of the total protein. It is the major structural component of the extracellular matrix (ECM) supporting cell proliferation, migration, and differentiation [3, 4]. Since collagen is present in the ECM of most tissues, it provides potential deposition sites for therapeutic agents. In addition, the detection of native collagen could be highly useful for monitoring progression and amelioration of diseases that are known to alter collagen remodeling activity such as fibrosis and arthritis.

A. Collagen binding peptides for enhancing growth factor efficacy and for modulating native collagen function

Growth factors (GF) are known to bind to the ECM for prolonged activity and timely function [5, 6]. ECM components, such as collagen acts as a natural repository that controls the presentation and release GFs. GF or GF-mimetics which can bind to collagen has the potential to improve *in vivo* stability and promote sustained release. Here we discuss collagen binding peptides (Table 1) that can mediate the attachment of GFs or other therapeutics moieties.

Many ECM-binding proteins contain specific domains that have affinity to collagen. Inspired by decorin, a collagen binding peptidoglycan was developed which contains a dermatan sulfate peptidoglycan (DS-GAG). DS-GAG utilized a peptide sequence derived from the collagen I platelet receptor [RRANAALKAGELYKSILYGC (SILY); Kd: 0.86 μ M] [7, 8]. The original sequence (RRANAALKAGELYKCILY; Kd: 10 nM) [9] has a higher binding affinity to collagen type I than the modified version, SILY, but the modification allowed dermatan sulfate to be conjugated to the terminus of the peptide. Similar to decorin which binds to collagen and inhibit lateral aggregation of collagen fibrils, DS-GAG modulated collagen fibril organization[8]. It is likely that the DS-GAG also had protective effects for collagens against MMP-mediated degradation by blocking MMP binding sites [10] [11]. In addition, DS facilitated cell proliferation by helping FGF receptors dimerize, since N-acetylgalactosamine (GalNAc) and iduronate (IdoA) can interact with FGFs (FGF-2 and FGF-10) and its receptor, FGFR2-IIIb [12, 13]. Further investigation of the original sequence of SILY revealed that only GELYKCILY was responsible for the collagen binding ability [14]. Goldberg research group conjugated collagen binding domain derived from decorin (residues 176-201, MIVIELGTNPLKSSGIENGAFQGMKK; Kd: 0.394 μ M) to a recombinant pig bone sialoprotein (BSP), P2S [15], which can bind to HA. The conjugate promoted mineralization of bone defects by binding to collagen and by inducing nucleation of HA [16]. Through mutagenic studies, glutamate (residue 180) was found to be critical for specific binding to collagen type I [17]. Recently, new decorin-based peptide that binds to type I collagen was developed. This peptide has a sequence of LRELHLNNN (Kd: 0.17 μ M) [18] and was found to increase the retention of molecules with a molecular weight of less than 2 kDa within type I collagen matrix (e.g., collagen gel). Upon matrix degradation in the biological system, controlled release of molecules was achieved. DARKSEVQK and KELNLVY, also derived from platelets, were found to specifically bind to type III collagen and inhibit type III collagen-induced platelet aggregation in a dose-dependent manner [19].

A collagen binding domain (CBD) WREPSFCALS, which was identified from von Willebrand Factor (vWF), exhibits low binding affinity (Kd: 100 μ M) for collagen type I [20].

Another CBD derived from the collagenase is TKKTLRT. Using the recombinant protein technique, Dai and coworkers fused TKKTLRT to various GFs *via* their C-termini, including brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), nerve growth factor β (NGF- β), basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), bone morphogenic protein 2 (BMP-2), neurotrophin 3 (NT3), and platelet-derived growth factor (PDGF). The TKKTLRT sequence restricted the GFs' diffusion and increased the tissue retention of the growth factors which led to enhanced tissue regeneration and wound repair. This sequence was found to promote vascularization and cellularization better than the WREPSFCALS because of its higher collagen binding affinity, which is presumably due to higher ratio of polar amino acids to non-polar amino acids [21]. The TKKTLRT sequence was even fused with single chain fragment variable (CBD-scFv) of cetuximab to target collagen rich carcinoma [22].

In addition to collagen specific domains derived from natural ECM binding proteins, there are other collagen targeting peptides that were identified using phage-display methods. A peptide WYRGRL that binds to $\alpha 1$ chain of type II collagen was discovered via phage-display and found to target articular cartilage [23-25]. Using this collagen binding sequence, Schultz and coworkers achieved targeted drug delivery of the cathepsin D inhibitor for the treatment of OA [24]. Three WYRGRL peptides and one cathepsin D inhibitor were tethered *via* a tetrapodal DOTAM [26] template, and the drug conjugate was administered intra-articularly to not only allow lipophilic drug molecules to penetrate joint compartments, but also to enhance the retention of inhibitor within cartilage through its multi-valent binding. Another collagen binding peptide, KLWVLPK, was discovered by phage-display and was found to specifically bind to collagen type IV. This peptide was attached to lipid-polymeric nanoparticles to target sites of balloon angioplasty [27]. It was also incorporated into a peptide amphiphile sequence in order to target areas of vascular intervention [28]. When conjugated to a β -sheet forming domain, self-assembled nanofibers were formed, which were shown to bind to exposed collagens on the injured blood vessels for up to two days. Besides the known slow clearance of nanofibers from circulation [29], the multivalent presentation of peptides also contributed to the increased binding to nanofibers.

Considering the importance of type I collagen in mediating homeostasis and biological development processes, the interactions of type I collagen with other extracellular matrix molecules and cell surface proteins have been mapped out [30-32]. For example, collagen has binding domains specific for fibronectin as well as integrins. Different types of glycoproteins and proteoglycans are involved in modulation of collagen fibrillogenesis and in cell attachment. More information about the interactions between collagen, ECM molecules and cells beyond the boundary of detecting and targeting of extracellular matrix molecules for therapeutic applications can be found in recently published review papers [33, 34]. These complex interactions provide a greater understanding about the relationship between ligand-binding sites and functional domains of type I collagen which will be highly useful in the design of collagen targeting molecules for therapeutic modulation of collagen function.

Collagen binding peptides	Source	Target	Kd (μ M)*	Application	Ref.
RRANAALKAGELYKSILYGC	Collagen I platelet receptor	Collagen type I	0.86	Modulate collagen fibrillogenesis and inhibit MMP-mediated collagen degradation	7,8
MIVIELGTNPLKSSGIENGAF QGMKK	Leucine-rich repeats (LRR) 4-5 of Decorin	Collagen type I	0.394	Induce collagen mineralization in bone defects	16
LRELHLNNN	Inner surface of LRR 10 of Decorin	Collagen type I	0.17	Promote controlled release of molecules ≥ 2 KDa	18
WREPSFCALS	Von Willebrand Factor	Collagen type I	100	Promote vascularization and cellularization	20, 21
TKKTLRT	Collagenase	Collagen type I	≤ 100	Promote vascularization and cellularization	21
WYRGRL	Phage display	Collagen type II	n.d.	Target articular cartilage	23-25
KLWVLPK	Phage display	Collagen type IV	n.d.	Target sites exposed collagen in blood vessels damaged by balloon angioplasty	27

*n.d. = not determined

Table 1. Collagen binding peptides and their applications.

B. Detection of collagen accumulation in fibrosis

Fibrosis is a common pathological outcome of many chronic inflammatory diseases affecting nearly all tissues in the body [35, 36]. Fibrosis is signified by the excessive accumulation of ECM components, particularly type I collagen, in and around the damaged tissues (**Fig. 1A**) [37, 38]. Tissue repair in response to injury or irritation can progress into an irreversible fibrotic response if the tissue injury is severe or repetitive, leading to permanent scarring, organ malfunction and, ultimately death, as seen in end-stage liver disease [39], kidney disease [40], idiopathic pulmonary fibrosis (IPF) [41] and heart failure [42]. In addition, fibrosis is a major pathological feature of many chronic autoimmune diseases, and is a common outcome of rejection of organ transplant and medical implants (**Fig. 1A**) [43, 44].

Figure 1. (A) Progression of fibrotic tissue formation caused by chronic inflammation or by incompatibility of biomaterial implants; **(B)** EP-3533, a Gd-bearing collagen-targeted MRI contrast agent (reprinted with permission from Ref. 55); **(C)** *In vivo* molecular imaging of pulmonary (top panel) and liver fibrosis (bottom panel) using EP-3533 (reprinted with permission from Ref. 59 and 60).

Currently, there is no single test that can diagnose fibrosis, and clinical methods for monitoring the disease progression are very limited. There is no established treatment for liver fibrosis [45], and only a few drugs were approved for treatment of other fibrotic diseases (e.g., both pirfenidone and nintedanib were approved for the treatment of IPF) [46-48]. Biopsy remains the most important, and often the only method for fibrosis detection and diagnosis (e.g., in liver fibrosis); however, biopsy is not suited for routine screening/monitoring because of its cost, and associated morbidity and mortality (rate between 0.01-0.1% in liver [49]), as well as inaccuracy caused by sampling variation. Therefore, there is an unmet need for accurate and non-invasive methods to detect fibrosis. Many imaging methods based on the morphologic or mechanical change of the fibrotic tissue in the late stage are under development, such as high resolution computed tomography (HRCT), magnetic resonance imaging (MRI) [50], ultrasonography (US) [51], as well as biochemical assays for identifying protein degradation fragments using ELISA [52, 53]. HRCT can provide cross-sectional images of the fibrotic tissues with a quality that depends on the radiation exposure [54]. Unfortunately, HRCT can induce cancer and genetic

mutations. MRI offers deep tissue penetration and high spatial resolution, however, its use is limited in diagnosing liver fibrosis as most contrast agents cannot distinguish the different stages of liver fibrosis [53]. US is typically used as a diagnostic tool in chest diseases, and as a guide for transthoracic needle biopsies [51]. Biochemical assays are non-invasive and suitable for high-throughput screening either in preclinical or clinical settings for estimating the safety and efficacy of certain therapies [52, 53], but they are neither site-specific nor disease-specific [55]. Scientists and engineers are addressing limitations of early stage detection (e.g., for MRI [56]) and inadequate correlation between the disease and serum markers [52].

Molecular imaging using a specific probe that targets excessive collagen is one appealing strategy for detecting fibrosis in its early stage when excess collagen molecules have been deposited in the tissue without apparent changes in the anatomy of the tissue. Among several collagen binding molecules that were tested [57, 58], EP-3533 (**Fig. 1B**) which employs a type I collagen-specific peptide (K_d : $1.8 \pm 1.0 \mu\text{M}$) has shown some promise in imaging fibrosis [56]. Because the concentration of collagen can increase up to 10-folds during fibrosis [59], EP-3533 was able to differentiate fibrotic tissues from the healthy ones. A combination of intensity strength of the contrast agent and Ultra-short Echo Time Magnetic Resonance (UTE-MR) allowed early-stage visualization of fibrotic tissue in the lungs and monitoring of the disease progression. The cyclic conformation (achieved by disulfide bridge formation) was responsible for the tight collagen binding. This agent was used to successfully visualize type I collagen in pulmonary [60] and liver fibrosis (**Fig. 1C**) [53, 61]. In addition, EP-3533 was conjugated to high-density lipoprotein nanoparticles to monitor compositional changes in atherosclerotic plaque regression [62].

CNA35, a 35 kDa peptide derived from an adhesion protein of *Staphylococcus aureus*, has moderate affinity to type I collagen ($K_d \sim 0.5 \mu\text{M}$) [63] and is frequently employed for collagen imaging [64-66]. For example, in vivo application of CNA35 allowed labeling [66] and imaging [67] of atherosclerotic plaques. By binding directly to both individual fibrillar and non-fibrillar collagens, CNA35 allows for detailed visualizations of different structural hierarchies of collagen organization for both early and mature collagens [63, 64].

C. Functional assemblies based on collagen binding molecules

Multivalent design is an effective way to enhance the affinity of collagen binding molecules. For example, an AB_5 dendritic wedge architecture (**Figure 2A**) was employed to enhance the binding affinity of the HVWMQAP which binds to type I collagen [68]. The dendritic display demonstrated a 100-fold improvement in collagen affinity compared to monovalent HVWMQAP. Significant improvement in the binding of CNA35 to collagen was achieved by employing micelle architecture (**Figure 2B**) [69]. By varying the ratio of CNA35 to lipid, different amounts of protein could be loaded in micelles. A fluorescent binding experiment showed that the most significant binding was observed with micelles having 5 and 17 proteins per micelle. A multivalent CNA35 micelle was also reported to target collagen type I, II, III, (fiber form) and IV (network-like structure).

Functionalization of nanoparticle surfaces with collagen binding molecules can lead to collagen-targeted drug delivery or improved molecular imaging of exposed collagen matrix *in vivo*. Targeting exposed collagen, especially in injured blood vessel, is particularly

important due to the fact that cardiovascular disease continues to be the leading cause of death worldwide [70]. Vascular intervention to treat the disease is also challenged by high failure rates from restenosis [71]. In addition, exposed collagen matrix is associated with angiogenesis in tumor formation as well as other angiogenesis-dependent diseases [72]. Langer and coworkers reported micelle-based paclitaxel delivery system that display HWGSLRA on micelle surface as “hooks” for binding to collagen (**Figure 2C**) [27]. Also, peptide amphiphiles can self-assemble into nanofibers (**Figure 2D**, top) or nanospheres (**Figure 2D**, bottom) which display high density of collagen binding peptides on their surfaces [28]. It was shown that nanofibers have preferential binding to injured vessels and are cleared at a slower rate relative to nanospheres of similar dimensions. Conjugation of peptide amphiphile with fluorescent labels provided molecular imaging of the injured tissues.

Figure 2. Multivalent display of collagen-targeting moiety: **(A)** HVWMQAP presented on AB₅ dendritic wedge (in pink; reprinted with permission from Ref. 68), **(B)** CNA35 micelle (reprinted with permission from Ref. 69), **(C)** HWGSLRA Nanoburrs (reprinted with permission from Ref. 27), **(D)** Peptide amphiphile nanostructures (reprinted with permission from Ref. 28).

Targeting and Monitoring Denatured Collagen (dn-collagen)

Collagen degradation is part of normal tissue development and maintenance; however, excessive collagen degradation is closely associated with a wide variety of diseases and pathological conditions. In cancer, collagen degradation caused by high MMP activity is responsible for angiogenesis and tumor progression [73-75]. In atherosclerosis, the thinning and weakening of the fibrous collagen cap due to MMP degradation renders the atherosclerotic plaques susceptible to rupture which can cause acute myocardial infarction and sudden cardiac death [76]. Degradation of type II collagen, the predominant protein in cartilage, is the key pathogenesis step of osteoarthritis [77, 78]. During persistent liver inflammation and injury, the hepatic stellate cells express MMPs to degrade normal liver matrix [79]. These examples highlight the pivotal role of collagen degradation in some of the

most prevalent and/or life-threatening diseases in current human population. (A list of diseases associated with collagen remodeling is found in **Table 2**).

Since the dn-collagen is present at the disease sites in all of the ailments listed in **Table 2**, it can be targeted similar to intact collagen for the purpose of imaging and drug delivery. The field of targeting dn-collagen is in its infancy; so far most targeting strategies are focused on MMP activities [80], or on targeting markers that signal collagen degradation [80]. Enzymatically digested collagen fragments have been used to develop several types of antibodies. Until recently, these antibodies were the only molecules that can directly target dn-collagen. Recently, researchers have discovered that collagen hybridizing peptide (CHP), also known as collagen mimetic peptides (CMP) can specifically target denatured collagen strands. CHP can recognize and hybridize with dn-collagen by re-forming the collagen triple helix [82]. Targeting dn-collagen is still a relatively new field, but it holds significant potential in advancing therapeutics and diagnostics for numerous human diseases (**Table 2**).

A. Collagen degradation

Collagen degradation can proceed either on the inside or outside the cells. Intracellular degradation occurs either in the lysosome or in the cisternae of the Golgi apparatus [83]. However, for the purpose of targeting and imaging disease, extracellular degradation is more relevant. Different types of MMPs or collagenases cleave collagen into asymmetrical fragments, which are susceptible to nonspecific proteolytic cleavage [84]. Proteolytic degradation unravels the triple helical structure of the native collagen and exposes additional bioactive sites that enhance cell attachment, migration, and proliferation [85]. Importantly, collagen cleavage also weakens the ECM leading to further degradation and many symptoms seen in diseases involving dn-collagen [86]. Due to the difficulty of directly measuring dn-collagen, MMP activity is often used as a surrogate to detect collagen degradation. Various methods have been developed to detect MMP activity, including reverse transcription polymerization chain reaction (RT-PCR) [87], ELISA assay, and zymography [88, 89]. There are also methods based on fluorescence measurements that employ FRET triple helical peptide (fTHP), and gelatin conjugated with fluorescein isothiocyanate (FITC) or 2-methoxy-2,4-diphenyl-3(2H)-

Distribution	Diseases and medical research areas
heart	<i>myocardial infarct</i>
blood vessels	<i>atherosclerotic plaques, blood vessel injury and failure (stroke)</i>
cartilage	<i>osteoarthritis, rheumatoid arthritis</i>
bone	<i>osteoporosis, bone fracture/healing, Marfan syndrome</i>
ligament & tendon	<i>mechanical injury, sports medicine, e.g., rotator cuff tear</i>
skin	<i>skin damage (photo, aging), skin cancer</i>
eye	<i>cornea injury & infection, corneal healing after LASIK, keratoconus, wet AMD</i>
lung	<i>lung inflammation, pulmonary fibrosis</i>
liver	<i>liver fibrosis, cirrhosis</i>
kidney	<i>nephritis, kidney fibrosis</i>
uterus	<i>uterine fibroid</i>
embryo	<i>ECM developmental biology, heritable connective tissue disorders</i>
teeth	<i>oral inflammation</i>
uterus	<i>uterine fibroid</i>
others	<i>cancer progression & metastasis, foreign body response to implants</i>

Table 2. Diseases associated with high level of collagen degradation and remodeling, and their location in the body.

furanone (MDPF), as well as colorimetric assay using horseradish peroxidase and biotinylated gelatin [90, 91]. Recently, our research group discovered that the dn-collagen strands can form triple helices with collagen mimetic peptides (CMP) [82].

B. Disease detection and therapeutics associated with collagen degradation

The ability to detect early stages of collagen remodeling is crucial for understanding the disease mechanism, prevention of the progression of disease, and evaluating the efficacy of available treatments. X-ray radiography, bone scan, magnetic resonance imaging, computed tomography, and histomorphometric analysis are considered gold-standard methods to measure the quality of bone and cartilage. These methods, however, have limitations in terms of sensitivity and reproducibility. Moreover, they lack the ability to detect diseases in their early stages. Therefore, there is a need for reliable methods with which early detection of changes in bone and cartilage quality can be measured with high fidelity.

There is a wealth of research on identifying biochemical markers associated with degradation of bone and cartilage [52, 55, 92-93]. In general, these biochemical markers, or neoepitopes, are by-products of enzymatic processes which are involved in the formation and degradation of bone and cartilage. Detection of these biomarkers in biochemical assays is usually done by measuring the level of the neoepitopes in body fluids, such as serum, plasma, synovial fluid, and urine [92]. There are several methods to detect these biomarkers including ELISA, radioimmunoassay (RIA), electrochemical luminescence, and HPLC analysis. Two notable epitopes for analyzing bone quality are serum C-telopeptide of type I collagen (CTX-I) and serum N-propeptide of type I collagen (PINP), which correlates with bone resorption and bone formation, respectively [55]. In contrast, cartilage quality can be measured by detecting the level of serum C-telopeptide of type II collagen (CTX-II). These are selected as reference markers in clinical settings due to their high sensitivity and reproducibility, as well as low cross-reactivity with other biomolecules. Nevertheless, full analysis of disease progression and selection of optimal treatment option can be best accomplished by a combination of imaging and biochemical techniques.

To date, development of therapeutics against bone diseases with high level of dn-collagen has largely focused on inhibiting the function of osteoclasts. One widely-used class of therapeutics for osteoporosis is bisphosphonates [94]. As anti-resorptive agents, bisphosphonates binds to bone minerals followed by uptake and apoptosis of osteoclasts [95]. Nitrogen-containing bisphosphonates, the most advanced drug in its class, disrupt the maturation of osteoclasts by inhibiting the function of Farnesyl Pyrophosphate Synthase (FPPS) [96]. Denosumab, a monoclonal antibody (mAb) which was recently approved by FDA for treating post-menopausal osteoporosis, binds to RANK-L and inhibits maturation and function of osteoclasts [97]. In 2010, FDA also approved denosumab for bone metastasis treatment. Certolizumab (phase III) is a PEGylated anti-TNF α that is approved for treatment of rheumatoid arthritis and Crohn's disease [98].

In conclusion, there are therapeutics developed for the treatment of diseases associated with high activity of collagen degradation; however, other than the bone therapy, discovery of drugs for such diseases has been hampered largely by the inability to verify the therapeutic efficacy in ECM remodeling. For example, there is no definitive bone remodeling markers to interpret clinical outcomes or treatment responses in patients with bone metastases [99]. Development of new biomarkers for collagen degradation would be

important not only to support the drug discovery processes, but also for detecting and managing disease in patients.

C. Antibody and phage display in discovering dn-collagen targeting molecules

Since the presence of dn-collagen is a symptom of many degenerative diseases, it is highly useful to have molecules that can recognize and specifically bind to the dn-collagen for the purposes of detecting disease or drug delivery. Using thermally denatured type I and IV collagens, in subtractive immunization technique, mAbs HUIV26 and HUI77 were developed and were found to specifically recognize enzymatically digested and denatured collagen [100]. HUIV26 specifically recognized thermally dn-collagen type IV, whereas HUI77 bound to thermally dn-collagens of type I, II, III, IV, and V with a range of affinities. In type IV collagen, the domain responsible for inducing angiogenesis exists as a hidden interaction site (cryptic site) that becomes exposed only after proteolytic degradation or denaturation of collagen triple helix. HUIV26 was reported to target such cryptic sites and was therefore used to inhibit tumor growth [101, 102].

D93 (K_d: 6.5 μ M), a humanized form of HUI77-derived from monoclonal IgG1 antibody, recognizes dn-collagen type IV by binding to a cryptic site of MMP-processed collagen [85]. The cryptic site was found to mediate adhesion, migration, and proliferation of tumor and endothelial cells [101, 103]. The cryptic site, located on the α 1(IV) chain, contained a peptide sequence of PGAKGLPGPPGPPGPY (P₁₃₃₇-Y₁₃₅₂). Binding assays revealed a sequence of OGAKGLPG-POGPOGPY to be the target of D93 binding. Under the name of TRC093 (developed by Tracoon Pharmaceuticals Inc.), D93 completed its phase I clinical trial in 2010.

In search for quantitative immunoassays for type II collagen, extensive screenings were performed against structurally different antigens of type II collagen [104]. Screening utilizing an ELISA assay yielded specific monoclonal antibodies E1E5 (IC₅₀: 400 ng/mL) and E4A11 (IC₅₀: 500 ng/mL) that interact with dn-collagen type II by binding to an epitope on the α 1 chain [105]. These mAbs can detect the presence of dn-collagen type II with a minimum concentration of 20 ng/mL.

The peptide sequence TLTYTWS which was discovered via phage-display was found to specifically bind to a cryptic site on MMP-2 processed type IV collagen [106]. This cryptic site was reported to serve as an angiogenic signal for endothelial cells. By binding to the cryptic site, TLTYTWS was found to reduce endothelial differentiation *in vitro* and inhibit angiogenesis *in vivo*. Nonetheless, the binding mechanism of TLTYTWS to the cryptic site requires further investigation.

Dn-collagen targeting molecules	Discovery method	Target	Kd (μM)*	Application	Ref.
mAb HUIV26	Subtractive immunization	Thermally dn-collagen type IV	n.d.	Inhibit angiogenesis and tumor growth	100-102
mAb HUI77	Subtractive immunization	Thermally dn-collagen type I - V	n.d.		100-102
D93	HUI77-derived humanized mAb	MMP-processed dn-collagen type IV	6.5	Inhibit tumor growth	85
mAb E1E5	Quantitative immunoassay	Dn-collagen type II	n.d.	Detect dn-collagen type II	105
mAb E4A11	Quantitative immunoassay	Dn-collagen type II	n.d.	Detect dn-collagen type II	105
TLTYTWS	Phage display	MMP2-processed collagen type IV	n.d.	Reduce endothelial differentiation and inhibit angiogenesis	106

*n.d. = not determined

Table 3. Dn-collagen targeting molecules and their applications.

Overall, dn-collagen targeting molecules selected by library approach have revealed new knowledge on how changes in collagen structure (which reveal cryptic sites) affect collagen's biological function. In addition, these molecules are expected to facilitate development of imaging agents and therapeutics that can specifically target dn-collagens.

D. Collagen Hybridizing Peptide

Collagen hybridizing peptide (CHP) is a rationally designed synthetic peptide that has strong propensity to fold into triple helix structure of collagen and is able to target dn-collagen strands both *in vitro* and *in vivo*. It is also known as collagen mimetic peptide (CMP) due to its resemblance to natural triple helical collagen. It is mainly composed of 6-10 repeats of triple helicogenic trimeric GlyProPro or GlyProHyp sequence. Since denatured collagen has unfolded single collagen strands, and since CHP has a strong propensity to fold into triple helix, CHP is able to hybridize to denatured collagen strands with high strength and specificity. This is similar to DNA fragments binding to complementary DNA strands [107].

Since CHPs self-assemble into homotrimers which have little driving force for collagen binding, the peptides need to be subjected to a condition that favor monomeric state before it can be allowed to bind to denatured collagen strands. In this regards, our

research group has developed two types of CHPs, one based on simple GPO sequence that needs to be heated to create single strands, and a photo-caged version which only hybridizes with dn-collagen after UV irradiation. The latter peptide is made of the same GPO sequence as the former but contains a photo-cleavable nitrobenzyl group attached to the single central glycine which prevents triple helical folding. (**Fig. 3A**) [108]. Heat inducible CHP can be used when the moiety (e.g. imaging modality or drug molecules) to be conjugate to the CHP is sensitive to light, whereas photo-inducible, caged CHP can be used when those moieties are heat sensitive (e.g. protein). Conjugating imaging agents can turn this peptide into diagnostic imaging agents, and attaching drug molecules can turn it into targeted drug delivery system [108-110].

The affinity of CHP to denatured collagen is driven by the availability of single strand collagen like chain (**Fig. 3B, C**). CHP can not only bind to fibrillar type (type I and II) of collagen, but also network-like collagen (type IV) as long as the triple helical domain of these collagens are denatured by proteolytic or other physico-chemical activity (e.g. heat, denaturants, mechanical stress). In addition, since CHP is hydrophilic and contains no charged amino acid, it is highly inert and shows almost no non-specific binding to other biomolecules (**Fig. 3D**) [109]. Most importantly, triple helical protein structure is an extremely rare supersecondary protein structure that is hard to find in proteins other than collagen [111]. For this reason, systemic administration of CHP conjugated with a near-IR dye demonstrated remarkably specific imaging of tumor as well as high bone remodeling activity in transgenic mouse with Marfan syndrome (**Fig. 3E**).

Therapeutic application of CHP was demonstrated by Raines and coworkers where (PPG)₇ was conjugated to substance P (SubP), a neuropeptide involved in wound healing [110]. Similar to peptides based on GPO sequence, the (PPG)₇ can hybridize with damaged collagen molecules at the wound site, allowing the SubP which is conjugated (PPG)₇ to be anchored in the wound bed and prevent scar tissue formation. In addition, a cancer-targeting delivery agent was developed by displaying (PPG)₇ on the surface of M13 bacteriophage. [112]. This work took advantage of the fact that cancerous tissues contain high level of denatured collagens due to fast tissue remodeling [113]. The bacteriophage displaying CHP had a 16-fold higher binding affinity to dn-collagen than the wild bacteriophage. In addition, CHP comprised of GPP sequence was used for gene delivery. By incorporating GEKGER peptide sequence into the middle of the CHP sequence [(GPP)₃GPRGEKGERGPR(GPP)₃GPCCG], Urello and coworkers demonstrated that the CHP could engage the $\alpha 2\beta 1$ integrin for gene internalization into the cell [114].

Figure 3. (A) Structures of fluorescently labeled CHP and fluorescently labeled and nitrobenzyl caged CHP, and schematic illustration of the two approaches (heat- vs photo-triggered) of generating single strand CHPs for dn-collagen hybridization (reprinted with permission from Ref. 109), (B) Schematics of CHP hybridizing to dn-collagen, (C) Specific binding of caged-CHP to gelatin after UV irradiation (reprinted with permission from Ref. 108), (D) SDS-PAGE loaded with collagen type I, II, IV, fibronectin, laminin, and endothelial cell lysates (2 μ g of each protein or lysates), and stained with CF-CHP and CB (reprinted with permission from Ref. 109), (E) Systemic injection of near-IR fluorophore conjugated CHP into transgenic mouse with Marfan syndrome (reprinted with permission from Ref. 108).

The CHP was also used to enhance the bioactivity of collagen-based tissue scaffolds [115-117]. Our research group has conjugated CHP, (ProHypGly)₉ to a proangiogenic peptide known as QK (KLTWQELYQLKYKGI) which is derived from VEGF. The QK-CHP enhanced early signs of angiogenic activity in HUVECs in scaffolds comprised of both gelatin and collagen which mimic the ECM of healing tissues [118, 119] suggesting that the QK-CHP can potentially be used to promote wound healing response of injured tissues.

Ultimately, CHP, with its simplicity and robust modularity, has the potential to facilitate the development of novel therapeutics targeting dn-collagen. Further discussion on assemblies and functionalization of CHP can be found in cited articles [82, 107, 120-122].

Conclusion

Since collagen is the major component of the ECM, providing structural support and key factors for tissue proliferation and regeneration, abnormal collagen remodeling activity can lead to conditions that result in excessive collagen build-up or collagen degradation, as in the case of fibrosis and arthritis, respectively. To understand, and ultimately remediate such pathologic conditions, researchers have focused on identifying marker molecules that can recognize and bind to either the intact collagens (of accumulated collagen) or the dn-collagen (of degraded collagen). Through the use of these biomarkers, a greater understanding of the disease states have been gathered, which in turn, is providing more effective steps in preventing the progression of the disease. In addition, these biological tools can be utilized in both delivering therapeutics and also evaluating their efficacy. They can be an excellent, so called, “theranostics” for common pathologic conditions with high collagen remodeling activity. Although there are many specific biological markers for intact collagen, there are only a few ones specific for dn-collagen, including the newly discovered CHP which is a small peptide that can bind to denatured collagen with high specificity. More efforts are needed from the research community to discover new biomarkers for denatured collagen and at the same time, develop existing markers into useful diagnostics and therapeutics that can address human disease associated with excess collagen degradation.

Acknowledgements

Development of collagen hybridizing peptide discussed in this paper was supported by grants from NIAMS/NIH (R01-AR060484 and R21-AR065124) and DOD (W81XWH-12-1-0555).

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