Investigating Interactions of Collagen Hybridizing Peptides with Fibrillar Collagen

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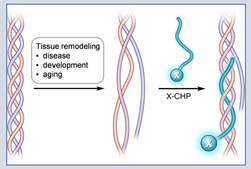
INTRODUCTION

Collagen is the major structural component of mammalian tissues and plays a critical role in cell proliferation, migration and differentiation [1]. This unique triple-helical protein undergoes extensive proteolytic remodeling during many life-threatening diseases including fibrosis, myocardial infarction, and cancer [1,2]. Detection of excessive degradation of collagen is an important marker of tissue damage, which is essential to diagnosis and treatment of disease [2]. Conventional collagen detection and targeting tools are difficult to use [1]; an alternative method of detection generating great interest is the use of fluorescent peptides to detect damaged collagen.

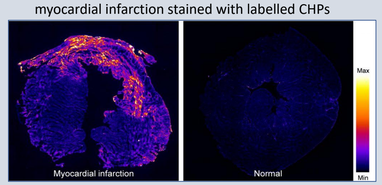
Collagen Hybridizing Peptide (CHP) is a synthetic peptide that specifically binds to denatured collagen. CHPs have a repeating Gly-X-Y sequence, the same structural motif found in natural collagen, enabling a strong capability to hybridize with unfolded collagen chains [2]. Labelled with fluorophores or other image-compatible markers, CHPs enable direct localization and quantification of collagen damage in tissues. However, much remains to be learned about the mechanism of interaction of CHPs with fibrillar collagen.

CHP integration into collagen strands



[1]

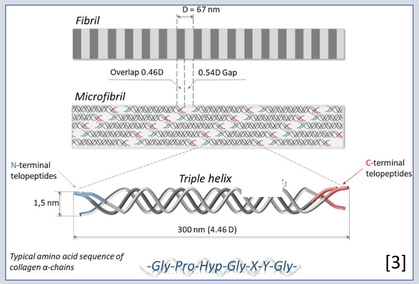
Fluorescence image of a mouse heart 7 days post myocardial infarction stained with labelled CHPs



[1]

FIBRIL FORMATION AND CHPs

- Collagen molecules self-assemble in vitro under optimal conditions to form banded fibrils [3].
- Rat-tail type I collagen molecules (Cultrex) are stored in acetic acid. Transfer of collagen into neutral buffer initiates the self-assembly of collagen fibrils.



[3]

CHP effects on collagen fibril formation are not well understood.

MEASURING FIBRIL FORMATION

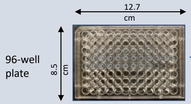
- Microplate readers can quantify collagen fibril growth by reading optical density (OD) - a measure of turbidity (light scattering).
- Fibril formation is initiated by diluting collagen molecules in acidic solution into fibril-forming buffer (273 mM NaCl, 5 mM KCl, 42 mM Na_2HPO_4 and 9 mM KH_2PO_4 pH 6.95) to a concentration of 0.5 mg/mL.

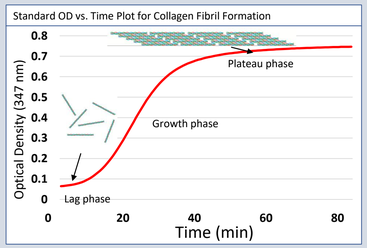
Synergy HTX Plate Reader

Agilent (2022)

Optical Density Reading

Agilent (2022)



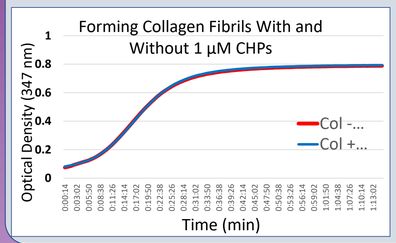


- Following nucleation during the lag phase, there is a rapid increase in optical density, signifying the lateral and longitudinal self-assembly of collagen.
- The plateau phase is observed when fibrils have fully formed. The reaction goes to completion; there are no monomers in solution [4].

FIBRIL FORMATION RESULTS

- While CHPs bind to damaged collagen fibrils, there is the question of whether they can affect the formation of new collagen fibrils.

Do CHPs induce collagen fibril growth?



- OD readings were taken at 25°C to monitor the assembly kinetics of fibril formation with and without CHPs.
- OD readings show that collagen assembles into fibrils with the same kinetics whether CHPs are present or absent, suggesting that CHPs do not affect fibril formation.

CHP BINDING MECHANISM

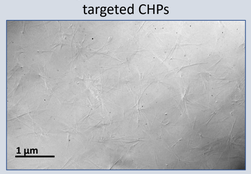
How do CHPs bind to collagen fibrils?

- To investigate the importance of the peptide sequence for binding to collagen, we compared targeted CHPs containing glycine-proline-hydroxyproline repeats $(GPO)_9$ (F-CHP, 3Helix) to scrambled control peptides ($G_9P_9O_9$) both labelled with 5-carboxyfluorescein.
- Collagen molecules were mixed with targeted or scrambled CHPs then assembled at 1 mg/mL into fibrils in 40 µL chambers.

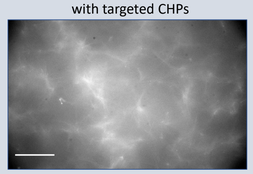
Glass slide with chamber

- Fibrils were washed with buffer then imaged with differential interference contrast (DIC) and fluorescence microscopy.

DIC image of fibrils formed with targeted CHPs

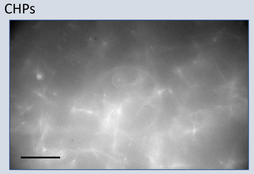


Fluorescence image of fibrils formed with targeted CHPs

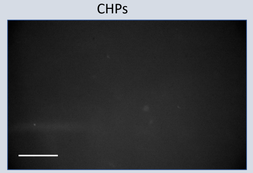


Comparing Fluorescence Images

Fibrils formed with targeted CHPs

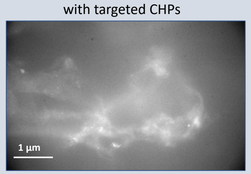


Fibrils formed with scrambled control CHPs

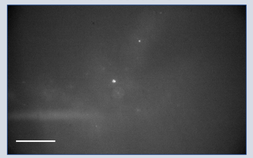


- Under the same imaging and editing parameters, there are significant qualitative differences in fluorescence intensities between targeted and control CHPs, suggesting that the peptide sequence with its helical structure is important for binding.
- Another method to investigate the binding mechanism is staining fully formed fibrils with CHPs. Fluorescence intensity is greater for fibrils stained with targeted CHPs, once again demonstrating the importance of the glycine-proline-hydroxyproline sequence for binding.

Fibrils formed then stained with targeted CHPs



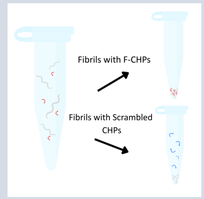
Fibrils formed then stained with scrambled control CHPs



QUANTIFYING CHP BINDING

Can we quantify the binding of F-CHPs to Fibrils?

- Fluorescence microscopy shows that CHPs bind to lab-grown collagen fibrils. To quantify this interaction, we developed a new centrifugation assay with the intention of quantifying the fraction of CHPs bound to collagen fibrils.
- We hypothesised unbound CHPs would remain in the supernatant after centrifuging the fibril samples.
- Fibrils were formed in Eppendorf tubes first before adding either F-CHPs or scrambled CHP to incubate overnight.
- After incubation, the fibrils were centrifuged to form pellets separated from the liquid supernatant.
- Once separated, the supernatant was measured to see the reduction in fluorescence signal caused by CHP associating with the pellet, referred to as depletion.



Hypothesis of the centrifugation assay.

Results of Centrifugation Assay

- This assay presented another way to test peptide sequence for binding to collagen, using the target F-CHPs and scrambled control.

CHP type (Mean: n=3)	Supernatant concentration (nM)	Depleted (%)
F-CHP (Mean)	**90.6**	**9.7**
Scrambled CHP (Mean)	**90.7**	**9.5**

- Depletion was calculated by subtracting the supernatant concentration from the starting concentration of CHPs (100nM).
- In both supernatant types, similar depletion levels were observed. This suggests the peptides are being trapped within the fibril network.

Limitations

- Reconstituted fibrils are known to have a different stability than native formed fibrils due to crosslinks in the latter.
- Fibrils imaged using this assay would have extra fluorescence from trapped CHPs in the fibrillar pellets.
  - Further washing to remove trapped, unbound peptides from the fibrillar network is needed.

CONCLUSIONS AND FUTURE DIRECTIONS

This preliminary investigation of the interactions between collagen hybridizing peptides and collagen fibrils provides the first steps to understanding the effects of CHPs on collagen fibril growth and remodeling. Through optical density experiments, our findings suggest that CHPs have no effect on collagen fibril formation. DIC and fluorescence images demonstrate that the peptide sequence is important for binding of CHPs to fibrillar collagen. Another quantifying assay is currently being developed to improve removal of unbound peptides. This will allow for comparison of binding to the collagen fibrils between different CHP types without background fluorescence. With CHPs being explored for therapeutics and cosmetic applications, the deeper understanding of the binding mechanism will enable development of novel approaches.

ACKNOWLEDGEMENTS

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