Detection of substrate binding of a collagen-specific molecular chaperone HSP47 in solution using fluorescence correlation spectroscopy

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Keywords

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Abstract

Heat shock protein 47 kDa (HSP47), an ER-resident and collagen-specific molecular chaperone, recognizes collagenous hydrophobic amino acid sequences (Gly-Pro-Hyp) and assists in secretion of correctly folded collagen. Elevated collagen production is correlated with HSP47 expression in various diseases, including fibrosis and keloid. HSP47 knockdown ameliorates liver fibrosis by inhibiting collagen secretion, and inhibition of the interaction of HSP47 with procollagen also prevents collagen secretion. Therefore, a high-throughput system for screening of drugs capable of inhibiting the interaction between HSP47 and collagen would aid the development of novel therapies for fibrotic diseases. In this study, we established a straightforward method for rapidly and quantitatively measuring the interaction between HSP47 and collagen in solution using fluorescence correlation spectroscopy (FCS). The diffusion rate of HSP47 labeled with Alexa Fluor 488 (HSP47-AF), a green fluorescent dye, decreased upon addition of type I or III collagen, whereas that of dye-labeled protein disulfide isomerase (PDI) or bovine serum albumin (BSA) did not, indicating that specific binding of HSP47 to collagen could be detected using FCS. Using this method, we calculated the dissociation constant of the interaction between HSP47 and collagen. The binding ratio between HSP47-AF and collagen did not change in the presence of sodium chloride, confirming that the interaction was hydrophobic in nature. In addition, we observed dissociation of collagen from HSP47 at low pH and re-association after recovery to neutral pH. These observations indicate that this system is appropriate for detecting the interaction between HSP47 and collagen, and could be applied to high-throughput screening for drugs capable of suppressing and/or curing fibrosis.

Introduction

HSP47, a heat shock protein with molecular mass of 47 kDa, is an ER-resident and collagen-specific molecular chaperone [1]. HSP47 belongs to the serine protease inhibitor (serpin) superfamily, and is also known as Serpin H1. It binds to collagen and promotes its triple-helical folding in the ER. HSP47 predominantly recognizes collagenous amino acid sequences, i.e., glycine-Xaa-Yaa (Gly-Xaa-Yaa) repeats, in which Xaa and Yaa are often proline (Pro) and hydroxyproline (Hyp), respectively. Hydroxylation of Pro residues is required to stabilize the triple-helical structure of collagen [2]. Since the surface of the collagen triple-helix region becomes hydrophobic, HSP47 must bind to the triple-helical form of procollagen to prevent its lateral aggregation in the ER [3]. The amino acid residues in HSP47 responsible for hydrophobic collagen binding have been identified [4].

The binding of HSP47 to collagen has been analyzed using several methods, including biochemical pull-down assay including immunoprecipitation [5], surface plasmon resonance (SPR) [6], fluorescence quenching measurement [7], Förster/fluorescence resonance energy transfer (FRET) [8], and bimolecular fluorescence complementation (BiFC) [8]. Those studies revealed that HSP47 binds to purified mature type I–V collagen, gelatin (denatured collagen), and peptides containing Gly-Pro-Pro repeats. The equilibrium dissociation constants (K_d) between HSP47 and collagen were determined using SPR and fluorescence quenching analysis [6, 7].

HSP47 is translocated into the ER *via* a signal sequence at the N-terminus and recycled from the Golgi to the ER *via* an ER-retention signal sequence (RDEL) at the C-terminus. During this cycle, dissociation between HSP47 and collagen at low pH promotes both the retention of HSP47 in the ER and appropriate trafficking of triple-helical collagen [1, 9].

Various fibrotic diseases, including liver cirrhosis and idiopathic pulmonary fibrosis, are characterized by abnormal chronic collagen accumulation in tissues [9-11]. Accordingly, it has been hypothesized that suppression of HSP47 activity would slow the progression of these diseases. Indeed, in mice, knockdown of HSP47 ameliorates liver fibrosis by inhibiting collagen secretion [12]. Therefore, the establishment of effective and high-throughput screening methods for discovering compounds capable of suppressing HSP47 would be clinically valuable.

Here, we demonstrate that the interaction between HSP47 and collagen in solution can be quantitatively and conveniently detected using fluorescence correlation spectroscopy (FCS) [13, 14]. This FCS system could be applied to high-throughput screening of reagents to identify molecules that inhibit the interaction between HSP47 and collagen, and could thus slow or curing of fibrotic disease.

Materials and Methods

Fluorescence labeling of proteins

Purified HSP47 in which Cys138 was replaced with alanine (C138A) as described previously [15], human protein disulfide isomerase (PDI) as described previously [16], and bovine serum albumin (BSA; purchased from Sigma-Aldrich, St. Louis, MO, USA) were incubated with Alexa Fluor 488 carboxylic acid and succinimidyl ester (A-20000, Thermo Fisher Scientific, Waltham, MA, USA), and covalently fluorescently labeled. For the labeling reactions, the molar ratios of protein to dye were 1:20, 1:10, and 1:5 for HSP47, BSA, and PDI, respectively. Proteins and dye were incubated overnight at 4°C. To stop the reaction, a 10% volume of 1.5 M hydroxylamine was added, and the samples were incubated for 1 h. Labeled proteins were purified and diluted in a 50 mM HEPES-KOH (pH 7.5) or 200 mM phosphate buffer at various pH values using a gel-filtration micro-spin column (#CS-900, Princeton Separations, Adelphia, NJ, USA).

Fluorescence correlation spectroscopy (FCS)

FCS measurements were performed using a ConfoCor 2 system combined with an LSM 510 (Carl Zeiss, Jena, Germany) through a C-Apochromat 40×/1.2 NA Korr water-immersion objective (Carl Zeiss). A confocal pinhole diameter was adjusted to 70 μm. Alexa Fluor 488 was excited at 488 nm, and emission signals were detected using a 505 nm long-pass filter. Measurements were performed in a cover-glass chamber (#155411, Thermo Fisher Scientific) in the absence or presence of acetic acid-solubilized type I and III collagen (Nitta Gelatin, Osaka, Japan). Obtained fluorescence autocorrelation function (ACF), $G(\tau)$, from which the lag time (τ), was analyzed using a two-component diffusion model including the triplet state was given by Eq. 1:

$$G(\tau) = 1 + \frac{1}{N}\left[\frac{T}{1-T}\exp\left(-\frac{\tau}{\tau_{\text{triplet}}}\right)\right]\left[\frac{1-F}{\left(1+\frac{\tau}{\tau_{\text{free}}}\right)^{-1}\left(1+\frac{\tau}{s^2\tau_{\text{free}}}\right)^{-\frac{1}{2}}} + \frac{F}{\left(1+\frac{\tau}{\tau_{\text{bound}}}\right)^{-1}\left(1+\frac{\tau}{s^2\tau_{\text{bound}}}\right)^{-\frac{1}{2}}}\right] \quad (1)$$

where τ_{free} and τ_{bound} are the diffusion times of free and bound molecules, respectively; F denotes bound ratio of Alexa Fluor 488–labeled HSP47; N is the average number of fluorescent molecules in the detection volume defined by the beam waist w_0 and the axial radius z_0; s is a structure parameter representing the ratio of w_0 and z_0; T is the triplet fraction; and τ_{triplet} is the relaxation time of the triplet state. $G(\tau)$s of samples were measured for 60 s. Following pinhole adjustment, the diffusion time (τ_{Rh6G}) and structure parameter (s) were determined before measurements using a 0.1 μM Rhodamine 6G (Rh6G) solution as a standard. The diffusion coefficient and molecular weight were determined using those of Rhodamine 6G according to a

previous study [17].

Calculation of K_d using FCS

Determination of the equilibrium K_d using FCS was performed using a modification of a previously reported procedure [13]. The fraction of the slow component was determined from curve-fitting analysis of ACF using a two-component diffusion model. After normalization, the binding fraction (F) and collagen concentration ([C]) were plotted. The plots were fitted using the Origin 2016 software (OriginLab) using Eq. 2:

$$F = \frac{[\mathrm{C}]^n}{{K_\mathrm{d}}^n + [\mathrm{C}]^n} \quad (2)$$

where n is the Hill coefficient.

Analysis of pH-dependent interaction between HSP47 and collagen

The binding ratio was obtained from a two-component diffusion model (Eq. 2). Half-binding pH was calculated according to the Boltzmann function (Eq. 3) using the Origin 2016 software:

$$B = 1 + \left[1 + \exp\left(\frac{x - x_0}{dx}\right)\right]^{-1} \quad (3)$$

where B is the binding ratio, x is a pH parameter, x_0 is the half-binding pH, and dx is the constant of the sigmoidal curve.

Results

Detection of single-molecule binding between HSP47 and collagen in solution

To detect the interaction between HSP47 and collagen in solution, we used FCS, which can determine the diffusion coefficients of fluorescence molecules with single-molecule sensitivity in solution. First, we obtained the ACF of Alexa Fluor 488–labeled HSP47 (HSP47-AF). The molecular weight (M_w) of HSP47-AF, calculated based on the diffusion coefficient of it (89.9 ± 3.52 μm^2/s), was 49.5 ± 3.36 kDa (mean ± SD; n = 4). This estimated M_w of HSP47-AF corresponds to the theoretical value obtained from the composition of amino acids of HSP47 (44.5 kDa), indicating that HSP47-AF exists as a monomer. Although a previous paper reported that a portion of HSP47 forms a trimer [18], our FCS analysis revealed no evidence for trimerization of

HSP47.

HSP47-AF diffused more slowly by titration of type I or III collagen (Fig. 1, A and B), whereas no change on Alexa Fluor 488–labeled BSA and PDI (BSA-AF and PDI-AF, respectively) were observed in the absence and presence of type I or III collagen (Fig. 1, C–F), indicating that HSP47-AF binds to type I or III collagen specifically.

High-ionic buffer has no effect on the interaction between HSP47 and collagen

To confirm that the interaction between HSP47 and collagen was hydrophobic in nature, we investigated whether the right shift of the ACF of HSP47-AF in the presence of type I collagen could be prevented by addition of 400 mM sodium chloride (NaCl). The shape of the ACF of HSP47 alone was not altered by the presence of NaCl (Fig. 1G). Similarly, the shape of the ACF of HSP47 with type I collagen was not affected by NaCl (Fig. 1G). Curve-fitting analysis using a two-component diffusion model revealed that the slow component of HSP47-AF with collagen did not differ between the absence (22.9% ± 1.7%; n = 3) and presence of NaCl (23.5% ± 1.0%; n = 3), again confirming that the presence of NaCl did not affect the binding. These results indicated that the interaction between HSP47 and collagen was not inhibited in the presence of high-ionic buffer, suggesting that the binding between HSP47 and collagen is not due to electrostatic interactions.

HSP47 and collagen do not interact cooperatively in solution

The interaction between HSP47 and collagen was detected as an apparent increase in M_w, as determined by FCS. However, to evaluate the strength of the interaction, it was necessary to obtain the K_d. The K_d values of the interaction between HSP47 and collagens, when the Hill coefficient parameter was fixed to 1, were 47.9 nM and 39.3 nM for type I and type III collagen, respectively (Fig. 2 and Table). When the Hill coefficient parameter was not fixed, the calculated K_d values were similar, and the Hill coefficients were calculated as 0.978 and 0.9 for type I and type III collagen, respectively (Table), indicating that the binding between HSP47 and collagen exhibited minimal cooperativity. A previous study, based on fluorescence quenching of tryptophan, reported cooperative binding between HSP47 and collagen with a Hill coefficient of 4.3 [7], but we observed no such effect in this analysis.

pH-dependent dissociation of collagen from HSP47

Collagen is released from HSP47 in a pH-dependent manner [19-21]. To characterize the pH-dependent shift in binding strength, we used FCS to measure the interaction between HSP47 and type III collagen in phosphate buffer with pH ranging from 5.5 to 7. With decreasing pH, HSP47 binding to collagen was gradually weakened (Fig. 3A). The half-binding pH was 6.33, typical of

the *cis*-Golgi compartment [21, 22]. These results suggest that collagen can be released from HSP47 in the *cis*-Golgi or ER–Golgi intermediate compartment.

Next, we investigated whether the binding activity of HSP47 is reversible after an increase in pH from 5.8 to 7.3. The ACF of HSP47-AF in the presence of type III collagen shifted to the right after the increase in pH (Fig. 3B), suggesting that the pH-dependent dissociation and association process of HSP47 and collagen is reversible. However, the right-shifted ACF after the increase in pH did not recover completely to the original value determined at the neutral pH (78.3% ± 5.1%; n = 3), possibly due to partial denaturation of HSP47 at low pH or during the pH change *in vitro*.

Discussion

Using FCS, we calculated that the biochemical K_d between HSP47 and type I or III collagen was ~40 nM in neutral pH solution (Table). These K_d values are at least 18 times smaller than those reported in a previous study using SPR analysis (1,143 and 712 nM for type I and III collagen, respectively) [6] and approximately 4 times smaller than those previously reported by a study using fluorescence quenching analysis (170 nM) [7]. The differences of K_d values in each assay would be due to the number of binding sites of HSP47 on collagen: 26 HSP47-recognition regions have been identified in the type III collagen chain [2].

Although HSP47 and collagen interact with each other in neutral pH, they are dissociated in low pH environments [21] due to ionization of histidine residues in HSP47, resulting in structural change [19, 20]. This dissociation is thought to be important for recycling of HSP47 from Golgi to the ER [23]. Our FCS-based measurement system reproduced the dissociation between HSP47 and collagen in low pH (Fig. 3). However, once HSP47 was returned to neutral pH after conditioned of low pH, a portion of HSP47 might have been denatured (Fig. 3). If denaturation of HSP47 is induced during recycling from Golgi to ER in cells, denatured HSP47 would be degraded *via* the ER-associated degradation (ERAD) system. However, the half-life of HSP47 in cells is at least 6 h [24], longer than the secretion time of type I and type III collagen (1–3 h) [25, 26]; therefore, such denaturation of HSP47 may not occur in cells. The detailed mechanism that prevents denaturation of HSP47 in cells should be investigated in a future study.

Our results demonstrate that FCS is a powerful tool for directly and quantitatively measuring the interaction between HSP47 and collagen in solution. Importantly, in contrast to pull-downs and SPR, this method does not rely on solid–liquid phase assays. The solution-based measurement and short measurement time (less than 1 min per sample) of FCS make it suitable for use in high-throughput screening for inhibitory drugs capable of blocking the interaction between HSP47 and collagen, potentially leading to therapies for diseases such as cystic or liver fibrosis.

Although the endogenous concentration of HSP47 in the ER has not been determined precisely, expression of HSP47 is upregulated by heat shock stress or elevated production of collagen [27]. These findings suggest that the balance between the concentrations of HSP47 and collagen is physiologically regulated.

We conclude that FCS is capable of detecting the interaction between HSP47 and collagen in solution. To directly detect a protein–protein interaction between HSP47 and a low–molecular weight substrate such as a triple-helical model peptide [2], two-color fluorescence cross-correlation spectroscopy (FCCS) [17, 28, 29] should be adopted. Because anti-aggregation drug screening has been performed using FCCS [30], both FCS and FCCS could be adopted to screen for drugs that inhibit the interaction between collagen and HSP47, thereby preventing collagen from being over-produced and accumulating in the extracellular matrix.

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Author contributions

Conceived and designed the experiments: AK YI HK KN. Developed the protein purification: YI SI TH KA. Performed the fluorescence correlation spectroscopy: AK YI CGP MK. Analyzed the data: AK YI CGP MK KN. Wrote the paper: AK YI SI KN.

Figure captions:

Figure 1　FCS measurement of fluorescently labeled HSP47, PDI, or BSA in the absence or presence of type I or III collagen

(A) Normalized ACFs of Alexa Fluor 488–labeled HSP47 as type I collagen is titrated (red to violet: 2.4, 6, 12, 30, 60, 180, 300, 480, and 600 nM). (B) Normalized ACFs of Alexa Fluor 488–labeled HSP47 as type III is collagen titrated (red to violet: 2.4, 6, 12, 30, 60, 180, 300, 480, 600, and 900 nM). (C) Normalized ACFs of Alexa Fluor 488–labeled bovine serum albumin (BSA) in the absence or presence of 0.6 μM type I or 0.9 μM III collagen. (D) Normalized ACFs of Rhodamine 6G in the absence or presence of 0.6 μM type I or 0.9 μM III collagen. (E) Normalized ACFs of Alexa Fluor 488–labeled protein disulfide isomerase (PDI) in the absence or presence of 0.9 μM type III collagen. (F) Normalized ACFs of fluorescently labeled HSP47 alone or with type I collagen in the absence or presence of sodium chloride.

Figure 2　Change in binding ratio of HSP47 as type I or III collagen is titrated

The values of the binding ratio are plotted against the concentration of free type I (A) and III (B) collagen. The gray curve in the plot shows the fitted Hill equation.

Figure 3　pH-dependent dissociation of HSP47 from type III collagen

(A) Binding ratio of HSP47 to type III collagen in buffers with the indicated pH (mean ± SD; n = 3). (B) Normalized ACFs of HSP47 in the absence or presence of type III collagen (magenta or blue, respectively). Normalized ACF of HSP47 with collagen in a buffer whose pH was changed from 5.8 to 7.3 (green).

Table　Dissociation constant and Hill coefficient obtained from FCS analysis

Dissociation constants obtained from FCS analysis when Hill coefficient was free or fixed to 1. † indicating fixed value during curve-fitting analysis.

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Table. Dissociation constant and Hill coefficient obtained from FCS analysis

		Hsp47	Hsp47
Type I collagen	K_d (nM)	47.9	47.7
	Hill coefficient	1†	0.978
Type III collagen	K_d (nM)	39.3	40.3
	Hill coefficient	1†	0.900

† indicates the fixed value during curve-fitting analysis.

Figure 1

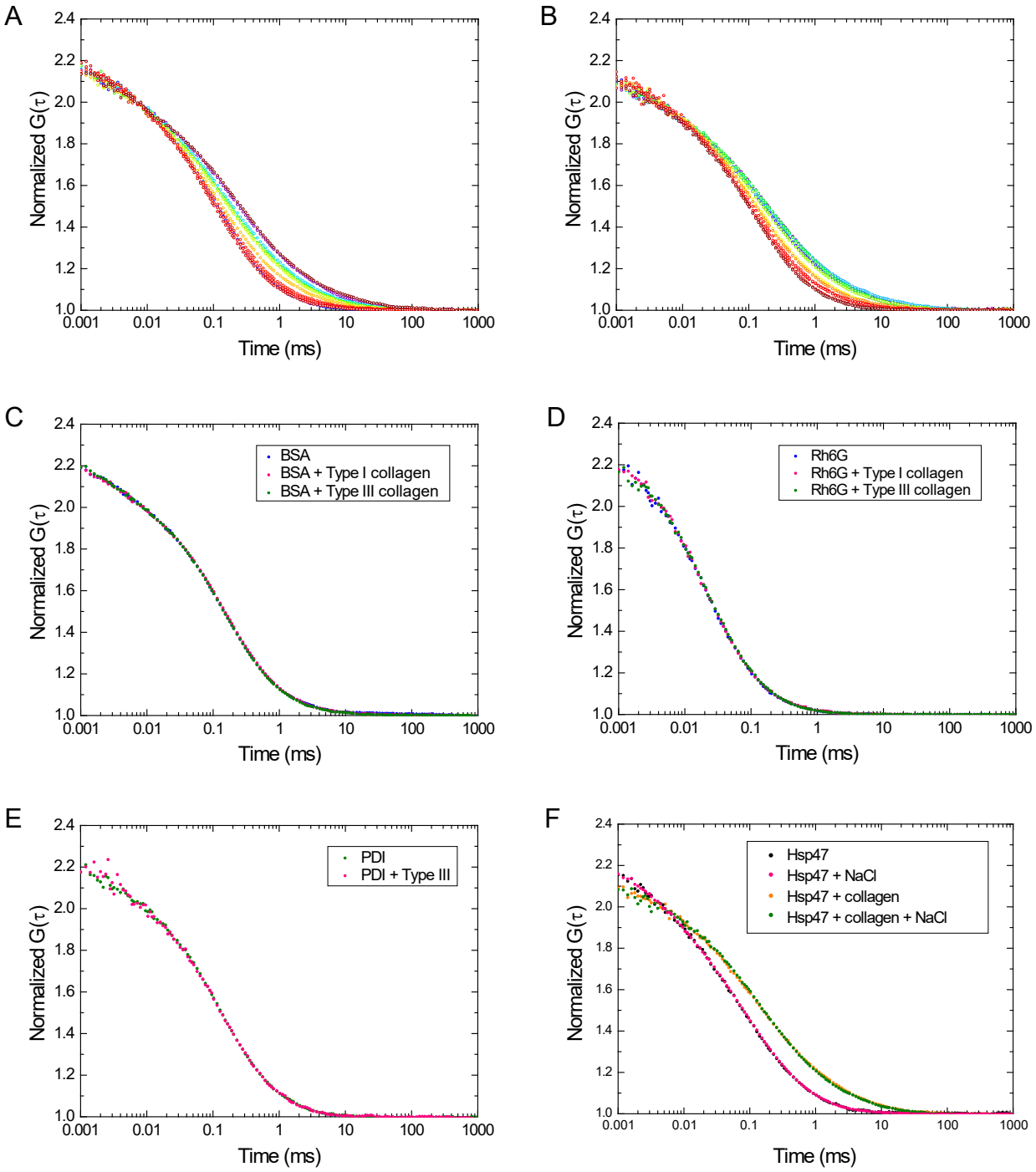

A
B
C
D
E
F
Normalized G(τ)
Time (ms)
BSA
BSA + Type I collagen
BSA + Type III collagen
Rh6G
Rh6G + Type I collagen
Rh6G + Type III collagen
PDI
PDI + Type III
Hsp47
Hsp47 + NaCl
Hsp47 + collagen
Hsp47 + collagen + NaCl

Figure 2

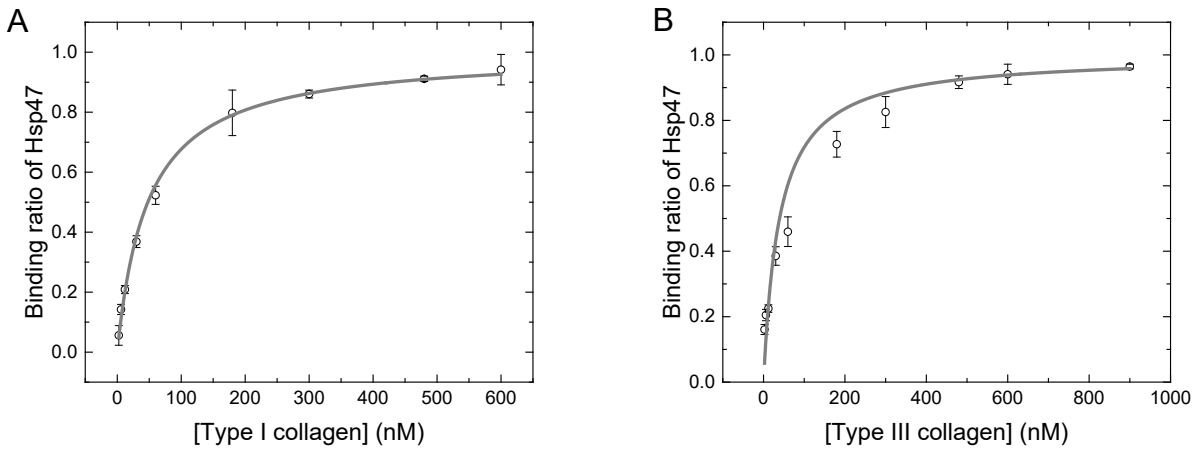
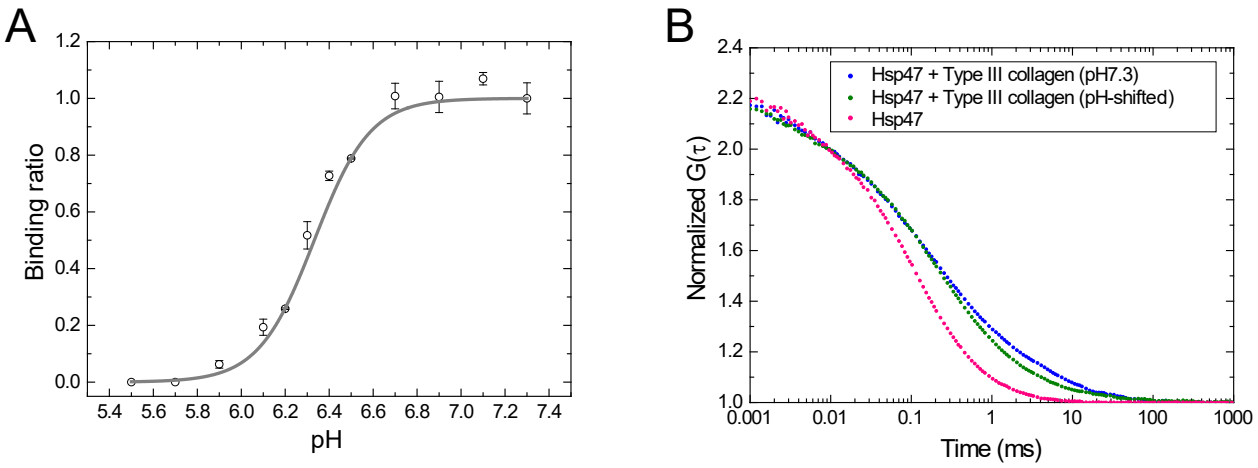

A
Binding ratio of Hsp47
0.0
0.2
0.4
0.6
0.8
1.0
0
100
200
300
400
500
600
[Type I collagen] (nM)
B
Binding ratio of Hsp47
0.0
0.2
0.4
0.6
0.8
1.0
0
200
400
600
800
1000
[Type III collagen] (nM)

Figure 3


A
Binding ratio
pH
B
Normalized G(τ)
Time (ms)
Hsp47 + Type III collagen (pH7.3)
Hsp47 + Type III collagen (pH-shifted)
Hsp47