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

3
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23
24 **Keywords**

25 Molecular chaperone, HSP47, collagen, fluorescence correlation spectroscopy

26

27

1 ***Abstract***

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

22

1 ***Introduction***

2
3 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) 4 superfamily, and is also known as Serpin H1. It binds to collagen and promotes its triple-helical 5 folding in the ER. HSP47 predominantly recognizes collagenous amino acid sequences, i.e., 6 glycine-Xaa-Yaa (Gly-Xaa-Yaa) repeats, in which Xaa and Yaa are often proline (Pro) and 7 hydroxyproline (Hyp), respectively. Hydroxylation of Pro residues is required to stabilize the 8 triple-helical structure of collagen [2]. Since the surface of the collagen triple-helix region 9 becomes hydrophobic, HSP47 must bind to the triple-helical form of procollagen to prevent its 10 lateral aggregation in the ER [3]. The amino acid residues in HSP47 responsible for hydrophobic 11 collagen binding have been identified [4]. 12

13 The binding of HSP47 to collagen has been analyzed using several methods, including 14 biochemical pull-down assay including immunoprecipitation [5], surface plasmon resonance 15 (SPR) [6], fluorescence quenching measurement [7], Förster/fluorescence resonance energy 16 transfer (FRET) [8], and bimolecular fluorescence complementation (BiFC) [8]. Those studies 17 revealed that HSP47 binds to purified mature type I–V collagen, gelatin (denatured collagen), and 18 peptides containing Gly-Pro-Pro repeats. The equilibrium dissociation constants (K_d) between 19 HSP47 and collagen were determined using SPR and fluorescence quenching analysis [6, 7].

20 HSP47 is translocated into the ER *via* a signal sequence at the N-terminus and recycled from 21 the Golgi to the ER *via* an ER-retention signal sequence (RDEL) at the C-terminus. During this 22 cycle, dissociation between HSP47 and collagen at low pH promotes both the retention of HSP47 23 in the ER and appropriate trafficking of triple-helical collagen [1, 9].

24 Various fibrotic diseases, including liver cirrhosis and idiopathic pulmonary fibrosis, are 25 characterized by abnormal chronic collagen accumulation in tissues [9-11]. Accordingly, it has 26 been hypothesized that suppression of HSP47 activity would slow the progression of these 27 diseases. Indeed, in mice, knockdown of HSP47 ameliorates liver fibrosis by inhibiting collagen 28 secretion [12]. Therefore, the establishment of effective and high-throughput screening methods 29 for discovering compounds capable of suppressing HSP47 would be clinically valuable.

30 Here, we demonstrate that the interaction between HSP47 and collagen in solution can be 31 quantitatively and conveniently detected using fluorescence correlation spectroscopy (FCS) [13, 32 14]. This FCS system could be applied to high-throughput screening of reagents to identify 33 molecules that inhibit the interaction between HSP47 and collagen, and could thus slow or curing 34 of fibrotic disease.

35 36 ***Materials and Methods***

1

2 *Fluorescence labeling of proteins*

3 Purified HSP47 in which Cys138 was replaced with alanine (C138A) as described previously [15],
 4 human protein disulfide isomerase (PDI) as described previously [16], and bovine serum albumin
 5 (BSA; purchased from Sigma-Aldrich, St. Louis, MO, USA) were incubated with Alexa Fluor
 6 488 carboxylic acid and succinimidyl ester (A-20000, Thermo Fisher Scientific, Waltham, MA,
 7 USA), and covalently fluorescently labeled. For the labeling reactions, the molar ratios of protein
 8 to dye were 1:20, 1:10, and 1:5 for HSP47, BSA, and PDI, respectively. Proteins and dye were
 9 incubated overnight at 4°C. To stop the reaction, a 10% volume of 1.5 M hydroxylamine was
 10 added, and the samples were incubated for 1 h. Labeled proteins were purified and diluted in a 50
 11 mM HEPES-KOH (pH 7.5) or 200 mM phosphate buffer at various pH values using a gel-
 12 filtration micro-spin column (#CS-900, Princeton Separations, Adelphia, NJ, USA).

13

14 *Fluorescence correlation spectroscopy (FCS)*

15 FCS measurements were performed using a ConfoCor 2 system combined with an LSM 510 (Carl
 16 Zeiss, Jena, Germany) through a C-Apochromat 40×/1.2 NA Korr water-immersion objective
 17 (Carl Zeiss). A confocal pinhole diameter was adjusted to 70 μm. Alexa Fluor 488 was excited at
 18 488 nm, and emission signals were detected using a 505 nm long-pass filter. Measurements were
 19 performed in a cover-glass chamber (#155411, Thermo Fisher Scientific) in the absence or
 20 presence of acetic acid-solubilized type I and III collagen (Nitta Gelatin, Osaka, Japan). Obtained
 21 fluorescence autocorrelation function (ACF), $G(\tau)$, from which the lag time (τ), was analyzed
 22 using a two-component diffusion model including the triplet state was given by Eq. 1:

23

$$24 \quad G(\tau) = 1 + \frac{1}{N} \left[\frac{T}{1-T} \exp\left(-\frac{\tau}{\tau_{\text{triplet}}}\right) \right] \left[\frac{1-F}{\left(1+\frac{\tau}{\tau_{\text{free}}}\right)^{-1} \left(1+\frac{\tau}{s^2\tau_{\text{free}}}\right)^{-\frac{1}{2}}} + \frac{F}{\left(1+\frac{\tau}{\tau_{\text{bound}}}\right)^{-1} \left(1+\frac{\tau}{s^2\tau_{\text{bound}}}\right)^{-\frac{1}{2}}} \right] \quad (1)$$

25

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

1 previous study [17].

3 *Calculation of K_d using FCS*

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

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

11 where n is the Hill coefficient.

14 *Analysis of pH-dependent interaction between HSP47 and collagen*

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

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

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

23 **Results**

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

26 To detect the interaction between HSP47 and collagen in solution, we used FCS, which can
27 determine the diffusion coefficients of fluorescence molecules with single-molecule sensitivity in
28 solution. First, we obtained the ACF of Alexa Fluor 488–labeled HSP47 (HSP47-AF). The
29 molecular weight (M_w) of HSP47-AF, calculated based on the diffusion coefficient of it ($89.9 \pm$
30 $3.52 \mu\text{m}^2/\text{s}$), was $49.5 \pm 3.36 \text{ kDa}$ (mean \pm SD; $n = 4$). This estimated M_w of HSP47-AF
31 corresponds to the theoretical value obtained from the composition of amino acids of HSP47 (44.5
32 kDa), indicating that HSP47-AF exists as a monomer. Although a previous paper reported that a
33 portion of HSP47 forms a trimer [18], our FCS analysis revealed no evidence for trimerization of

1 HSP47.

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

6
7 *High-ionic buffer has no effect on the interaction between HSP47 and collagen*

8 To confirm that the interaction between HSP47 and collagen was hydrophobic in nature, we
9 investigated whether the right shift of the ACF of HSP47-AF in the presence of type I collagen
10 could be prevented by addition of 400 mM sodium chloride (NaCl). The shape of the ACF of
11 HSP47 alone was not altered by the presence of NaCl (Fig. 1G). Similarly, the shape of the ACF
12 of HSP47 with type I collagen was not affected by NaCl (Fig. 1G). Curve-fitting analysis using a
13 two-component diffusion model revealed that the slow component of HSP47-AF with collagen
14 did not differ between the absence ($22.9\% \pm 1.7\%$; $n = 3$) and presence of NaCl ($23.5\% \pm 1.0\%$;
15 $n = 3$), again confirming that the presence of NaCl did not affect the binding. These results
16 indicated that the interaction between HSP47 and collagen was not inhibited in the presence of
17 high-ionic buffer, suggesting that the binding between HSP47 and collagen is not due to
18 electrostatic interactions.

19
20 *HSP47 and collagen do not interact cooperatively in solution*

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

31
32 *pH-dependent dissociation of collagen from HSP47*

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

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

3 Next, we investigated whether the binding activity of HSP47 is reversible after an
4 increase in pH from 5.8 to 7.3. The ACF of HSP47-AF in the presence of type III collagen shifted
5 to the right after the increase in pH (Fig. 3B), suggesting that the pH-dependent dissociation and
6 association process of HSP47 and collagen is reversible. However, the right-shifted ACF after the
7 increase in pH did not recover completely to the original value determined at the neutral pH
8 ($78.3\% \pm 5.1\%$; $n = 3$), possibly due to partial denaturation of HSP47 at low pH or during the pH
9 change *in vitro*.

11 **Discussion**

13 Using FCS, we calculated that the biochemical K_d between HSP47 and type I or III collagen was
14 ~ 40 nM in neutral pH solution (Table). These K_d values are at least 18 times smaller than those
15 reported in a previous study using SPR analysis (1,143 and 712 nM for type I and III collagen,
16 respectively) [6] and approximately 4 times smaller than those previously reported by a study
17 using fluorescence quenching analysis (170 nM) [7]. The differences of K_d values in each assay
18 would be due to the number of binding sites of HSP47 on collagen: 26 HSP47-recognition regions
19 have been identified in the type III collagen chain [2].

20 Although HSP47 and collagen interact with each other in neutral pH, they are dissociated in
21 low pH environments [21] due to ionization of histidine residues in HSP47, resulting in structural
22 change [19, 20]. This dissociation is thought to be important for recycling of HSP47 from Golgi
23 to the ER [23]. Our FCS-based measurement system reproduced the dissociation between HSP47
24 and collagen in low pH (Fig. 3). However, once HSP47 was returned to neutral pH after
25 conditioned of low pH, a portion of HSP47 might have been denatured (Fig. 3). If denaturation
26 of HSP47 is induced during recycling from Golgi to ER in cells, denatured HSP47 would be
27 degraded *via* the ER-associated degradation (ERAD) system. However, the half-life of HSP47 in
28 cells is at least 6 h [24], longer than the secretion time of type I and type III collagen (1–3 h) [25,
29 26]; therefore, such denaturation of HSP47 may not occur in cells. The detailed mechanism that
30 prevents denaturation of HSP47 in cells should be investigated in a future study.

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

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

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

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19 20 **Author contributions**

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

1 **Figure captions:**

2
3 **Figure 1 FCS measurement of fluorescently labeled HSP47, PDI, or BSA in the absence or**
4 **presence of type I or III collagen**

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

14
15 **Figure 2 Change in binding ratio of HSP47 as type I or III collagen is titrated**

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

18
19 **Figure 3 pH-dependent dissociation of HSP47 from type III collagen**

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

24
25 **Table Dissociation constant and Hill coefficient obtained from FCS analysis**

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

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13
14

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

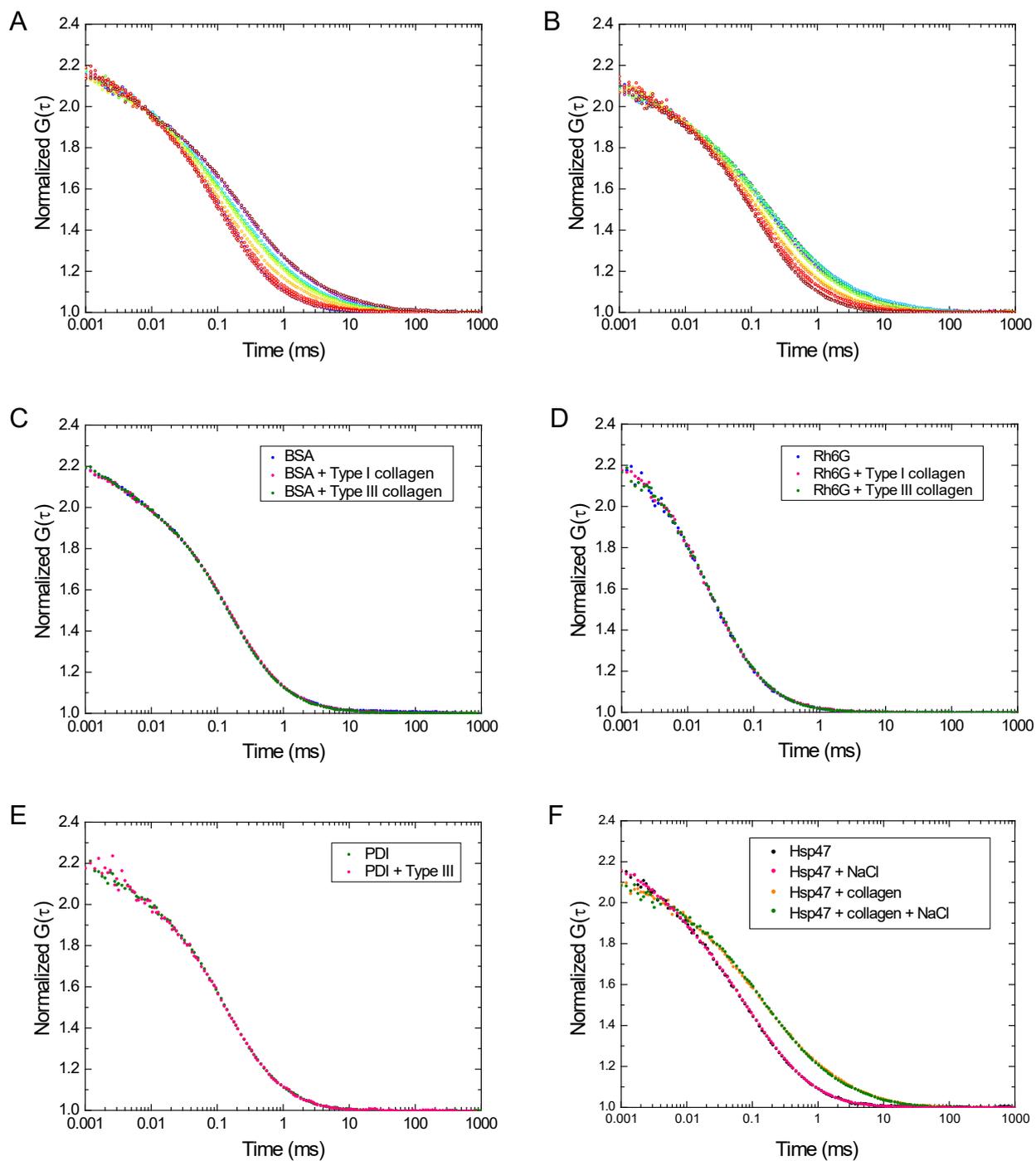


Figure 2

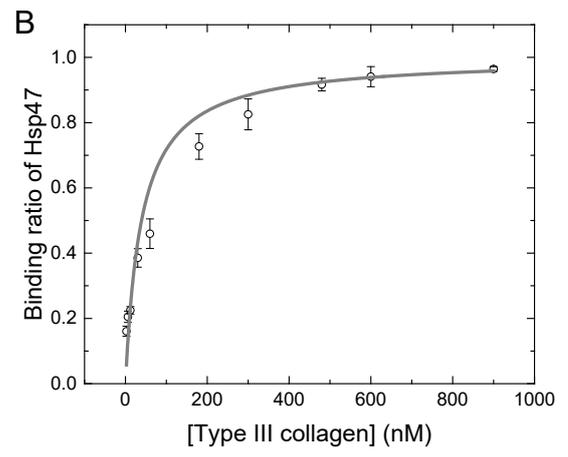
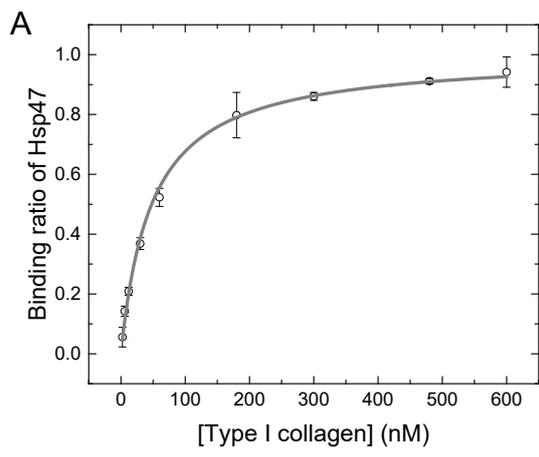


Figure 3

