Optimized Methods for the Surface Immobilization of Collagens and Collagen Binding Assays

Nadia Chaher, Giuseppe Digilio, Sara Lacerda, René Botnar, Alkystis Phinikaridou

TITLE:

Optimized Methods for the Surface Immobilization of Collagens and Collagen Binding Assays

AUTHORS AND AFFILIATIONS:

Nadia Chaher[1], Giuseppe Digilio[2]*, Sara Lacerda[3]*, René M Botnar[1,4,5,6], Alkystis Phinikaridou[1,4+]

[1]School of Biomedical Engineering and Imaging Sciences, King's College London, St Thomas' Hospital, London, UK

[2]Dipartimento di Scienze e Innovazione Tecnologica, Università del Piemonte Orientale, Vercelli, Italy

[3]Centre de Biophyisique Moléculaire, CNRS UPR 4301, Université d'Orléans, Orléans, France

[4]BHF Centre of Research Excellence, Cardiovascular Division, King's College London, London, UK

[5]Escuela de Ingeniería, Pontificia Universidad Católica de Chile, Santiago, Chile

[6]Instituto de Ingeniería Biológica y Médica, Pontificia Universidad Católica de Chile, Santiago, Chile

*These authors contributed equally.

Email addresses of the co-authors:

Nadia Chaher (nadia.chaher@kcl.ac.uk)

Giuseppe Digilio (giuseppe.digilio@uniupo.it)

Sara Lacerda (sara.lacerda@cnrs.fr)

René M Botnar (rene.botnar@kcl.ac.uk)

[+]Corresponding author:

Alkystis Phinikaridou (alkystis.1.phinikaridou@kcl.ac.uk)

SUMMARY:

This work presents an optimized protocol to reproducibly immobilize and quantify type I and III collagen onto microplates, followed by an improved *in vitro* binding assay protocol to study collagen–compound interactions using a time-resolved fluorescence method. The subsequent step-by-step data analysis and data interpretation are provided.

ABSTRACT:

Fibrosis occurs in various tissues as a reparative response to injury or damage. If excessive, however, fibrosis can lead to tissue scarring and organ failure, which is associated with high morbidity and mortality. Collagen is a key driver of fibrosis, with type I and type III collagen being the primary types involved in many fibrotic diseases. Unlike conventional protocols used to immobilize other proteins (e.g., elastin, albumin, fibronectin, etc.), comprehensive protocols to reproducibly immobilize different types of collagens to produce stable coatings are not readily available. Immobilizing collagen is surprisingly challenging because multiple experimental conditions may affect the efficiency of immobilization, including the type of collagen, the pH, the temperature, and the type of microplate used. Here, a detailed protocol to reproducibly immobilize and quantify type I and III collagens to create stable and reproducible gels/films is provided. Furthermore, this work demonstrates how to perform,

analyze, and interpret *in vitro* time-resolved fluorescence binding studies to investigate the interactions between collagens and candidate collagen-binding compounds (e.g., a peptide conjugated to a metal chelate carrying, for example, europium [Eu(III)]). Such an approach can be universally applied to various biomedical applications, such as in the field of molecular imaging to develop targeted imaging probes, drug development, cell toxicity studies, cell proliferation studies, and immunoassays.

INTRODUCTION:

The accumulation of fibrous connective tissue as part of the natural wound-healing process following tissue injury is known as fibrosis. However, if the deposition of fibrous tissue fails to terminate and continues beyond what is needed for tissue repair, then fibrosis becomes excessive[1,2]. Excessive fibrosis impairs organ physiology and function and could lead to organ damage and potentially organ failure[3–5]. Two main drivers of fibrosis are the extracellular matrix (ECM) proteins collagen type I and type III[6]. Collagen is a structural protein found in various organs that makes up approximately one-third of the total protein content of the human body[1]. There are 28 different types of collagens identified by human genome sequencing, and the most abundant are the fibrillar collagens[7]. The primary fibrillary collagen is type I collagen, which provides the ECM with tensile strength and resistance to deformation[8]. Type III collagen is a structural component that provides elasticity and colocalizes with type I collagen. It is expressed during embryogenesis and is naturally found in small amounts in adult skin, muscle, and blood vessels[9].

In vivo collagen synthesis begins with an intracellular process in which mRNA is transcribed in the nucleus and then moves to the cytoplasm, where is it translated. After translation, the chain formed undergoes post-translational modification in the endoplasmic reticulum, where pro-collagen (the precursor of collagen) is formed. The pro-collagen then travels to the Golgi apparatus for final modification before being excreted to the extracellular space[10]. Through proteolytic cleavage, the procollagen is transformed into tropocollagen. This is then cross-linked either *via* an enzymatic-mediated cross-linking pathway catalyzed by the enzyme lysyl oxidase (LOX) or *via* a non-enzymatic-mediated cross-linking pathway involving the Maillard reaction[11]. However, *in vitro* protocols to immobilize collagen mainly rely on the ability of collagen to self-assemble. Collagen is extracted from tissues based on its solubility, which largely depends on the extent of cross-linking of individual collagen fibrils[7]. Fibrillar collagen is dissolved in acetic acid, and fibrils can reform when the pH and temperature are adjusted[12]. *In vitro*, the fibrillogenesis of collagen can be viewed as a two-stage process[7]. The first stage is the nucleation phase, where collagen fibers form dimers and trimer fibrils before they are rearranged to form a triple helical structure. The second phase is the growth phase, whereby the fibrils start to grow laterally and result in the characteristic D-band formation, which is generally observed by changes in turbidity[7]. Atomic force microscopy (AFM) studies have also revealed that type I and type III collagen have different characteristics (Erreur ! Source du renvoi introuvable.)[13].

To study the binding interactions between collagen and other compounds, collagen needs to be reproducibly immobilized into the wells of microplates. There are various protocols for immobilizing soluble collagen[14–16]. Commercially available microplates that are pre-coated with collagen are typically used for cell culture. However, pre-coated microplates have a very thin layer of an unknown amount of collagen coated onto the wells, which makes them

unsuitable for *in vitro* binding assays. There are several challenges when immobilizing collagen onto the plate wells. One of the key challenges is choosing a suitable type of microplate, because different types of collagens (e.g., type I and III) have different chemical properties and, therefore, immobilize more stably and effectively depending on the material of the microplate. Another challenge is the experimental conditions of the immobilization protocol, as the process of fibrillogenesis depends on multiple factors, including temperature, pH, the stock concentration of collagen, and the ionic concentration of the buffer[7].

For studying the interactions between the collagen (the target) and other compounds (i.e., a targeting peptide), it is also necessary to develop a robust screening assay to investigate the specificity and selectivity of the compound toward the target by measuring the dissociation constant, K_d. The position of the equilibrium of formation of a bimolecular complex between a protein (collagen) and a ligand is expressed in terms of the association constant K_a, whose magnitude is proportional to the binding affinity. However, most commonly, biochemists express affinity relationships in terms of the equilibrium dissociation constant, K_d, of the binary complex, which is defined as $K_d = 1/K_a \cdot K_d$ and is the inverse of K_a; the lower the K_d value, the stronger the binding strength between the protein and the ligand. The advantage of using K_d to compare the binding affinity of different ligands for the same protein (and the other way around) is linked to the fact that the units of K_d for a bimolecular complex are mol/L (i.e., concentration units). Under most experimental conditions, the K_d value corresponds to the ligand concentration that leads to 50% saturation of the available binding sites on the target at the equilibrium[17,18]. The dissociation constant is typically extracted by analyzing the receptor fractional occupancy (FO), which is defined as the ratio between the occupied binding sites and total available binding sites, as a function of ligand concentration. This can be done provided that an analytical assay able to distinguish and measure the amount of bound ligand is available.

In vitro ligand binding assays can be performed using various bioanalytical methods, including optical photometry, radioligand methods, inductively coupled plasma mass spectrometry (ICP-MS), and surface plasmon resonance (SPR). Amongst the photometric methods, those based on fluorescence emission typically require the labeling of ligands or proteins with fluorophores to increase the sensitivity and to improve the detection limit of the assay. Chelates of certain lanthanides(III) ions, such as Eu(III), are very attractive as fluorophores as they have large Stokes' shifts, narrow emission bands (providing a good signal-to-noise ratio), limited photobleaching, and long emission lifetimes. Importantly, the latter property enables the use of time-resolved fluorescence (TRF) from Eu(III) fluorophores to abolish background autofluorescence[19]. In the dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) version of the Eu(III)-based TRF assay, ligands labeled with a non-luminescent Eu(III)-chelate are incubated with the receptor immobilized onto microplates. The labeled ligand/receptor complex is separated from the unbound ligand, and Eu(III) fluorescence is activated by dissociation of the Eu(III) complex at an acidic pH, followed by re-complexation with a fluorescence-enhancing chelator to form a micelle-embedded, highly fluorescent Eu(III) complex[20].

The decomplexation step can be reasonably achieved with chelators, such as diethylenetriamine pentaacetate (DTPA), that show fast decomplexation kinetics. However,

Eu(III) complexes with certain macrocyclic chelators, such as DOTA (1,4,7,10-tetraazacyclododecane1,4,7,10-tetraacetic acid) and its monoamide derivatives (DO3AAm), show high thermodynamic stability and very high kinetic inertness. In this case, the decomplexation steps must be accurately optimized to achieve sufficient and reproducible activation of Eu(III)-based TRF[21]. It is worth noting that lanthanide (Ln(III))-DOTA and Ln(III)-DO3AAm complexes are those most commonly employed as contrast agents for *in vivo* molecular imaging by magnetic resonance imaging (MRI) techniques[22]. Thus, the Ln(III)-based TRF assay is the tool of choice to study *in vitro* the binding affinity of MRI molecular probes with their intended biological targets. Currently, comprehensive and reproducible protocols for immobilizing type I and type III collagen and a reproducible pipeline for performing *in vitro* binding Eu(III) TRF experiments are lacking. To overcome these limitations, reproducible methods to self-assemble and immobilize type I and type III collagen and generate stable gels and films, respectively, with the sufficient concentration of collagen required for *in vitro* binding assays, were developed. An optimized protocol for Eu(III) TRF of highly inert Eu(III)-DO3Aam-based complexes is presented. Finally, an optimized *in vitro* microplate Eu(III) TRF assay to measure the K_d of Eu(III)-labeled ligands toward immobilized type I and type III collagen is demonstrated (**Figure 1**).

PROTOCOL:

NOTE: All product information used for this work is presented in the **Table of Materials**.

1. Collagen immobilization

NOTE: Ensure each well in the microplate used during the binding assay has adjacent wells free to avoid cross-fluorescence. Carry out this part of the protocol on ice because collagen self-assembles at rising temperatures and pH levels. Perform this procedure in a tissue culture hood and under sterile conditions because the microplates are subsequently incubated in a tissue culture (TC) incubator.

1.1. Immobilization of type I collagen on the 96-well microplates (**Figure 2: Workflow to immobilize type I collagen**)

Day 1

1.1.1. Prepare a silicone tray with ice. Place the vial containing type I collagen, the cold 10x phosphate-buffered saline (PBS), and the microplates on ice, and spray everything with 70% ethanol. Place the material under the TC hood.

1.1.2. Neutralize the collagen using equal volumes of type I collagen and 10x PBS (pH 7.4).

1.1.3. Invert the solution a few times, ensuring that no bubbles form.

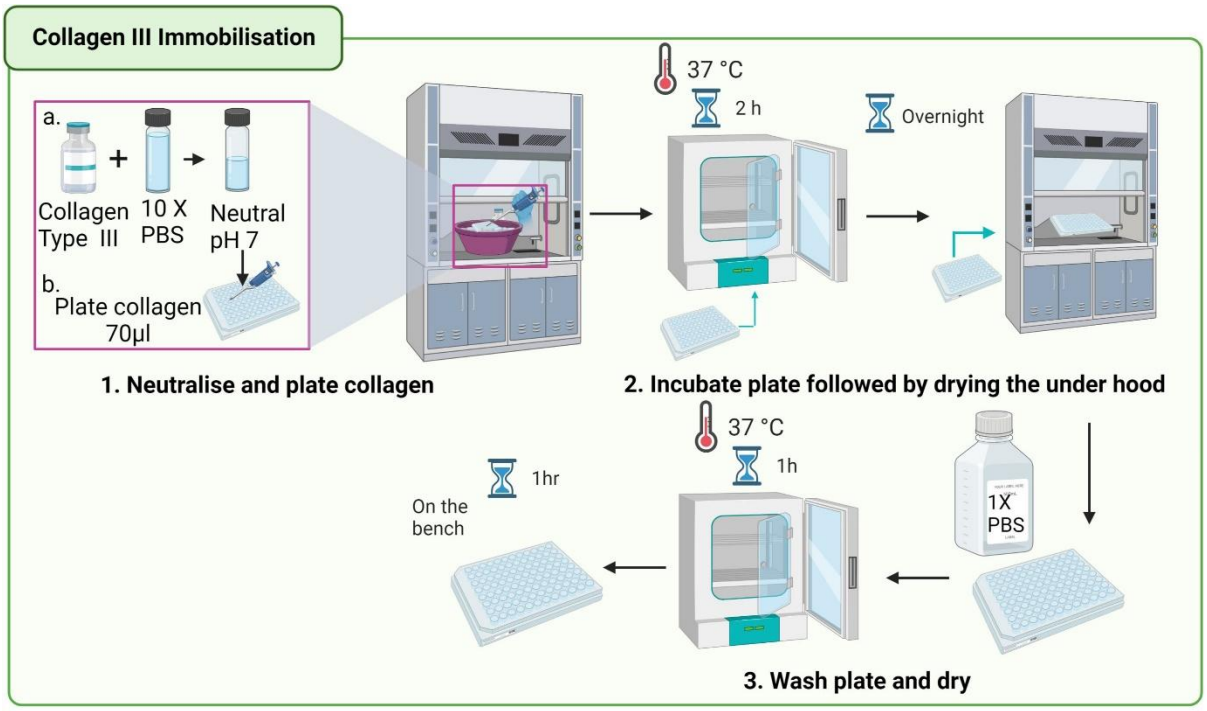
1.1.4. Add 100 µL of the neutralized collagen to every other well of the microplate, and incubate at 37 °C for 18–20 h to evaporate the collagen to dryness.

Day 2

1.1.5. Wash the microplates with 100 µL of 1x PBS, pH 7.4, twice to remove any unbound collagen.

1.1.6. Transfer the microplates into the incubator at 37° C for another 2 h to dry before using them for further binding experiments.

1.2. Immobilization of collagen type III on the 96-well microplates (



1.3.

1.4. **Figure *3***)

Day 1

1.2.1. Prepare a silicone tray with ice. Place the vial containing type III collagen, the cold 10x PBS, and the microplates on ice, and spray everything with 70% ethanol. Place the material under the TC hood.

1.2.2. Neutralize the collagen using equal volumes of type III collagen and 10x PBS (pH 7.4).

1.2.3. Add 70 µL of the neutralized collagen to every other well of the microplate, and incubate at 37 °C for 2 h by placing the microplate under the tissue culture hood to evaporate the collagen to dryness.

Day 2

1.2.4. Wash the microplates with 70 µL of 1x PBS, pH 7.4, twice to remove any unbound collagen.

1.2.5. Transfer the microplates to the incubator for 1 h at 37 °C, and then transfer the microplates to the bench, and allow them 1 h to dry before using them in further binding experiments.

2. Assessment of the stability of the immobilized collagen gels/films

2.1. Incubation with PBS for 1 h

NOTE: During the binding experiment, incubate the immobilized collagen with the compound of interest. It is important to investigate the stability of the resulting collagen gel or film. To do this, measure the stability of three conditions: **no wash** = measures the immobilized collagen directly after incubation; **washing** = measures the immobilized collagen after washing the plate twice with 100 µL of PBS; and **1 h PBS mimic & wash** = measures the immobilized collagen acter incubating for 1 h with PBS followed by two rounds of washes with PBS. Below, the PBS incubation method is explained.

2.1.1. Add 70 µL of PBS (1x) to each of the wells coated with collagen, and incubate the microplate at room temperature for 1 h.

2.1.2. Aspirate the excess liquid from each well using a pipette, and wash with PBS (1x) twice before carrying out the protein quantification assay described below.

2.2. Quantification of the amount of immobilized collagen using a bicinchoninic acid assay (BCA)

NOTE: Use the Pierce BCA Protein Assay Kit (**Table of Materials**) following the manufacturer's instructions. Make respective collagen standards for this assay. The concentration range for collagen I is from 0–3,000 µg/mL and for collagen III from 0–750 µg/mL. In total, make 11 standards per collagen.

2.2.1. Prepare the total volume of working reagent (WR) needed by following the manufacturer's instructions.

2.2.2. Add 25 µL of each of the collagen standard into the microplate wells (in duplicate). These solutions are used to draw the standard curve.

2.2.3. Add 200 µL of working reagent solution to each of the wells containing the standards and the wells coated with unknown concentrations of collagen.

2.2.4. Place the microplate on a plate shaker for 30 s. Cover the microplates, and incubate at 37 °C for 30 min.

2.2.5. Remove the microplates, and allow to cool at room temperature. Measure the absorbance at 560 nm using a plate reader.

2.2.6. Plot a calibration curve by plotting the A_{560} (AU) versus the concentration (µg/mL) of the 11 standard solutions, and use the calibration curve to calculate the amount of collagen.

3. Europium(III) TRF ligand binding assay (Figure 1)

NOTE: The compound used is a candidate collagen-binding peptide (CBP) labeled with a single Eu(III)-DO3AAm complex, referred to as Eu(III)-DO3AAm-CBP (

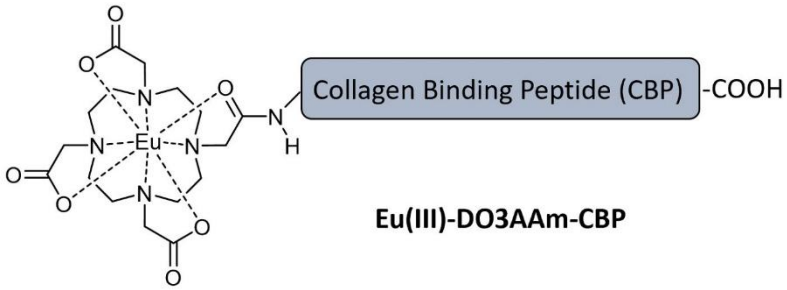


Figure *4*).

3.1. Incubation of the collagen-coated plates with the Eu(III)-DO3AAm-CBP compound

3.1.1. Prepare solutions of the Eu(III)-DO3AAm-CBP compound with concentrations ranging between 0.1–15 µM (0.1 µM, 0.5 µM, 1 µM, 3 µM, 5 µM, 7 µM, 10 µM, and 15 µM) in 1x PBS.

3.1.2. Add 75 µL of each concentration of compound into the collagen-coated wells (Plate A). Perform the experiment in triplicate to calculate the amount of compound that binds to the collagen.

3.1.3. Use a second uncoated plate (Plate B), and add 75 µL of each compound to the empty wells to calculate the non-specific binding of the compound to the plate. Use triplicates for each concentration.

3.1.4. Incubate the microplates for 1 h at room temperature.

3.1.5. Using a pipette, aspirate and discard the excess solution from each well, and wash the wells with 1x PBS twice to remove excess, unbound compound. Perform this step using both the collagen-coated and uncoated microplates.

3.1.6. To a third uncoated plate (Plate C), add 10 µL of the same range of Eu(III)-DO3AAm-CBP concentrations (in duplicate). Use the fluorescence reading from the Eu(III)-DO3AAm-CBP in solution to make a calibration curve.

NOTE: Do not wash or aspirate the solution from this plate.

3.2. Acid extraction of Europium(III) and time-resolved fluorescence (TRF) readings

NOTE: Please refer to the supplemental information about the preparation and calibration of the volumes of the acidic solution (AS) and the buffering solution (BS). The volumes of the AS and BS required to reproducibly achieve an optimal pH were 54 µL and 46 µL, respectively, in this work. Carry out the following operation on plate A, plate B, and plate C.

3.2.1. Add 54 µL of acidic solution (AS) to each well, and place the plate in the incubator at 37 °C for 90 min, covering the microplates with foil to avoid evaporation. The temperature and incubation time must be carefully controlled to achieve reproducible decomplexation.

3.2.2. Add 46 µL of buffering solution (BS) to each well, and gently shake the plate for 30 s.

3.2.3. Add 100 µL of enhancement solution (ES), and shake the plate for 30 s.

3.2.4. Wait for 30 min before reading the plate using a TRF plate reader. Use the parameters listed in Erreur ! Source du renvoi introuvable..

4. Data analysis

4.1. Quantification of the concentration of collagen immobilized on the wells

4.1.1. Obtain the equation of the calibration curve of the A_{560} (AU) versus the concentration (µg/mL) of the 11 standard solutions.

4.1.1.1. Use the absorbance readings acquired from the wells containing the collagen standards.

4.1.1.2. Tabulate the average values from the duplicate wells, and plot the mean absorbance against the known protein (collagen) concentrations (µg/mL) to obtain the equation for the standard curve.

4.1.2. Use the absorbance values to calculate the mass (µg) and concentration (M) of immobilized collagen.

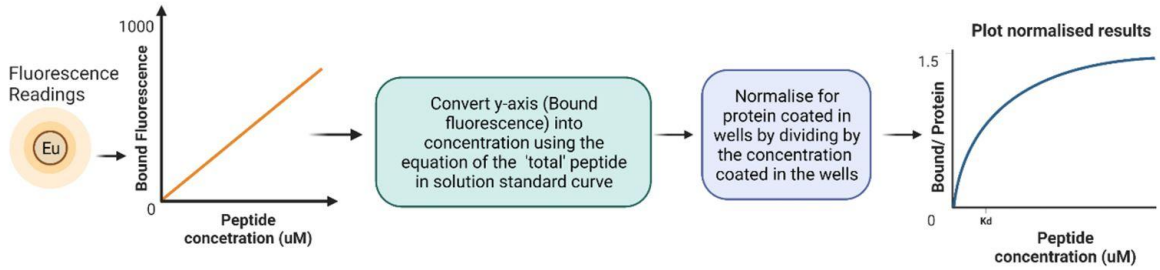
4.1.2.1. Compute the average absorbance values across the three wells that contained immobilized collagen, and record the standard deviation.

4.1.2.2. Use the standard curve equation obtained from the collagen standard curve (step 2.2.6) to convert the absorbance measured from the collagen-coated wells into concertation. From this, calculate the concentration of collagen that was immobilized within the experimental wells in µg/mL.

4.1.2.3. Convert the concentration calculated in step 4.1.2.2 (µg/mL) first to grams/liter and, based on the molecular weight of the collagen, into molar (M).

4.1.2.4. Finally, calculate the mass of the collagen immobilized in each well by dividing the concentration by the volume of collagen added to the well (100 µL for type I collagen and 70 µL for type III collagen).

4.2. Calculation of the dissociation constant (K_d) (



4.3.

4.4. **Figure 5**)

4.4.1. Extract the fluorescence readings.

4.4.1.1. Export the fluorescence readings from the plate reader to a spreadsheet.

NOTE: In binding assays, it is important to account for the potential non-specific binding of a compound to the plastic surface of the plates

4.4.1.2. Calculate the mean values of triplicate measurements from each compound concentration for the three different plates: the specific binding readings from the coated wells (Plate A), the non-specific binding from the uncoated wells (Plate B) and the total Eu(III)-DO3AAm-CBP in solution in the uncoated wells (Plate C).

4.4.1.3. Determine the fluorescence values for the bound compound by subtracting the fluorescence readings of the uncoated wells (Plate B) from that of the coated wells (Plate A).

Equation 1: Determining the bound fluorescence[17]:

$$Bound\ fluorescnece = Specific\ (coated\ wells) - Unspecific\ (uncoated\ well)$$

4.4.1.4. Generate a calibration curve using the readings from the Eu(III)-labeled compound in solution (Plate C). Plot the fluorescence readings obtained against the concentration of the Eu(III)-labeled compound. Perform a linear regression fit.

4.4.2. Convert the fluorescence readings to concentrations.

4.4.2.1. Convert the readings of the bound fluorescence (step 4.2.3) into concentration using the standard fluorescence curve from the data generated using the compound concentrations in solution (step 4.2.1.4).

NOTE: When comparing the binding properties of one compound toward different target proteins that immobilize at different concentrations, the latter will need to be considered when calculating the amount of compound bound to the target (i.e., bound compound/protein).

4.4.2.2. Divide the concentration of the bound compound by the concentration of the protein immobilized in the well.

NOTE: For this calculation, use the concentration of immobilized collagen that was calculated after the wells were incubated with PBS for 1 h (so-called PBS mimic experiment; section 2.1 above). This is to account for potential losses of collagen during the incubation step and washing step so that those losses will not contribute to the final fluorescence signal.

4.4.2.3. Plot the data using a scatter plot that has the concentrations of the compound on the x-axis (µM) and the bound compound/protein on the y-axis.

4.4.3. Obtain the K_d values.

4.4.3.1. Fit the data acquired in step 4.2.2.3 using two possible binding kinetic models: one-site binding and one-site binding with a hill slope. The equations for each model are shown in Erreur ! Source du renvoi introuvable..

4.4.3.2. Choose the model that provides a non-ambiguous fitting with the highest R-squared value when fitting the data.

4.4.3.3. Exclude the outliers (s) for each set of fluorescence readings per concentration per plate.

4.4.3.4. Calculate the final K_d value, and present the data as the mean ± standard deviation of independent experiments.

NOTE: For robust results, perform triplicate measurements within each plate and at least three independent experiments with different microplates.

4.4.4. Calculate the fractional occupancy (FO).

NOTE: From Equation 2, the concentration of the target is unknown, and, therefore, by using algebra and the K_d, from Equation 3, a workable equation for calculating the fractional occupancy arises in the form of Equation 4.

Equation 2: Definition of fractional occupancy[17]:

$$Fractional\ Occupancy = \frac{()}{(target) + (compound \cdot target)}$$

Equation 3: The dissociation constant, K_d, which is the concentration at which the compound occupies 50% of the target at equilibrium[17]:

$$\frac{(compound) \cdot (target)}{compound \cdot target} = \frac{Koff}{Kon} = Kd$$

Equation 4: Rearranged equation to calculate the FO equation[17]:

$$Fractional\ Occupancy = \frac{(compound)}{(compound) + Kd}$$

4.4.4.1. Calculate the FO using the independent K_d values obtained for each individual plate. Plot the results, mean, and standard deviations of the FO against the concentration of the compound.

4.4.4.2. Report the FO with values ranging from 0 to 1 or as a percentage with values ranging from 0%–100%.

REPRESENTATIVE RESULTS:

Assessing the stability and concentration of type I and type III collagen immobilized in gels/films

The quantification of the collagen concentration immobilized per well was carried out using three different conditions: a) in wells without washing with PBS after immobilizing the proteins (no wash); b) in wells with a wash step (twice with PBS) after immobilization to remove any uncoated protein; c) in wells after incubation with PBS for 1 h (PBS mimic experiment). The PBS incubation mimicking step was performed to understand the stability of the collagen gel/film following incubation with a solution containing the candidate compound during the actual binding assay. Quantifying how much collagen is immobilized per well is crucial for accurately calculating and comparing the bound fraction and dissociation constant (K_d) of a compound toward different targets, especially when those targets have different coating efficiencies. Erreur ! Source du renvoi introuvable. shows a representative stability assay for type III collagen, from which it is evident that the amount of collagen that remained immobilized on the wells decreased when washing and/or incubation steps were included. The results demonstrate that a greater loss of collagen from the gel/film occurs during the washing step and less loss occurs during the incubation step. However, washing is a crucial step during an *in vitro* binding assay to ensure that the unbound compound is removed. For this reason, it is important that the amount of collagen that remains coated within the well, and not the amount of collagen initially immobilized, is calculated and used for the normalization of the results of the *in vitro* binding assay. The optimized conditions that resulted in the reproducible immobilization of collagen and the corresponding amount of collagen that was immobilized after following the washing and incubation steps are reported in Erreur ! Source du renvoi introuvable..

***In vitro* Eu(III) TRF binding assay to characterize the binding of Eu(III)-DO3AAm-CBP toward type I and type III collagen**

A representative analysis and results obtained from an *in vitro* Eu(III) TRF experiment to investigate the affinity of the Eu(III)-DO3AAm-CBP compound toward collagen type I and type III are shown in Erreur ! Source du renvoi introuvable.. The bound fluorescence readings and corresponding K_d values of the candidate collagen binding compound are shown in **Figure 8A**. The results show that the candidate compound bound non-specifically to type I collagen (as the curve does not reach saturation), and, therefore, no K_d value was reported. In contrast, the candidate compound was shown to bind to type III collagen with a K_d value of 4.2 µM ± 0.7 µM. As the coating efficiencies of type I and III collagen are different (as shown in **Table 3**), a normalization step was implemented to account for the amount of

immobilized collagen per well. To achieve that, the standard curve obtained from the total compound in solution (**Figure 8B**) was used to convert the bound fluorescence readings into concentration. These values were then normalized by dividing the bound fluorescence readings by the concentration of collagen coated onto the wells to obtain the plot shown in **Figure 8C**, in which the bound protein data for type I and III collagen could be presented on the same plot. It must be noted that although the K_d values were not altered by the normalization, in this particular example, the results could be plotted and directly compared using the same graph. The fractional occupancy results, shown in **Figure 8D**, demonstrated that 71% of the collagen was occupied by the compound at the highest concentration of 10 µM.

FIGURE AND TABLE LEGENDS:

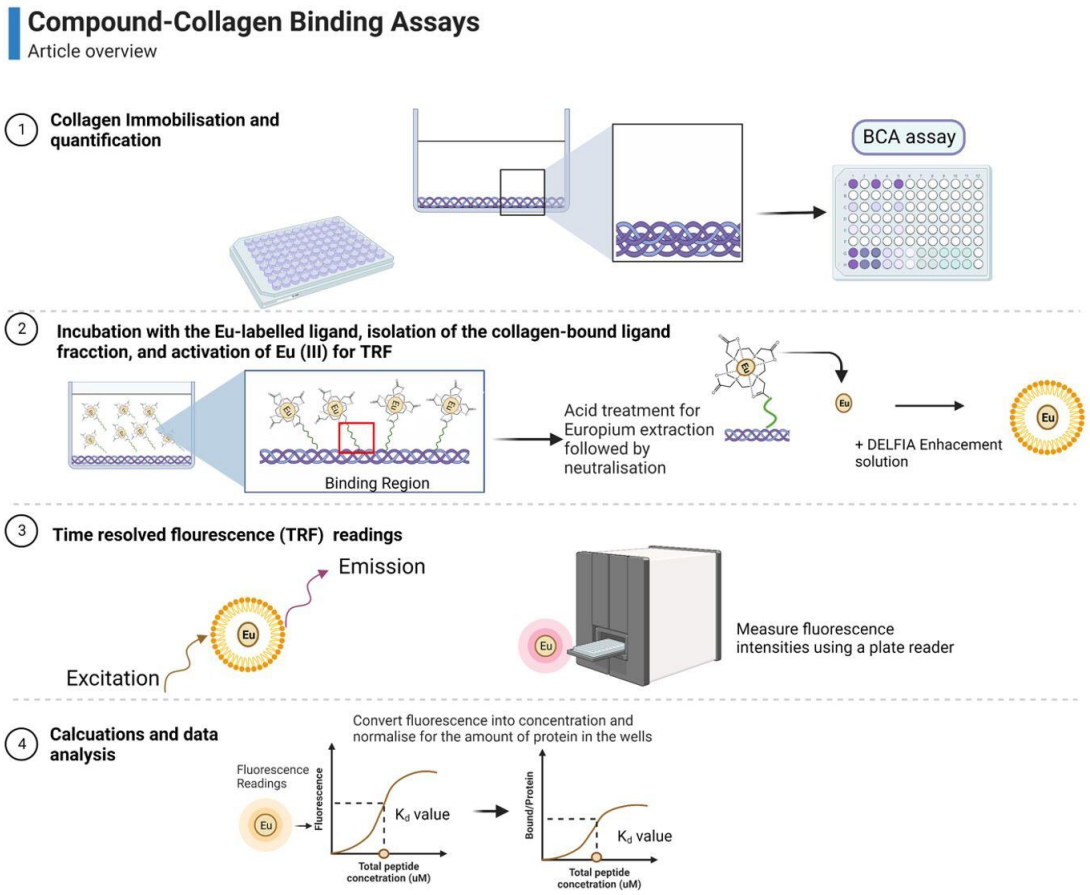


Figure 1: Overview of the methodology used to study compound–protein binding interactions using Eu(III) TRF. Created with BioRender.com.

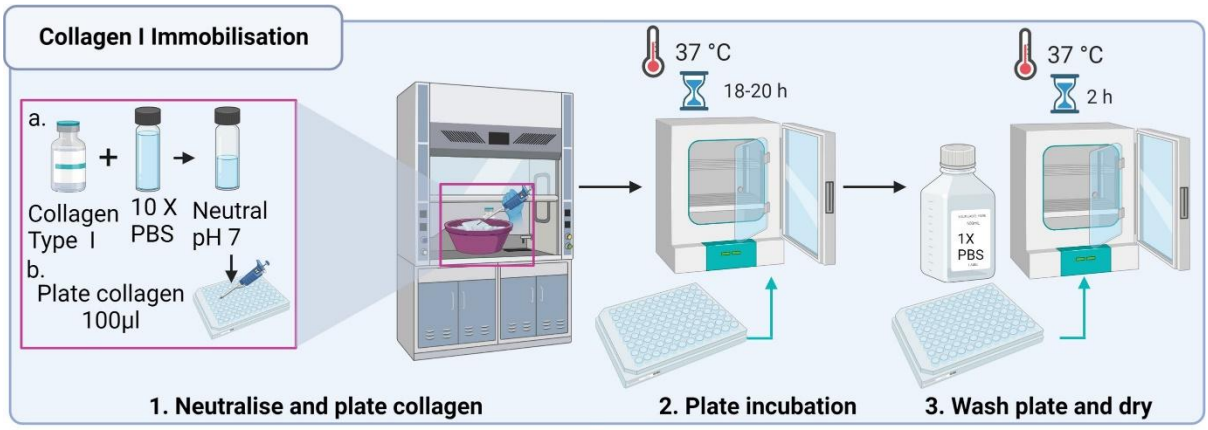


Figure 2: Workflow to immobilize type I collagen. Created with BioRender.com.

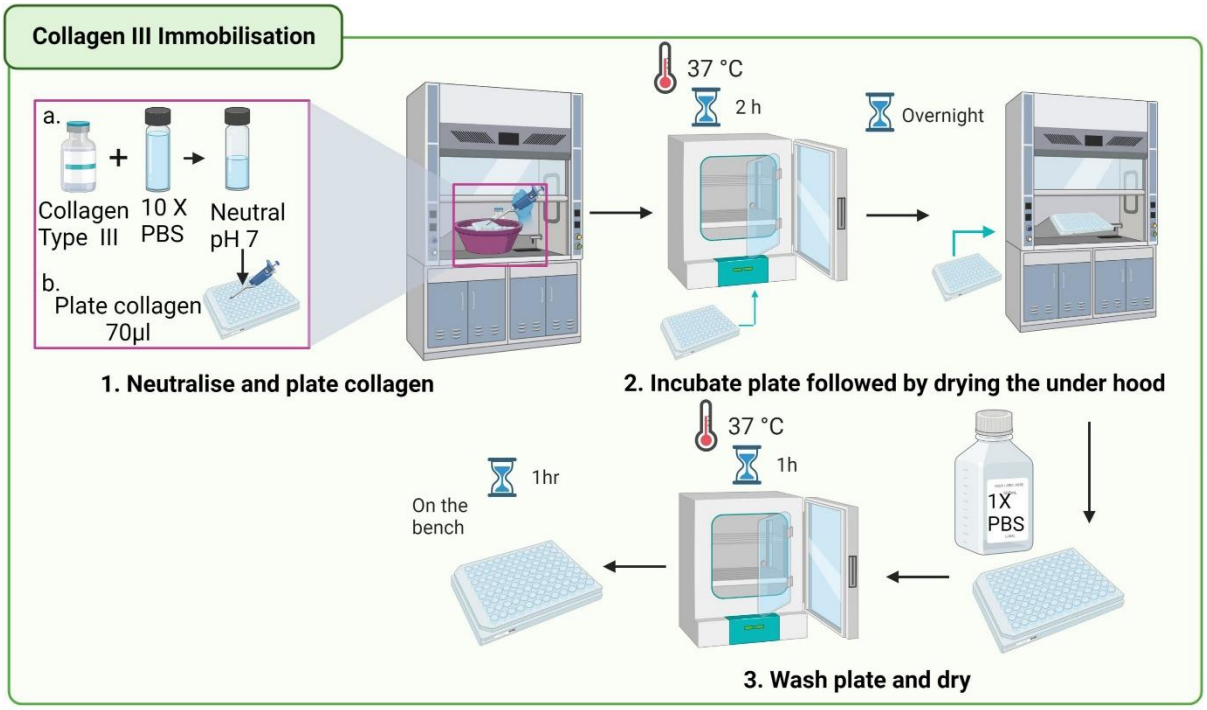


Figure 3: Workflow to immobilize type III collagen. Created with BioRender.com.

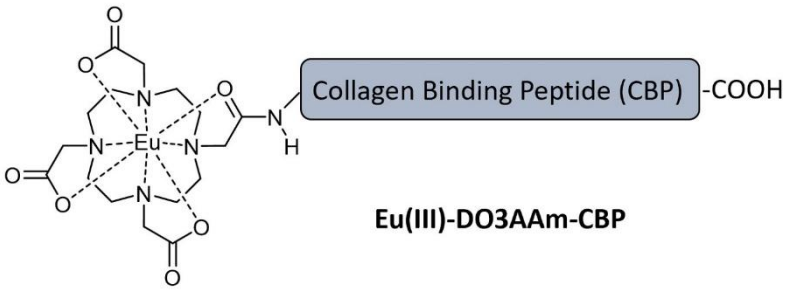


Figure 4: Chemical structure of the Eu(III)-DOTA-monoamide derivative carrying a collagen-binding peptide (CBP) used for the *in vitro* binding studies.

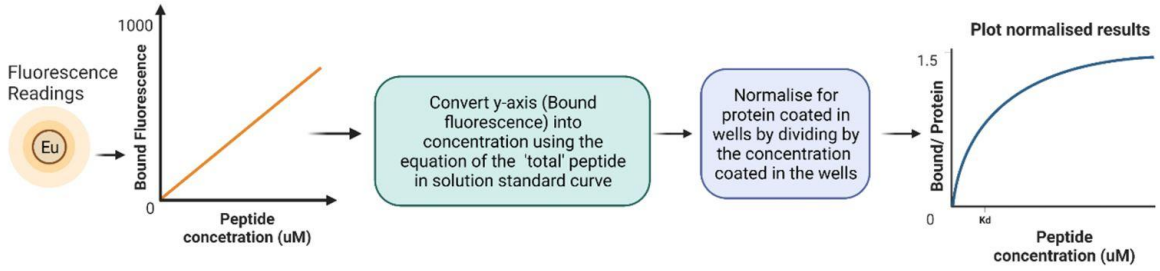


Figure 5: Overview of the data analysis pipeline to calculate the dissociation constant following the *in vitro* binding assay. Created with BioRender.com.

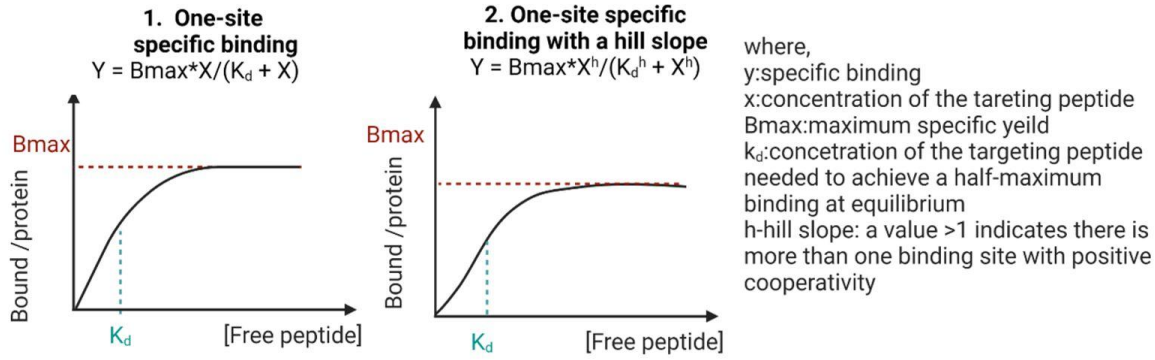


Figure 6: Fitting models used in the binding assays to obtain the K_d Values. Created with BioRender.com.

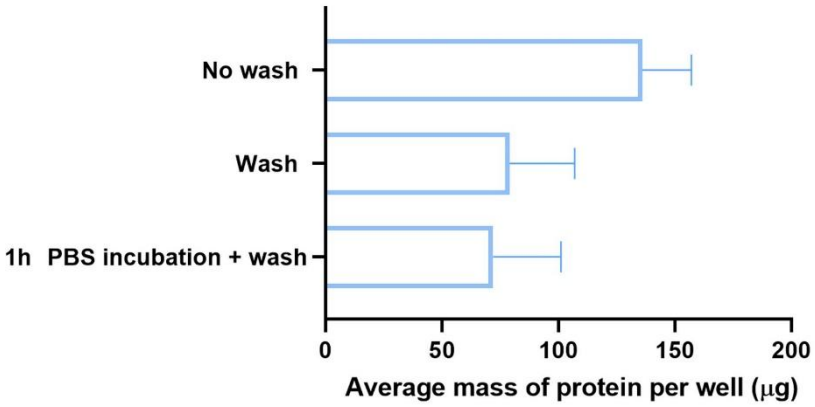


Figure 7: Amount of immobilized type III collagen in three experimental conditions. No wash = measuring the immobilized collagen directly after incubation; wash = washing the plate twice with 100 µL of PBS; 1 h PBS mimic & wash = incubation for 1 h with PBS followed by two rounds of washes with PBS to study the stability of the film before using the microplates for further *in vitro* binding experiments (n = 4 represented as the mean from duplicate readings and two independent experiments; error bars = standard deviation)

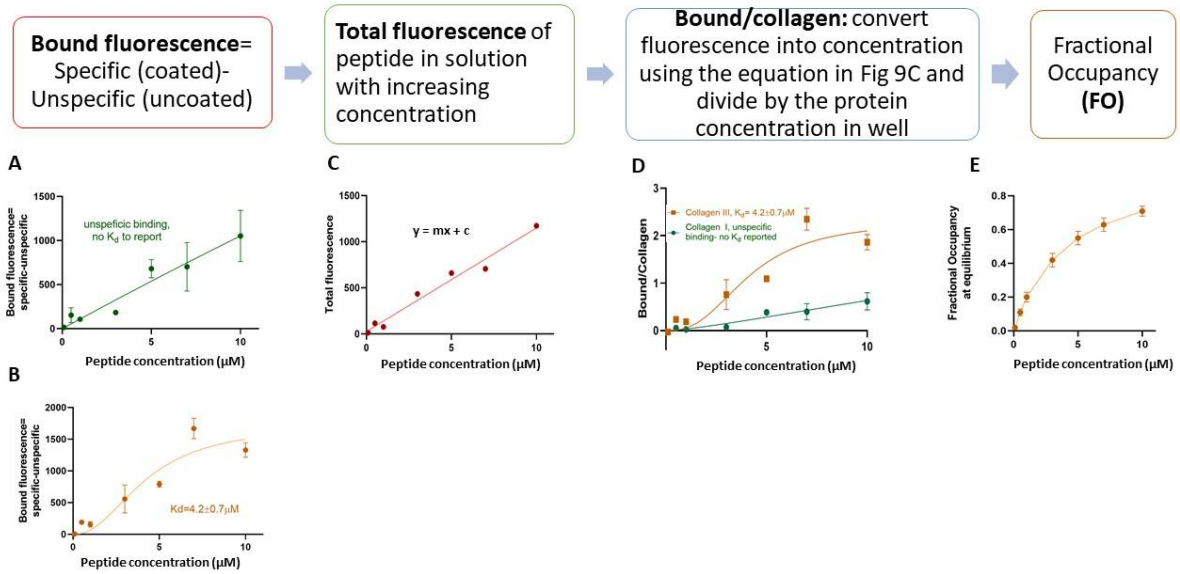


Figure 8: *In vitro* binding analysis pipeline. (**A**,**B**). *In vitro* Eu(III) TRF binding curves using Eu(III)-DO3AAm-CBP and immobilized type I and type III collagen, respectively. (**C**) Total fluorescence readings of the Eu(III)-DO3AAm-CBP in solution. (**D**) Calculation of the bound compound/protein by rearranging the equation from **Figure 8C** to convert the fluorescent values into concentration and then dividing by the concentration of the immobilized protein in the wells. (**E**) The fractional occupancy analysis of the candidate compound against type III collagen gave an FO of 71 %. The results reported are the mean values of triplicate readings within the same plate and from two independent plate readings (error bars = standard deviation).

Table 1: Summary of the main differences between the intrinsic characteristics of type I and type III collagen. Adopted from Eryilmaz et al.[13].

Characteristic	Collagen Type I	Collagen Type III
Branches	Linear	Globular with branch formation likely to collapse
Polarity	Non-polar	More polar
Fibril assembly	Rapid	Slower transition from globular to extended-high nucleation rate but slow assembly
Fibrils	Straight, lateral growth	Lower prosperity to nucleate straight fibrils-interconnected

Table 2: Parameters for Eu(III) TRF measurements.

Excitation Wavelength	340 nm
Emission Wavelength	619 nm
Integration Time	400 µs
Lag Time	400 µs

Table 3: Optimized conditions for the immobilization of type I and type III collagen and the quantified amount immobilized.

Protein	Immobilisation protocol	Coating characteristic	Volume added per well	Mass of collagen coated post-PBS mimic (µg)
Collagen Type I	37 ° tissue culture incubator overnight	Gel	100µl	240
Collagen Type III	37° for 2hrs; laminar flow under the hood overnight	Clear film	70µl	70

Supplemental File 1: Preparation of the acidic solution (AS) and buffering solution (BS) and calibration of the volume.

DISCUSSION:

This work presents a reproducible method for immobilizing type I and type III collagen. It also demonstrates a protocol for acquiring, analyzing, and interpreting *in vitro* Eu(III) TRF binding data to characterize the binding properties of a candidate ligand toward type I and III collagen. The protocols for immobilizing type I and type III collagen presented here were developed and optimized considering previously published work on type I and type III collagen fibrillogenesis *in vitro*[13,16]. Specifically, the reproducible coating of type I and III collagen was achieved by optimizing critical conditions, including the type of microplates, the pH, the temperature, and the ionic concentration of the buffer. Given that collagens self-assemble with increasing pH values and temperature, the neutralization and plating

steps for immobilizing collagen must be completed on ice and in the cold room. This ensures that the nucleation rate of collagen, the first step of fibrillogenesis, does not start before the collagen comes into contact with the well but rather occurs within the well after the temperature is increased during the incubation step. By quantifying the amount of protein in each well, it was found that type I collagen coated reproducibly and stably using non-binding specific microplates that were coated with a non-ionic hydrophilic surface. In contrast, type III collagen, showed reproducible and stable coating using tissue culture polystyrene- microplates that were exposed to plasma gas, making the microplate more hydrophilic. Immobilization protocols for type III collagen are less explored compared with those available for type I collagen, and, therefore, this protocol is unique. Although the immobilization protocols used for type I and type III collagen follow similar steps, there are crucial differences in the duration and temperature of the incubation due to the different intrinsic characteristics of the two collagen types. It is known that type III collagen has a faster nucleation rate and a slower growth rate than type I collagen, and these differences were considered when establishing this protocol (**Table 1**)[13]. For this reason, type III collagen requires incubation at 37 °C for only 2 h, whereas type I collagen is incubated for 18–20 h.

The coating efficiency—how much of each collagen was coated onto the wells—was also studied, and the stability of the coating under different protocols, especially after washing and after incubating with PBS for 1 h, was investigated. The stability of the coated protein is crucial for the reproducible screening of the binding properties of a compound toward the target and the accurate calculation of the K_d and FO values. This is a step that is rarely performed, and binding protocols typically only quantify the concentration of the immobilized protein at the start of the experiment (after immobilization but prior to the incubation)[16]. However, because both the coating efficiency and stability of the coated protein may vary between different proteins, it is important to determine how much of the initial protein remains stably coated within the well at the end of the binding experiment and, therefore, contributes toward the final measurement. Knowing that there is a reproducible amount of protein coated on the plates is important when studying the interactions of these proteins with binding molecules, as this contributes directly to the fluorescence reading from the Eu(III)-labeled compound. Normalizing the bound fluorescence measurements with the amount of protein immobilized in the well (bound protein) does not affect the K_d or FO per se. However, such normalization leads to the rescaling of the data plotted on the y-axis of the binding curve, enabling the direct comparison of the binding profile of the same compound toward different targets with different immobilization efficiencies.

In the analysis, it is also important to account for the non-specific binding of the compound to the experimental matrix (i.e., the plastic plate) rather than assuming that the readings acquired from the coated wells solely represent compound bound to collagen. This was accounted for by subtracting the non-specific binding of the compound to the plate (uncoated wells) from the specific reading, represented as “bound fluorescence” (collagen-coated wells), under the same TRF measurement conditions.

Eu(III) TRF-based methods have been widely used for *in vitro* binding studies[23–25]. It is a flexible assay that can be used in multiple applications, such as immune assays, cell cytotoxicity studies, enzyme assays, and binding studies, as demonstrated here and in

previous work[23,26]. It is also highly sensitive and takes advantage of time-resolved fluorescence properties to eliminate any non-specific background fluorescence signals that may be observed in conventional direct readings[21]. In standard DELFIA applications, users are able to use the standard enhancement solution to efficiently extract Eu(III) from its complexes. However, the decomplexation of Eu(III) from Eu(III)-DO3AAm-like complexes is difficult because of the very high kinetic inertness of such complexes, which means that decomplexation will be far from being complete and reproducible. A protocol to achieve Eu(III) decomplexation from such complexes has been proposed by De Silva et al.[21]. Such a protocol involves the incubation of the Eu(III)-DO3AAm complex with a very acidic solution (AS, 2 M HCl) for 2 h, followed by the addition of a neutralizing solution (NS, 2 M NaOH) to bring the pH back to neutrality. The enhancement solution is then added to activate the Eu(III) and to adjust the pH to be in the optimal range (3.0–3.5) for TRF readings. However, it was found that small volume inaccuracies in the amounts of acid and neutralizing solutions added to microwells could lead to significant fluctuations in the final pH, which could affect the intensity of the Eu(III) TRF signal. Thus, the protocol was modified by adding a strong glycine buffer to the NaOH solution to obtain the buffering solution (2 M NaOH and 2 M glycine). After treatment with the acid solution to extract Eu(III), the pH is adjusted directly to 3.2 in a very consistent and reproducible manner by the addition of a calibrated amount of the buffering solution. Finally, the enhancement solution can be added for Eu(III) re-coordination and self-association into TRF active micelles.

A limitation of the Eu(III) TRF assay is that the maximum concentration of the Eu(III)-labeled compound that can be used in the assay is 10–15 µM because, at higher concentrations, the extraction of Eu(III) and the formation of micelles are no longer linear. Another method that has been used to measure K_d values is by using compounds labeled with other lanthanides, such as Gd(III). In this approach, the Gd(III) concentration is measured in the supernatant collected after the incubation of the Gd(III)-labeled compound with the target using ICP-MS. The ICP-MS of the supernatant collected after the incubation of the compound provides an indirect measurement of the bound fraction remaining in the well by subtracting the amount of Gd(III) in the supernatant from the “total gadolinium” concentration that was initially added in the well. Despite the indirect measurement of the “bound fraction”, this approach yields comparable binding affinities to those measured with high-performance liquid chromatography (HPLC)[16]. The ICP-MS method is useful in cases where the K_d may be high, meaning concentrations of the compound higher than the 10–15 µM limit of the DELFIA assay are required in order to reach the plateau (saturation) of the binding curve and, thus, accurately calculate the K_d. Another technique to study the interactions between molecules is SPR[27]. This is an optical technique based on the immobilization of a target protein on a gold film and the analysis of the binding of another protein/molecule in a mobile state. The advantage of SPR is that it measures the interactions directly with no need for any fluorophore or lanthanide complexation to measure the binding response. However, the limitations of this technique include the complexity of optimizing the immobilization process (which can compromise the binding of the epitope), the difficulty of determining non-specific binding, and the cost[28].

This study presents an optimized protocol that enables the reproducible immobilization of type I and type III collagen, a detailed methodology for performing Eu(III) TRF assays to

study compound–collagen interactions, and a clear workflow for analyzing and interpreting the results.

ACKNOWLEDGMENTS:

We are grateful to the following funders for supporting this work: (1) the UK Medical Research Council (MR/N013700/1) and King's College London member of the MRC Doctoral Training Partnership in Biomedical Sciences; (2) BHF program grant RG/20/1/34802; (3) BHF Project grant PG/2019/34897; (4) King's BHF Centre for Research Excellence grant RE/18/2/34213; (5) the ANID Millennium Science Initiative Program – ICN2021_004; and (6) ANID Basal grant FB210024.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

REFERENCES:

1. Distler, J. H. W. et al. Review: Frontiers of antifibrotic therapy in systemic sclerosis. *Arthritis and Rheumatology*. **69** (2), 257–267 (2017).
2. Wynn, T. A. Fibrotic disease and the TH1/TH2 paradigm. *Nature Reviews Immunology*. **4** (8), 583–594 (2004).
3. Saha, P. et al. Magnetic resonance T_1 relaxation time of venous thrombus is determined by iron processing and predicts susceptibility to lysis. *Circulation*. **128** (7), 729–736 (2013).
4. Mirshahi, M. et al. Defective thrombolysis due to collagen incorporation in fibrin clots. *Thrombosis Research*. **8**, 73–80 (1988).
5. Comerota, A. J. The ATTRACT trial: Rationale for early intervention for iliofemoral DVT. *Perspectives in Vascular Surgery and Endovascular Therapy*. **21** (4), 221–225 (2009).
6. Bateman, E. D., Turner-Warwick, M., Adelmann-Grill, B. C. Immunohistochemical study of collagen types in human foetal lung and fibrotic lung disease. *Thorax*. **36** (9), 645–653 (1981).
7. Pawelec, K. M., Best, S. M., Cameron, R. E. Collagen: A network for regenerative medicine. *Journal of Materials Chemistry B*. **4** (40), 6484–6496 (2016).
8. Frantz, C., Stewart, K. M., Weaver, V. M. The extracellular matrix at a glance. *Journal of Cell Science*. **123** (24), 4195–4200 (2010).
9. Copes, F., Pien, N., Van Vlierberghe, S., Boccafoschi, F., Mantovani, D. Collagen-based tissue engineering strategies for vascular medicine. *Frontiers in Bioengineering and Biotechnology*. **7**, 166 (2019).
10. Veis, A. The biochemistry of collagen. *Annals of Clinical and Laboratory Science*. **5** (2), 123–131 (1975).
11. Bielajew, B. K., Hu, J. C., Athanasiou, K. A. Collagen: Quantification, biomechanics, and role of minor subtypes in cartilage. *Nature Reviews Materials.* **5**, 730–747 (2020).
12. Zhao, Z. et al. Structural and functional plasticity of collagen fibrils. *DNA and Cell Biology*. **38** (4), 367–373 (2019).
13. Eryilmaz, E., Teizer, W., Hwang, W. In vitro analysis of the co-assembly of type-I and type-III collagen. *Cellular and Molecular Bioengineering*. **10** (1), 41–53 (2017).
14. Jagnow, J., Clegg, S. *Klebsiella pneumoniae* MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. *Microbiology*. **149** (9), 2397–2405 (2003).
15. O'Sullivan, D., O'Neill, L., Bourke, P. Direct plasma deposition of collagen on 96-well

polystyrene plates for cell culture. *ACS Omega*. **5** (39), 25069–25076 (2020).
16. Caravan, P. et al. Collagen-targeted MRI contrast agent for molecular imaging of fibrosis. *Angewandte Chemie - International Edition*. **46** (43), 8171–8173 (2007).
17. Copeland, R. A. *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*. John Wiley & Sons, Ltd. Hoboken, NJ (2000).
18. Salahudeen, M. S., Nishtala, P. S. An overview of pharmacodynamic modelling, ligand-binding approach and its application in clinical practice. *Saudi Pharmaceutical Journal*. **25** (2), 165–175 (2017).
19. Bünzli, J. C. G., Piguet, C. Taking advantage of luminescent lanthanide ions. *Chemical Society Reviews*. **34** (12), 1048–1077 (2005).
20. Hemmilii, I. Luminescent lanthanide chelates - A way to more sensitive diagnostic methods. **225** (1–2), 480–485 (1995).
21. De Silva, C. R., Vagner, J., Lynch, R., Gillies, R. J., Hruby, V. J. Optimization of time-resolved fluorescence assay for detection of europium-tetraazacyclododecyltetraacetic acid-labeled ligand-receptor interactions. *Analytical Biochemistry*. **398** (1), 15–23 (2010).
22. Digilio, G., Lacerda, S., Lavin Plaza, B., Phinikaridou, A. Extracellular matrix targeted MRI probes. *Analysis & Sensing*. **3** (1), e202200039 (2022).
23. Phinikaridou, A. et al. Tropoelastin: A novel marker for plaque progression and instability. *Circulation. Cardiovascular imaging*. **11** (8), e007303 (2018).
24. Guzaeva, T. V. et al. Protein A used in DELFIA for the determination of specific antibodies. *Immunology Letters*. **35** (3), 285–289 (1993).
25. Nasiri, A. H., Nasiri, H. R. Polymerase assays for lead discovery: An overall review of methodologies and approaches. *Analytical Biochemistry*. **563**, 40–50 (2018).
26. Capuana, F. et al. Imaging of dysfunctional elastogenesis in atherosclerosis using an improved gadolinium-based tetrameric MRI probe targeted to tropoelastin. *Journal of Medicinal Chemistry*. **64** (20), 15250–15261 (2021).
27. Drescher, D. G., Drescher, M. J., Ramakrishnan, N. A. Surface plasmon resonance (SPR) analysis of binding interactions of proteins in inner-ear sensory epithelia. *Methods in Molecular Biology*. **493**, 323–343 (2009).
28. Murali, S., Rustandi, R. R., Zheng, X., Payne, A., Shang, L. Applications of surface plasmon resonance and biolayer interferometry for virus–ligand binding. *Viruses*. **14** (4), 717 (2022).