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Research article

Physico-chemical characterization studies of collagen labelled with Ru(II) polypyridyl complex

P. Anithabanu [a,b], Surabhya Balasubramanian [a,b], P. David Dayanidhi [a,b], T. Nandhini [a,b], V.G. Vaidyanathan [a,b,*]

[a] Advanced Materials Laboratory, CSIR-Central Leather Research Institute, Adyar, Chennai 600020, India
[b] Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India

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ABSTRACT

The rich luminescence behaviour exerted by transition metal complexes has found significant role in the development of biomolecular and cellular probes. The conjugation of fluorophore to a protein has its own advantage over the label-free system due to its high sensitivity. While numerous proteins have been labelled with either organic or inorganic fluorophores, the conjugation of luminescent transition metal complexes with collagen has not yet been attempted. Here, in this study, the conjugation of a Ru(II) polypyridyl complex with collagen was carried out and its physico-chemical characterization was studied. The conjugation of Ru(II) to collagen was characterized by UV-Visible, fluorescence and ATR-FT-IR spectroscopy. The conjugation of Ru(II) did not alter the triple helical structure of the collagen as evidenced from CD spectral data. The luminescence behaviour of the Ru-tagged collagen was found to be similar to that of the commercially available fluorescein isothiocyanate (FITC) tagged collagen with increase in luminescence upon addition of collagenase. Gel-based collagenase assay showed that the digestion of collagen can be vizualized using UV light due to intrinsic fluorophore tag without carrying out the staining-destaining processes. Energy dispersive X-Ray analysis (EDAX) confirms the presence of Ru in Ru-collagen fibrils. To the best of our knowledge, this is the first report on the conjugation of a Ru(II) complex with the fibrous protein collagen that exhibits similar property as of FITC-collagen and can be used as an alternative.

1. Introduction

Radiolabelling is a technique commonly used in biochemical research and other related fields to track chemical or biological events [1]. The use of radioactive isotopes, despite having tremendous advantages due to its sensitivity, also poses serious environmental risk. One of the most commonly used organic fluorophores for the labelling of proteins or DNA is fluorescein isothiocyanate (FITC) [1, 2, 3, 4, 5, 6, 7]. However, fluorescein has its own drawbacks such as Stokes' shift of 30 nm, shorter lifetime and higher light-scattering effects [8]. To overcome these drawbacks, transition metal complex-based probes have been developed for biological applications.

In recent years, there is a surge in the use of transition metal complexes as probes for biological molecules. Among the transition metal systems, lanthanides, Ru(II) and Ir(III) have been extensively studied as cell imaging dyes due to its strong luminescence behavior [9, 10]. In contrast to organic fluorophores, metal complex based systems exhibit large Stokes shifts and prevents self-quenching and homo-fluorescence resonance energy transfer [9, 10]. One of the major advantages of metal-based probes is its photostability which is crucial for monitoring biological events through fluorescence or microscopy. Further, the application of the metal complexes can be easily tuned by varying ancillary ligands thereby altering the structural, electronic and coordination behaviour [10, 11, 12, 13, 14, 15, 16].

Covalent tagging of metal complexes has been studied for cell lineage tracing in embryonic cells, to understand the dynamic activities of living cells, probes of phagocytosis and immunoassay and immunofluorescence [8, 17, 18, 19, 20, 21, 22, 23]. The labelling of the proteins has been carried out either by using isothiocyanate complexes for albumins or maleimide complexes for conjugation with cysteine based biomolecules [24]. Aldehyde-based multiple reactive sites in the ligands of cyclometalated Ir(III) complexes have been used for the crosslinking of primary amines of two proteins [10]. Similarly, rhenium-based complexes also have been used to crosslink proteins [9]. Apart from the tagging of the proteins, metal complexes have also been used as staining agents by

* Corresponding author.
E-mail address: vaidyanathan@clri.res.in (V.G. Vaidyanathan).

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covalent labelling of cellular components through reactive aldehyde groups [9].

Ru(II) polypyridyl complexes have been extensively used for conjugation with biomolecules such as poly(L-lysine), albumins and immunoglobulin G [8, 13, 16, 22]. The conjugation of Ru(II) polypyridyl complexes did not reduce the activity of IgG and have been considered as potential candidates for immunoassays. Adamson et al. have studied the RGD labelled Ru(II) polypyridyl conjugates as reporters for integrin conformation [16]. Lin et al. demonstrated the application of sulfhydryl reactive Ru(II) polypyridyl complex that conjugates with protein G as a universal agent for fluorescent immunoassays.

So far, the conjugation of metal complexes has been carried out for globular proteins. However, reports on the conjugation of fibrous proteins by either organic or inorganic fluorophores are very limited. Various researchers have demonstrated the activity of collagenase using fluorescently-tagged truncated or synthetic peptides. In this study, we have explored the conjugation of Ru(II) (**1**) with a fibrous protein, i.e., collagen. To our knowledge this is the first report on metal complex-collagen conjugation that exhibits similar behaviour to commercially available FITC-collagen. The advantage of this approach is to study the collagenase activity using fluorescence spectroscopy as well as gel imaging by avoiding the staining-destaining process.

2. Experimental section

$RuCl_3.xH_2O$, 1,10-phenanthroline, 4-formylbenzoic acid and fluorescein isothiocyanate tagged collagen were purchased from Sigma-Aldrich India Limited. All the solvents used in this study were used as received. *cis*-$Ru(phen)_2Cl_2$, 2-(4-carboxyphenyl)imidazo[4,5-*f*] [1,10] phenanthroline (PIC) and $[Ru(phen)_2(PIC)]^{2+}$ (**1**) were prepared according to previously reported protocols [16]. The active ester $[Ru(phen)_2(PIC\text{-}NHS)]^{2+}$, was prepared by stirring $[Ru(phen)_2(PIC)]^{2+}$ (0.06 mmol), N-hydroxysuccinimide (0.045 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.41 mmol) in acetonitrile medium for 6 h at room temperature. The product was collected by centrifugation and dried in an oven.

2.1. Conjugation of Ru(II) with collagen

To the acetate solution of collagen (0.08 mg/mL) $[Ru(phen)_2(PIC\text{-}NHS)]^{2+}$ (0.2 mg/mL) was added. The pH of the solution was adjusted to 7.5 using PBS buffer and stirred for 30 min at 0 °C. After the completion of the reaction, the pH was lowered to 4.5 using acetate buffer. The unreacted Ru(II) was removed by dialysis against acetate buffer (pH 4.5). Dialysis was carried out for 2 days with replacement of fresh buffer every six hours. The concentration of collagen in Ru-collagen was estimated using hydroxyproline assay. The bound Ru in collagen was calculated using the standard curve of Ru(II) complex with the absorption at 450 nm.

2.2. Fibrillation kinetics assay

The effect of Ru(II) conjugation on fibril formation of collagen(0.5 μM) was measured at 313 nm using a Shimadzu UV1800 spectrophotometer attached with a circulating water bath [25]. The fibril formation was measured in terms of turbidity after adjusting the pH to alkaline by using 0.5 M NaOH. The turbidity ($t_{1/2}$) was used to determine the rate of fibril formation.

2.3. Circular dichroic studies

The impact of covalent conjugation of Ru(II) on the secondary structure of collagen were studied using a JASCO J-815 spectropolarimeter under N_2 atmosphere. CD spectra of collagen and Ru-collagen in 10 mM acetate buffer were measured using quartz cell of path length 0.1 cm at 25 °C. The samples were scanned thrice and accumulated data was averaged and smoothened.

2.4. ATR-FT-IR analysis

The samples of collagen and Ru-collagen were further characterized using PerkinElmer FTIR Two model spectrometer attached with ATR accessory. FT-IR spectra for the collagen and Ru-collagen were recorded over the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}.

2.5. Luminescence studies

In order to understand the stability and reactivity of Ru-collagen on collagenase, luminescence studies were performed by reacting the collagenase and Ru-collagen. The results were compared with the luminescence profile of FITC-collagen. The emission spectra were recorded using Hitachi F-7000 fluorescence spectrophotometer equipped with Peltier temperature control. The luminescence spectra were recorded at two different temperatures (25 and 37 °C) for both FITC-collagen (9 μg/mL) and Ru-collagen (9 μg/mL) samples at 0 and 60 min after incubation with the enzyme (0.04 μg).

2.6. Microscope studies

Microscope images of collagen and Ru-collagen were recorded using CLARA GMU field emission scanning electron microscope (FESEM) in ultra-high resolution mode from M/s. TESCAN Brno. Czech. The samples were diluted and drop casted on a mica surface and dried under room temperature overnight. The dried samples were coated with gold using a sputter coater (M/s. Quorum Technologies). To determine elemental composition in the collagen/Ru-collagen samples, energy dispersive X-Ray analysis (EDAX) was performed using EDAX Octane Plus probe.

2.7. PAGE analysis

To study the collagenase activity on the Ru-collagen and unmodified collagen, SDS-polyacrylamide gel electrophoresis (PAGE) was carried out. The samples containing either collagen (9 μg) or Ru-collagen (9 μg) were incubated with collagenase (0.02 μg and 0.04 μg) for 1 h at 25 and 37 °C. The samples were loaded on 10% resolving gel and run at 100 V for 5 h. The samples were fixed and either stained with Coomassie blue dye or directly scanned under UV light.

3. Results and discussion

The preparation of Ru complex (**1**) was performed using the reported procedure (Figure 1) [16]. The formation of the complex was characterized by ESI-MS and ^1H NMR spectroscopy (Figure 2 and Figure 3). UV-visible spectrum of the complex shows intense ligand-centered (LC) transitions between 330-350 nm and low-energy MLCT band was observed at 450 nm. The emission spectrum of the complex exhibited the characteristic ^3MLCT band at 600 nm. The conjugation of Ru complex with collagen was carried out using the NHS ester of Ru complex. The reaction mixture was stirred for 3 h followed by dialysis for 48 h with

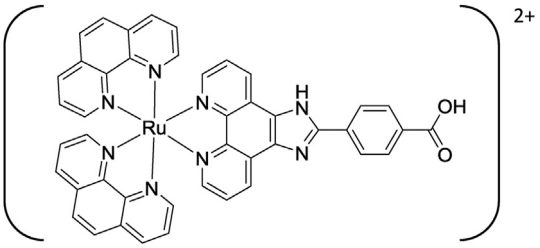

Figure 1. Chemical structure of $[Ru(phen)_2(PIC)]^{2+}$ (**1**).

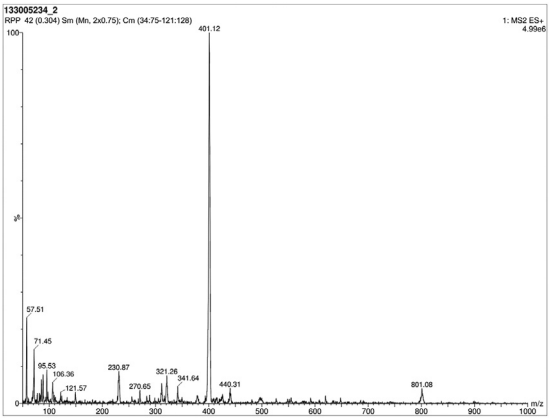

Figure 2. ESI mass spectrum of $[Ru(phen)_2(PIC)]^{2+}$(Theoretical mass M^{2+}: 402.580).

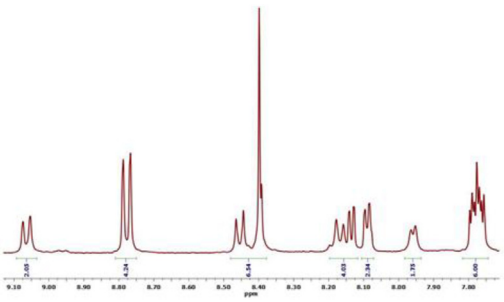

Figure 3. ^1H NMR spectrum of $[Ru(phen)_2(PIC)]^{2+}$.

buffer change every six hours to remove the unbound Ru molecules. The extent of conjugation was measured using the standard curve plot.

To study the impact of Ru(II) conjugation on the collagen triple helical structure, circular dichroic studies were carried out. Typically, collagen exhibits a positive band at 222 nm and a negative dip at 190 nm. A similar profile was observed for the Ru(II) conjugated collagen (Figure 4)which leads us to conclude that conjugation of the Ru(II) with collagen did not alter the triple helical nature of the protein. Vibrational spectroscopic studies showed that Ru(II) conjugation did not alter the amide bonds but leads to a 79 cm^{-1} shift of the amide A from 3437 to 3358 cm^{-1} (Figure 5). However, in the amide II band, a marginal shift of

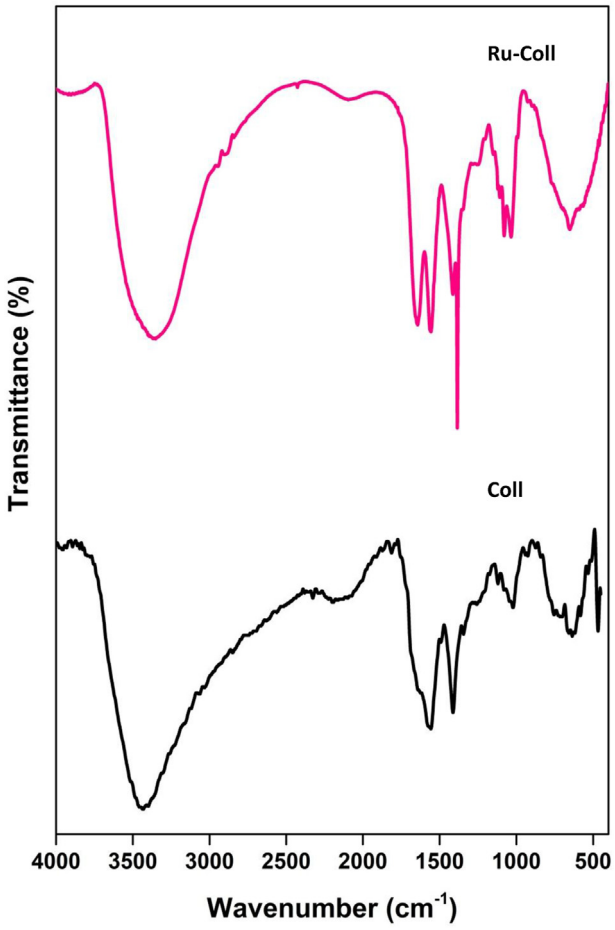

Figure 5. ATR-FT-IR spectra of collagen (bottom) and Ru-collagen (Top).

5 cm^{-1} was observed after Ru(II) conjugation. The IR data further confirms the conjugation of Ru to the collagen.

Fibrillation assay was carried out to study the impact of Ru(II) conjugation on the collagen fibril. As seen from Figure 6, Ru(II) conjugation delayed the fibril formation compared to unmodified collagen control. From the turbidity measurements, $t_{1/2}$ of Ru-collagen was found to be 444 s while for the control it was 261s under similar conditions.

Luminescence studies were carried out by exciting Ru-collagen at 450 nm and the emission maxima was observed at 590 nm, characteristic of

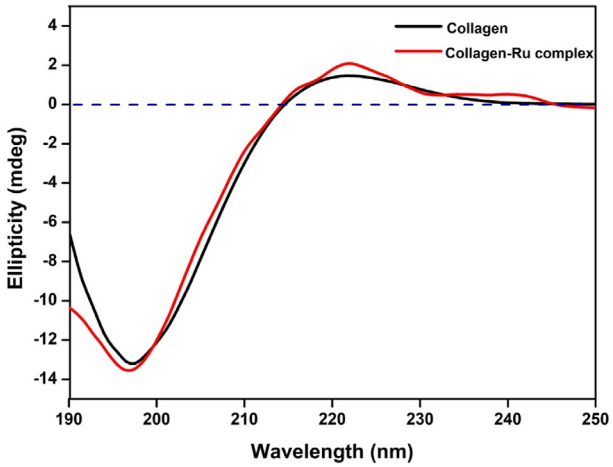

Figure 4. CD spectra of collagen and Ru-collagen.

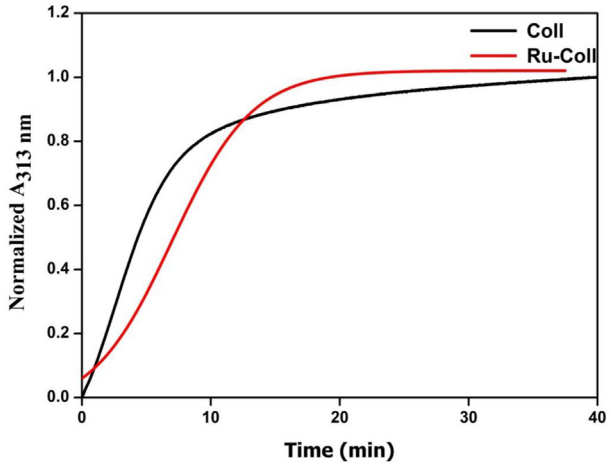

Figure 6. Fibrillation kinetics of collagen and Ru-collagen.

the ^{3}MLCT of Ru(II). On the other hand, unlabelled collagen did not exhibit any luminescence at 600 nm. The luminescence of collagenase treated Ru-collagen was also recorded and compared with the FITC-collagen luminescence profile (Figures 7 and 8). For FITC-collagen, the emission maxima was observed at 525 nm when excited at 490 nm. Upon collagenase treatment, both FITC-collagen and Ru-collagen exhibited similar behaviour where a significant enhancement in the luminescence was observed. Collagenase treated Ru-collagen exhibited a near 2-fold increase in emission compared to untreated control (Ru-collagen) while collagenase treated FITC-collagen exhibited a 3-fold increase compared to the untreated one. Further, collagenase treated Ru-collagen did not show any significant enhancement in emission at 25 °C compared to a 2-fold increase at 37 °C. On the other hand, no significant increase in emission was observed for collagenase treated FITC-collagen at both temperatures. The results obtained in this study suggest that the enhancement in the emission profile after enzymatic digestion could be due to the release of fluorophore from the entangled collagen. In contrary to FITC-collagen, Ru-collagen is highly sensitive to temperature as luminescence was found to increase with temperature.

Electron microscopic studies have been carried out to understand the morphology of collagen after conjugation with Ru(II). In the control (unlabelled) collagen, the fibrils are closely knitted as seen in Figures 9a and 9b. After conjugation with Ru(II), the highlighted areas (red circles) were found to be almost uniformly distributed in the collagen fibre network. EDAX analysis of the SEM micrograph revealed the presence of Ru(Figure 9c).

Further, the analysis of collagenase activity was carried out using SDS-polyacrylamide gel electrophoresis (PAGE). Unlabelled and Ru-labelled collagen were subject to collagenase digestion at two different temperatures viz., 25 and 37 °C. After electrophoresis, the gel was fixed and exposed to UV-light. Due to the excellent photoluminescence behaviour of Ru(II), the digestion of Ru-collagen was monitored and a

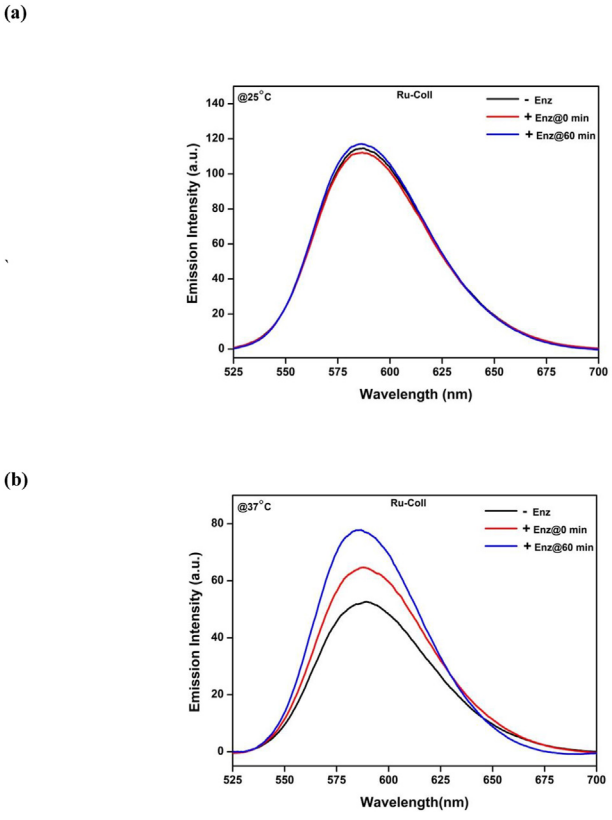

Figure 7. Luminescence spectra of Ru-collagen without and with collagenase (Enz) at (a) 25 °C and (b) 37 °C (λ_{ex}: 450 nm) in 10 mM acetate buffer (pH 4.5).

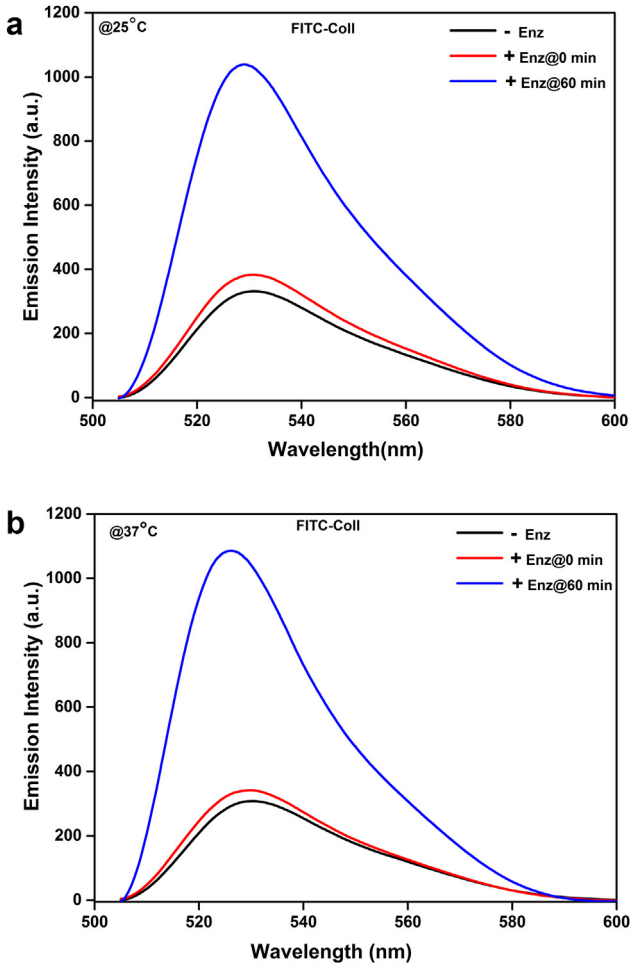

Figure 8. Luminescence spectra of FITC-collagen without and with collagenase (Enz) at (a) 25 °C and (b) 37 °C (λ_{ex}: 495 nm) in 10 mM acetate buffer (pH 4.5).

decrease in intensity of the bands were seen due to fragmentation of the collagen (Figure 10a). However, in the non-labeled one, collagen or digested fragments could not be observed under UV light. After staining with Coomassie blue followed by the destaining process, both collagen and Ru-collagen exhibited well-defined and fragmented bands (Figure 10b).

The labelling of proteins generally involves the modification of tyrosines and ε-amino groups of lysine, carboxyl groups of glutamic and aspartic acids and –SH groups from the reduction cysteine residue [15]. The site at which modification takes place is also important as some amino acid modification leads to partial or complete loss of activity. The conjugation of metal complexes to various proteins especially globular proteins have been previously explored as biological tools in cell imaging experiments and kinetic assays [26]. Several phosphorescent metal complexes have been used as organelle or cellular compartment dyes while Ru(II) and Ir(III) complexes have been used as probes for DNA and protein conformational studies [10]. Researchers have used covalent modification of proteins with relatively low molecular weight metal complexes as an alternative for bulkier probes such as GFP [8]. Further, the conjugation is mostly carried out using activated NHS ester and targeting the lysine residues at alkaline pH (pH 8). There are other modes such as targeting the cysteine residues through maleimide chemistry. Since globular proteins are less prone to gel formation at pH 8.0 or above, conjugation of small molecules is quite simple. Commercially, only two fluorophores are having the Stokes shift of 30 nm or more, i.e. 5-carboxyfluorescein (36 nm) and Nile Red (70 nm) and the cost of NHS esters is also quite expensive.

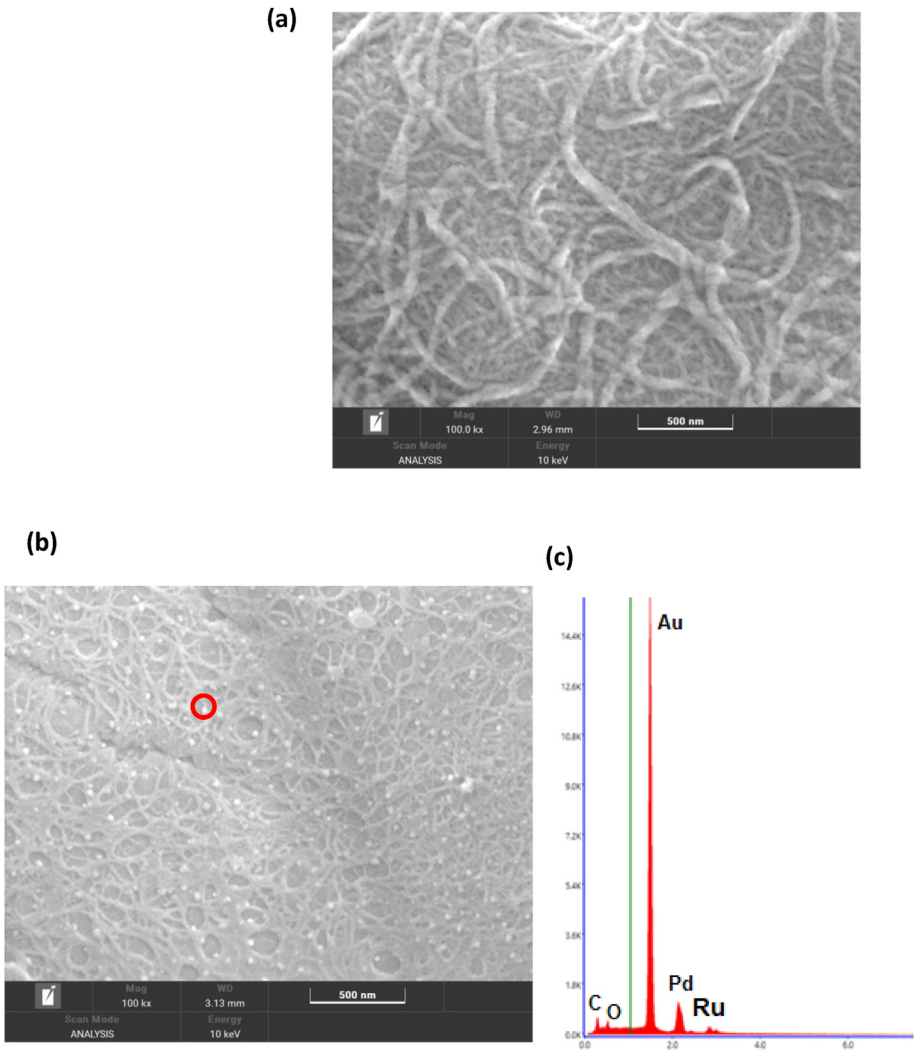

Figure 9. Scanning electron microscopic images of (a) collagen, (b) Ru-collagen and (c)EDAX analysis of the Ru-collagen fiber at the red circle. Red circle represents the content of Ru.

In contrast to globular proteins, the conjugation of fibrous proteins is tricky and tedious. Being insoluble in water, collagen is stable and soluble in acetate buffer (pH 4.5). As the pH of the solution increases, collagen starts to form a gel at pH 8.0. Due to this reason, only very few fluorophores or fluorescence-based substrates are used for collagenase assay. Fluorescein tagged collagen is commercially available and the process of conjugation is not known but it is presumed to be freely loaded dye on the collagen matrix. During the digestion of collagen with collagenase, the dye is released and fluorescence intensity increases. Further the organic dye is not as stable as seen for various metal complexes and it undergoes photobleaching. However, these issues are addressed with metal based fluorophores that are highly stable with minimal photobleaching behaviour. Apart from commercial availability of FITC collagen as substrate for collagenase, there are two other fluorogenic substrates available for Matrix metalloproteinase (MMP) that are based on dinitrophenyl tagged oligopeptides *viz.*, MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2[Dpa = 3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Nva = L-norvaline] for MMP-2 and MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 ·TFA [Dpa = N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; MCA: (7-methoxycoumarin-4-yl)acetyl for MMP-2 and MMP-7 enzymes [27, 28, 29, 30, 31]. Using capillary gel electrophoresis with laser induced dynamic fluorescence detection, three collagenase assays for MMPs were also studied with triple-helical collagen as substrates and a non-covalent fluorescent dye, NanoOrange [32, 33]. All these assays used fluorescent resonance energy transfer (FRET) by conjugating donor/acceptor with a triple helical peptide. Upon enzymatic cleavage, the donor and acceptor are separated and lead to increase in the fluorescence intensity of the dye.

To explore the conjugation of Ru(II) to collagen, the pH of the collagen was brought to 7.5 slowly before the addition of the active ester of Ru(II). After the completion of the reaction, the pH was brought back to 4.5 in order to prevent gel formation. Vigorous dialysis for 48 h and monitoring of the elution of Ru-complex through UV-Visible spectroscopy confirms the removal of unbound Ru. The slow addition of Ru to collagen with appropriate measure in maintaining the pH of the solution is the key element in the conjugation process. CD, FT-IR, fibrillation kinetics assay and SEM analysis further confirms that the conjugation of Ru(II) took place on the collagen fibre without perturbing the structure significantly. With the advantage of large Stokes shift (>120 nm) and better photostability, Ru based probes can be utilized for biological assays.

In conclusion, Ru(II) polypyridyl complex was conjugated with collagen and studied as collagenase substrate. Upon conjugation of Ru, the secondary structure of collagen remains unaltered. Luminescence studies showed that upon addition of collagenase, an enhancement in luminescence was observed as similar to FITC-collagen. Conjugation of collagen with a fluorophore allows for the omission of staining and de-staining process in gel-visualization. EDAX studies confirm the presence of Ru in collagen fibrils. The present study paves way for the

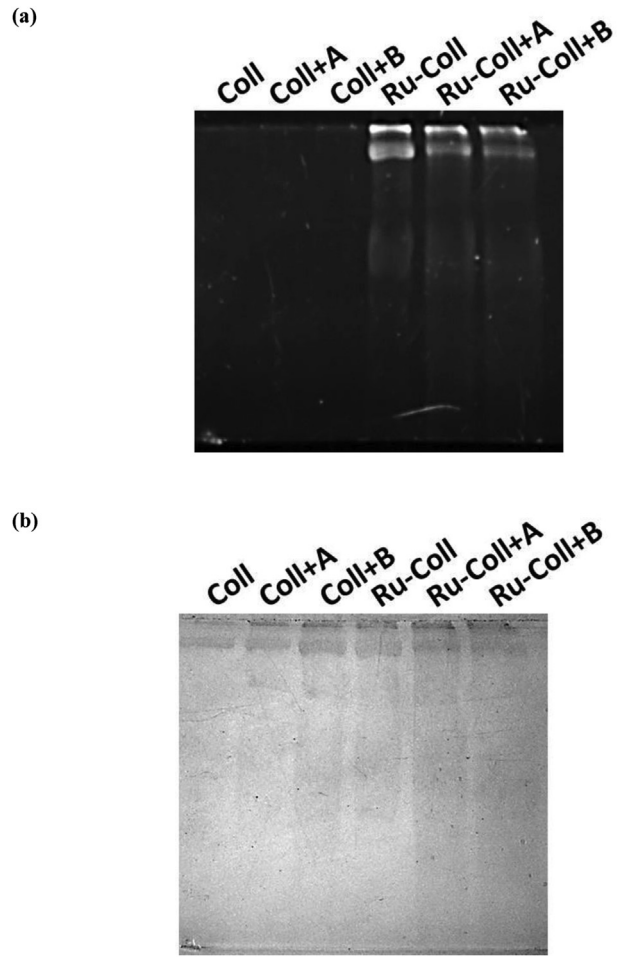

Figure 10. Gel electrophoresis of collagen and Ru-collagen with and without collagenase. (a) Under UV light without staining and (b) stained with Coomassie blue. [Coll + A: Collagen+ 0.02 μg/mL of collagenase; Coll + B: Collagen+ 0.04 μg/mL of collagenase; Ru-Coll + A: Ru-Collagen+ 0.02 μg/mL of collagenase; Ru-Coll + B: Collagen+ 0.04 μg/mL of collagenase].

labelling of fibrous protein that can be explored for monitoring the formation of collagen in live tissue culture.

Declarations

Author contribution statement

Anithabanu, P., Surabhya Balasubramanian, David Dayanidhi, P.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Nandhini, T.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Vaidyanathan, V.G.: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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