Oxygen sensing in a 3D cell culture using an oxygen-sensitive dye based on a Ru(II) complex

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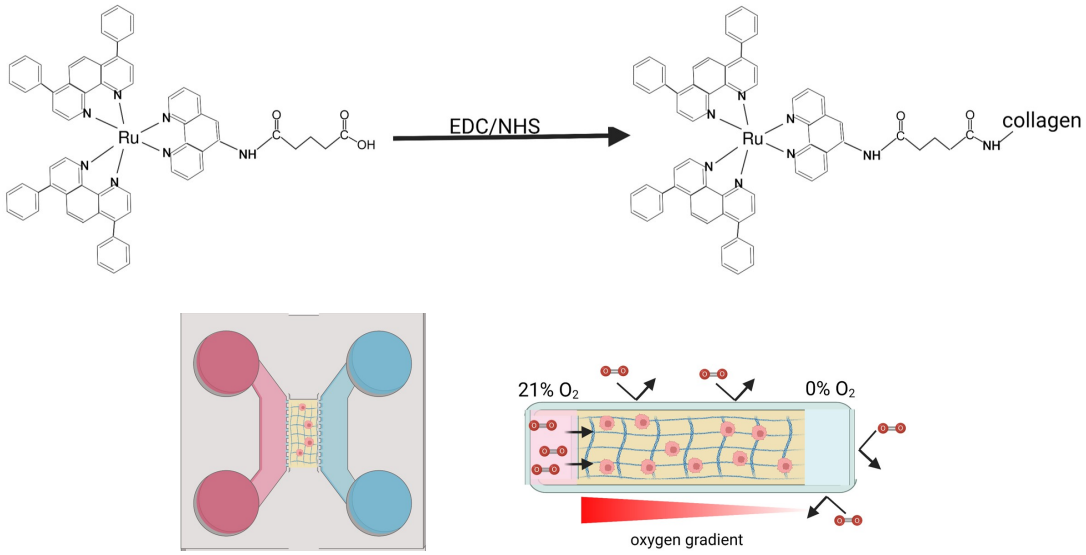

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Fienie Pouw

Abstract **– While oxygen plays a major role in cell viability and function it is often neglected and/or underestimated in cell or tissue based studies. 3D oxygen mapping makes it possible to map the oxygen concentration and measure metabolic cell activity more accurately. In this study, the applicability of a Ru(II) complex based dye, [Ru(BPhen)2]2+ (BPhen=batophenanthroline) with a terminated carboxylic acid group (Ru-complex), for oxygen mapping in a 3D cell/cluster-laden collagen hydrogel was validated based on optical properties, binding efficiency and toxicity.**

The Ru(II) based dye quenches linearly by increasing oxygen concentration (when dissolved in Milli-Q with a small percentage of DMSO). The carboxylic acid group of the Ru-complex can be covalently attached to collagen type I through 1-Ethyl-3- [3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) chemistry. Optimalisation of the coupling protocol can increase binding efficiency and make it more time efficient.

Unattached dye is toxic to MCF-7 cells (common breast cancer lineage) when used in a cell- and dye-laden collagen matrix, which was more significant upon excitation of the dye, which makes it unsuitable for cell applications. Additionally, the dye was insoluble in aqueous media and precipitation was observed when phosphate buffered saline (PBS) buffer or N-Hydroxysulfosuccinimide (Sulfo-NHS) was used in combination with the dye. In future studies, a dye with for example a negative charge could be considered to decrease toxicity and increase solubility.

I. Introduction

Oxygen is an essential marker for various processes in the cell as, for example, cell signaling, enzyme activity and oxidative processes in which oxygen acts as electron acceptor [1]. For instance, oxygen plays a key role in Cellular respiration in which the mitochondria consume oxygen to produce adenosine triphosphate (ATP). Also, oxygen is involved in stabilizing proteins in the endoplasmic reticulum (ER) by forming disulfide bonds via oxidation [2]. Due to these processes in the cell, oxygen influences the cell function and viability [1] [3]. Organs need different oxygen concentrations varying from for example 1.3% in the superficial skin to 14.5% in the alveoli [4]. When cells or tissues are exposed to different oxygen concentrations than preferably needed, it can lead to oxidative stress in cells. Molecules on cells will be attacked by reactive oxygen species (ROS), which causes damage to bio-molecules and might lead to apoptosis of the cells [5].

For these reasons, oxygen concentration is an important parameter to be taken into account when culturing cells to create in vivo-like conditions. A way of creating 3D in vitro conditions to reproduce and study physiological functions of tissues or organs is the use of organ-on-a-chip (OoC) platforms. OoCs are microfluidic platforms that allow additional control and include gradients of chemical parameters in 3D cell cultures as for example the oxygen concentration [6]. Various OoC platforms have been developed over the last years and show great ability to mimic in vivo conditions in a cost-saving way [3] [4] [7]. In vivo, vascularization networks provide the cells the needed amount of oxygen. In cell cultures in microfluidic devices, the oxygen supply has to be delivered by the medium flow or by diffusion through the chip walls [8]. This makes it more complicated to control the oxygen supply accurately in the cell culture, as there are several parameters involved between the oxygen diffusion through the chip wall and the oxygen consumption in the platform [9]. Examples of involved parameters are the diffusive flux/diffusion coefficient and permeability of the chip wall [10]. By monitoring the oxygen concentration throughout the cell culture, the oxygen concentration can be controlled more accurately, as the concentration can be adapted based on the measurements values. In addition, by monitoring the oxygen concentration in a 3D cell culture, the oxygen consumption of the cells can be determined. As a result, metabolic cell activities and cell behavior can be better controlled in OoCs when the oxygen concentration is monitored and controlled properly.

There are several ways to measure the oxygen concentration in 3D cell cultures, in which optical and electrochemical sensors are the most common [7]. Electrochemical sensors measure the oxygen concentration in a solution and give an electrical current proportional to the oxygen concentration. Oxygen can be reduced in hydroxide ions when a potential is applied, resulting in a current [11] [3] [12]. These sensors can measure the oxygen concentration in a 3D cell culture precisely and quickly, but they show multiple disadvantages [13]. The sensors consume oxygen, are invasive/toxic to cells and cannot give 3D information (in XYZ-space) of the oxygen concentration [3]. Optical sensors are based on luminescence quenching in presence of oxygen. The relationship between the luminescence intensity and oxygen concentration is determined by the Stern-Volmer relation [14]:

$$\frac{I0}{I} = 1 + Ksv[O_2] \quad (1)$$

For the luminescence intensity (I), fluorescence or

phosphorescence intensity can be used in the relationship. The Ksv is the Stern-Volmer constant and the $[O_2]$ is the oxygen concentration. Optical sensors can measure the oxygen concentration precisely without influencing the oxygen gradient, still they can be complicated to integrate in a 3D culture and can be prone to dye bleaching [7] [8]. New methods for oxygen sensing are developed over the last years, still better oxygen sensing methods to access 3D information about the oxygen concentration in 3D cell cultures are required.

One optical approach to sense oxygen relies on the use of metal coordination complexes based on Ru, Ir and Pt, for instance [15]. Their luminescence quenches in presence of oxygen molecules, which can be monitored and employed to derive the oxygen concentration. Two different mechanisms are involved in the luminescence quenching (Figure 1). First, quenching of the luminescence occurs when the excited triplet state of the metal complex falls back to the ground state after energy transfer with oxygen molecules. Secondly, electron transfer occurs from the triplet excited state of the metal complex to the oxygen molecule, which creates a positive charged ground state metal complex with energy loss [15]. In both mechanisms, reactive oxygen species will eventually form.

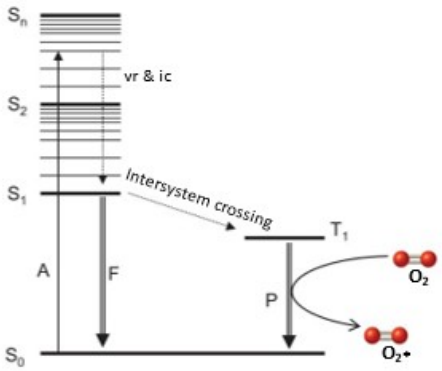

Fig. (1). *Jablonski diagram of the energy levels of a metal coordination complex in presence of the quencher oxygen. A=absorption, F=fluorescence, P=phosphorescence,T1= triplet state, S_0=ground state, $S_{1\text{-}n}$=singlet state, O_2*=oxygen molecule after energy transfer, vr & ic= vibrational relaxation & internal conversion. Based on [15].*

In this study, the oxygen sensing abilities of the following Ru(II) complex, $[Ru(BPhen)_2]^{2+}$ (BPhen=batophenanthroline) with a terminated carboxylic acid group (Ru-complex), will be validated for the use of 3D oxygen sensing in a 3D cell culture (Figure 2). To access 3D information about the oxygen concentration, the dye should be (evenly) distributed throughout the 3D cell culture. Collagen is commonly used in OoCs as a model for the extracellular matrix (ECM). The carboxylic acid moieties on the Ru-complex enable using 1-Ethyl-3- [3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) chemistry to covalently bind to the amine groups on the collagen matrix. We will study basic characteristics (absorbance and fluorescence properties and molecule structure) of the dye, the binding efficiency of the dye, the toxicity of the dye to cells and the relationship between the fluorescence intensity of the dye and oxygen concentration via the Stern-Volmer relationship. Eventually, the dye will be integrated in a 3D cell culture to evaluate the potential of oxygen sensing in 3D.

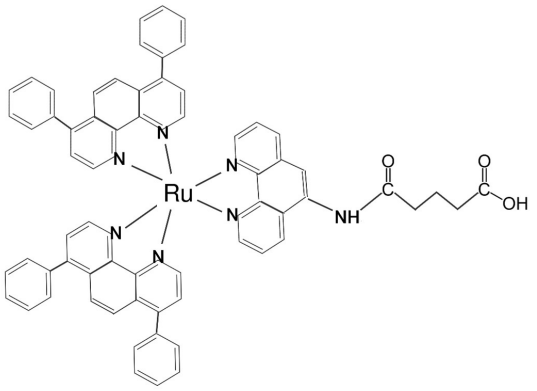

Fig. (2). *$[Ru(BPhen)_2]^{2+}$ (BPhen=batophenanthroline) with a terminated carboxylic acid group (Ru-complex), with a spacer and a terminal carboxylic acid group. Cartoon created by BioRender.com.*

II. Materials and Methods

A. Materials

The Ru-complex dye was synthesised by Dr. A. Ruggi, department of chemistry at University of Fribourg.

The used collagen stock was Collagen type I from rat tail, 3 mg/mL and pH 3.0. It was purchased from FUJIFILM Wako Chemicals U.S.A. Corporation.

Dimethylsulfoxide (DMSO,D8418) was purchased from Merck.

MCF-7 (ATCC #p32) cells (breast cancer cell lineage) were used. DMEM (11584486), Fetal Bovine Serum (FBS, 11573397), Penicillin-Streptomycin (Pen/Strep, 11548876) were purchased from ThermoFisher Scientific.

The used 96-wells plates for absorbance and fluorescence are the Nunc™ MicroWell™ 96-Well Microplates (260860) and the Nunc™ F96 MicroWell™ Black and White Polystyrene Plate(237105) from ThermoFisher Scientific. The used 96-wells plate for cell culturing are the Greiner CELLSTAR® 96 well plates, Merck.

B. Characterization of Ru-complex

Absorbance & fluorescence characteristics

A 1 mM Ru-complex stock solution (including 3.8% DMSO) was made by dissolving the Ru-complex in dimethylSulfoxide (DMSO)(107 mg/mL) after which Milli-Q was added drop-wise (5.37 mg/mL). A series of concentrations 0-400 μM dye in a Milli-Q (with DMSO from Ru-complex stock) solution were prepared in triplicates.

The absorption spectrum (between 300 and 600 nm), and emission spectrum (between 450 and 850 nm) of the dye at 50 and 400 μM were recorded using a

well plate spectrophotometer (VarioskanLUX, ThermoFisher Scientific).

The absorbance was measured at 434 nm for these samples. The Beer-Lambert law was used to find the absorption coefficient (ε) for the dye in Milli-Q (and DMSO).

The effect of temperature (25 and 37° C) on fluorescence intensity was determined.

To study the effect of the collagen matrix on the dye absorbance and fluorescence properties, the same measurements were performed in 2 mg/mL collagen hydrogels. A 5 mM Ru-complex stock solution was made the same way as previously described (11.5 % DMSO in stock solution). The collagen hydrogels with dye were made at 4°C by mixing the collagen stock, Milli-Q, dye stock and 10x PBS together. After this, the pH was adjusted to $\pm$ 8.5 with NaOH buffer (260 mM $NaHCO_3$, 20 mM Hepes and 0.05 M NaOH) and pH paper (pH-box pH 0.5-13, Merck, 1095650001). 60 μL of the hydrogel with unactivated dye was pipetted on a 96-well plate and the plate was afterwards incubated for 30min at 37°C in 5% CO_2 afterwards for 1h to set the gel in a 96-well plate.

NMR spectrometry Ru-complex

The C-NMR and H-NMR spectra were performed to determine the molecular structure of the Ru-complex. The H-NMR was performed with the Bruker 400MHz (Robot) and the C-NMR was performed with the Bruker 600MHz. The Ru-complex was dissolved in d4-MeOH for these analyses.

C. Coupling of the Ru-complex

The dye was coupled to the collagen backbone with EDC chemistry. To determine the binding efficiency, the concentration of attached dye was measured and compared to the concentration of dye which was initially added.

A Ru-complex solution of 1.87 mM was prepared in DMSO (2.01 mg/mL) after which 2-(N-morpholino)ethanesulfonic acid buffer (MES buffer)(10 mM, pH=5) and EDC (20 mM) was added. The mixture was placed on a roller mixer (Stuart™ analogue rocker & roller mixers, Merck) for 2h at RT to activate the carboxylic acid groups. After this, the activated dye was added to 3 mg/mL collagen stock with final dye concentrations 100, 200 and 400 μM. The pH of the mixture was adapted to pH 6 and placed on a roller mixer at T=4°C to react overnight. Next day, Milli-Q, 10x PBS and NaOH buffer were added to set the gel as previously described. For the purification after the coupling reaction, the wells with collagen were washed with Milli-Q by pipetting 200 μL on the hydrogel and placing it back in the incubator. The Milli-Q on the hydrogel was refreshed 2x a day for 2 days until no more dye came out of the hydrogel. This was determined by measuring the absorbance at 434 nm of the Milli-Q used for washing. Collagen hydrogels with unactivated dye (effectiveness of washing) and collagen hydrogels without purification (initially added (activated) Ru-complex concentration) were used as a controls.

Absorbance (λ= 469 nm) was measured with a well plate spectrophotometer.

D. Toxicity of Ru-complex in MCF-7 cells

The toxicity of the unattached dye was determined by making use of a PrestoBlue Assay (A13261, ThermoFisher Scientific) for the following concentrations of dye a 2 mg/mL collagen hydrogel:0 ,10 ,25 and 50 μM. MCF-7 cells (human breast cancer cell line) were used, with a seeding density of 16,000 cells/mL in the hydrogels.

The cell and dye laden hydrogels were prepared the same way as before. After adapting the pH, the cell suspension (2% of total volume) was added and the matrixes were incubated for 1h at 37°C with 5% CO_2.

The effect of direct toxicity (the presence of the dye) and the indirect toxicity (effect of excitation and emission of the dye) was distinguished by determining the cell viability for both conditions. The plate used to determine the indirect toxicity was illuminated at 485nm (bandwith 15nm) with excitation energy 2000 eV (due to limited filter and setting options) with a pulse length of 1s every 2 min for 1h, by making use of a well plate spectrophotometer (VICTOR3, PerkinElmer). Illumination of the plate was performed to see if excitation of the dye would lead to more cell death compared to the well-plate where the dye was not excited.

Negative control wells contained cells in a collagen with medium and positive control wells contained cells collagen with 70% EtOH (100% cell death). A control for the effect of the solvent of the dye was included; these wells contained cells in medium with the same concentration of Milli-Q and DMSO as used to dissolve the dye. At last, a control without cells containing the same dye concentrations was included to determine the fluorescence intensity coming from the dye. All conditions were performed in triplicates.

The PrestoBlue assay was performed 24h after the hydrogels were made for both wells plates. The hydrogels in the wells were washed with phosphate buffered saline (PBS) once and afterwards 100 μL medium with 10% PrestoBlue stock was added. The plates were covered from light and incubated for 3h. 90 μL was collected from every well and placed on a fluorescence 96-well plate. The fluorescence intensity was quantified at 590 nm with excitation at 570 nm, using the VarioskanLUX (ThermoFisher Scientific).

Uptake of unattached Ru-complex

The Ru-complex is going to be covalently coupled to the collagen hydrogel, which will be remodeled by cells. As a result, cells can eventually take up the dye.

The uptake of unattached dye was determined in 2D. MCF-7 cells were seeded on a 96-well plate with a seeding density of 16,000 cells/mL.The medium with Ru-complex (10 and 50 μM, 0.14% DMSO & 4.86% Milli-Q) was added approximately 15h after the cells were seeded. Also a control of medium without dye was included as a control. After 24h, the medium with dye was removed and the cells were washed 5 times with PBS. After washing, the well plate was imaged using the the EVOS microscope with RFP filter (excitation: 542/20 nm and emission: 593/40 nm), as the wavelength of the RFP filter is within the absorption and emission spectrum of Ru-complex.

E. correlation between oxygen concentration and luminescence intensity

The 96 well plate with Ru-complex in Milli-Q (and DMSO) from Materials and Methods B.was re-used to measure the fluorescence intensity at 3, 6, 9 and 21% oxygen in the VarioskanLUX. The oxygen concentration was set by attaching a N_2 source to the Varioskan and the plate was incubated at each oxygen concentration for (at least) half an hour at 37°C. After each incubation period, fluorescence intensity was measured at 631 nm ($\lambda_{excitation}$= 450 nm).

F. statistical analysis

Data analysis was performed in Matlab. Error bars were computed with the standard deviations for each measured condition with the function std and statistical/possible errors were passed on according to the calculation rules. The function *ttest* was used to determine statistical significance between two conditions. P-values were considered statistically significant when $p \leq 0.05$. A linear fit for a set of measurements was determined with the function *refline* and the slope (to calculate the absorptivity and the Stern-Volmer constant) and R^2 were determined with the toolbox basic fitting.

III. Results

A. Absorbance and fluorescence properties Ru-complex

The absorbance and fluorescence spectra were measured for the Ru-complex 50 and 400 μM in Milli-Q (with DMSO) and collagen (with DMSO).

The absorption spectrum shows a plateau peak absorption between $\lambda_{maxabsorbance}$ 434nm and 463nm (Figure 3). The $\lambda_{maxfluorescence}$ was 631 nm in Milli-Q and 637 nm in the collagen hydrogel (Figure 4).

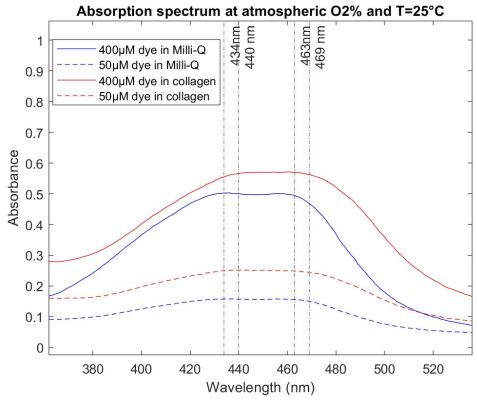

Fig. (3). *The absorption spectrum for 50 and 400µM Ru-complex in Milli-Q (with DMSO) and collagen (with DMSO) at room temperature. The $\lambda_{maxabsorbance}$ are shown with a dotted line for the Ru-complex in Milli-Q (and DMSO) and collagen (and DMSO).*

The intensity of the absorbance and fluorescence/emission spectra increased when the concentration of dye was increased in both Milli-Q and collagen. Also, the fluorescence intensity and absorbance of the spectra were higher for the dye in collagen hydrogels (with DMSO) compared to the dye in Milli-Q (with DMSO) Figure 4 3. Both absorption and emission spectra show a minimal redshift for dye in collagen compared to dye in Milli-Q (approximately 6 nm).

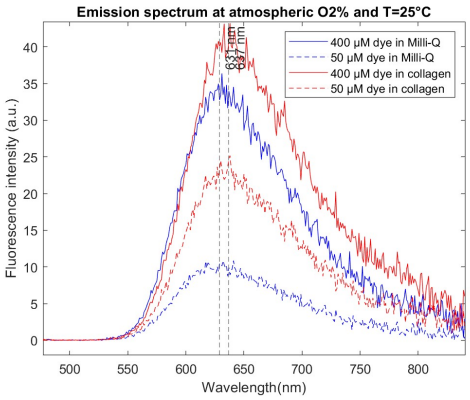

Fig. (4). *The fluorescence spectrum for 50 and 400 µM Ru-complex in Milli-Q (with DMSO) and collagen (with DMSO) at room temperature. The $\lambda_{maxfluorescence}$ are shown with a dotted line for the Ru-complex in Milli-Q (with DMSO) and collagen (with DMSO)*

Effect of temperature on the fluorescence intensity

The fluorescence intensity of the Ru-complex decreased in collagen(with DMSO) and Milli-Q (with DMSO) when the temperature was increased from 25 to 37°C (Figure5). The fluorescence intensity was for each concentration higher in collagen than in Milli-Q. Furthermore, the linear range for the relationship between concentration and fluorescence intensity was 0-250 μM for Ru-complex in Milli-Q and 0-50 μM in collagen.

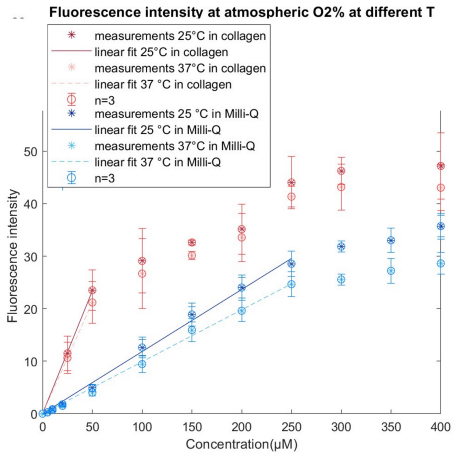

Fig. (5). *The fluorescence intensity for 50-400 µM of Ru-complex in Milli-Q and collagen. 463 nm for excitation and 631 nm for emission in Milli-Q (and DMSO), 469 nm for excitation and 637 nm for emission in collagen (and DMSO).*

Absorptivity

The absorption coefficient (ε) was determined for the Ru-complex in Milli-Q (with DMSO) and collagen (with DMSO) by computing the linear fit in Matlab, in which the slope is equal to the absorption coefficient according the Beer-Lambert law. An absorptivity of 12423 $M^{-1}cm^{-1}$ was measured in Milli-Q (with DMSO) and 7746 $M^{-1}cm^{-1}$

in collagen (with DMSO)(Figure 6). A difference in path length was consequently visible between measurements in collagen and Milli-Q due to meniscus forming of the collagen matrix in the wells. The path length in the wells (for both collagen and Milli-Q) was back calculated by performing measurements in cuvettes (n=1) with constant and known path length (1 cm) for 50, 100 and 150 μM of Ru-complex. The difference in path length was corrected by dividing the absorbance by optical path length, which is equal to 0.17 cm in collagen and 0.18 cm in Milli-Q).

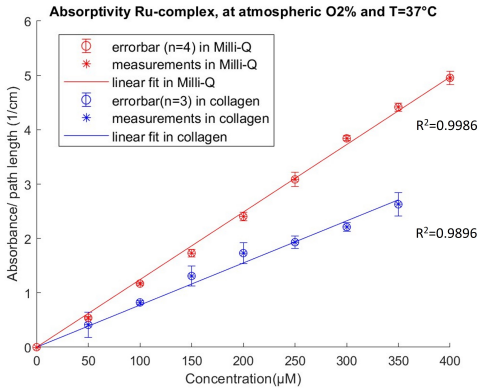

Fig. (6). *Correlation between Ru-complex concentration and absorbance/path length measured at 434 nm in Milli-Q (with DMSO) and 469 nm in collagen (with DMSO). The slope of the linear fit is equal to the absorptivity, according the Beer-Lambert law.*

H-NMR and C-NMR

The H-NMR(Figure 9) and C-NMR(Figure 8) spectra both showed molecular groups from the spacer and the benzene complex. The carboxylic acid group (C-atom and H-atom) is in both the H-NMR and C-NMR expected at a higher chemical shift than the measured range. Two peaks around 4.5 ppm in H-NMR and 61 and 72 ppm in the C-NMR could not be assigned to the Ru-complex structure. In the C-NMR, the chemical shift at 35ppm refers to the CH2 group neighboring a carbonyl or carboxyl group (group b or d).

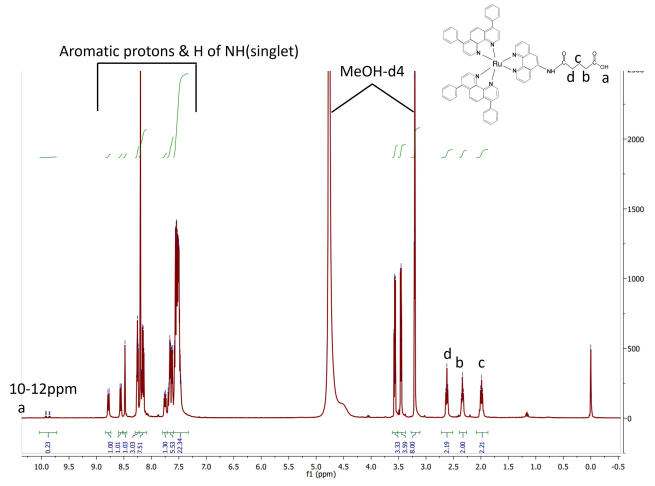

Fig. (7). *1H-NMR spectrum of Ru-complex. Groups of the spacer are indicated with alphabetic letters.*

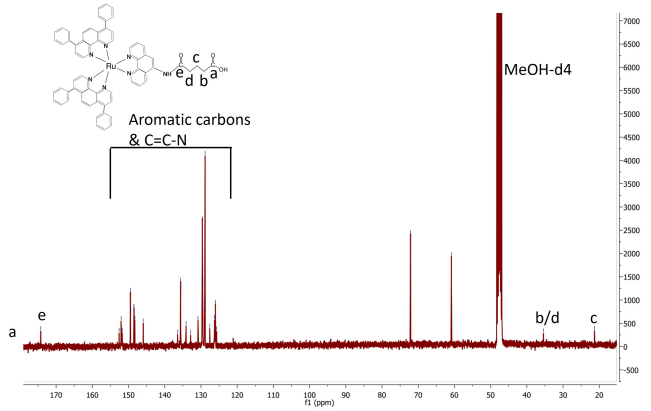

Fig. (8). *13C-NMR spectrum of Ru-complex. Groups of the spacer are indicated with alphabetic letters.*

B. binding efficiency Ru-complex to collagen

Absorbance was measured at 469 nm for 100 and 200 μM initially added Ru-complex concentrations. Absorbance of the collagen blank was subtracted from the condition with inactivated dye after washing and activated dye without washing (initially added concentration of Ru-complex), so an absorbance of 0 is equal to the collagen blank. The absorbance of inactivated dye after washing was subtracted from the condition with remaining activated dye after washing to correct for remaining dye after purification and absorbance of the collagen (blanco). The 400 μM conditions showed a heterogeneous contribution of Ru-complex and were not measured. For different conditions, one measurements (of the triplo) was not used, as a result of detachment of the collagen matrix from the well or formation of air bubbles.

A significantly higher Ru-complex concentration was measured for the conditions with 200 μM added activated Ru-complex compared to the inactivated Ru-complex (p=0.002). The absorbance of added activated 200 μM dye before washing was equal to 0.65 ($\pm$ 0.27) and after washing was equal to 0.096 ($\pm$ 0.0074), so 14.7 ($\pm$ 8) % was covalently attached to the collagen matrix (n=3).

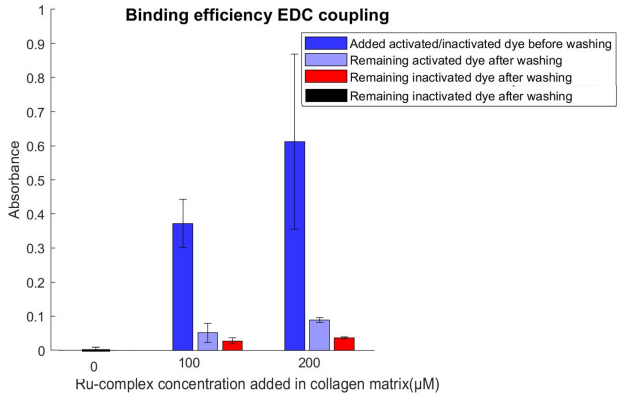

Fig. (9). *Binding efficiency of Ru-complex with EDC coupling for 100 and 200 μM initially added dye concentrations. The 0 μM represents the collagen condition. n=2 for 100 μM and n=3 for 200 μM.*

C. Toxicity towards MCF-7 cells

The MCF-7 cells and dye (for each condition) were homogeneously distributed through the collagen hydrogel when observed with a microscope. After the PrestoBlue was added to the wells and an incubation time of 4h, no visible difference in medium colour was visible between the conditions.

Differences in colour and fluorescence of the medium indicate differences in the degree of reduction of resazurin (non-fluorescent blue) to resorufin (fluorescent red) by viable cells. The quantitative results of the fluorescence measurements are shown in Figure 10. Results were plotted relative to the positive control without illumination, where 100% indicates fluorescence intensity equal to the control. The blanco (medium+10% PrestoBlue) was subtracted from every condition.

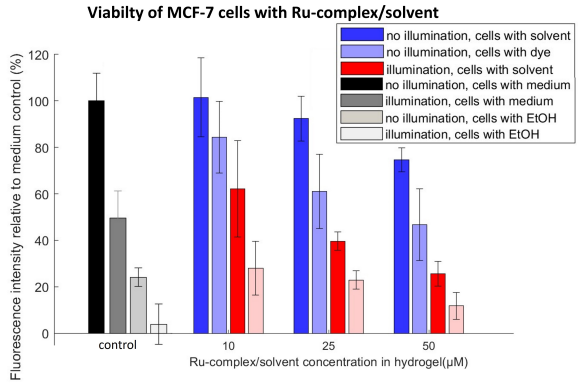

Fig. (10). *Fluorescence intensity measurements of the PrestoBlue Assay of each condition relative to the negative control of cells with culture medium without illumination, n=3.*

A higher dye concentration leads to a lower fluorescence intensity of the measured PrestoBlue (590 nm), which indicates a lower cell viability. This difference was not statistically significant due to the large standard deviations in these measurements. The same holds for the cells with corresponding solvent (Milli-Q and DMSO from the Ru-complex stock solution).

Cells showed for each concentration a statistically significant decrease in viability for dye with illumination compared to cells in dye without excitation ($p \leq 0.003$ for all conditions). Also cells in solvent with illumination showed a lower viability compared to this condition without illumination ($p \leq 0.04$ for all conditions).

All concentrations of solvent without illumination did not show a statistically significant difference compared to the medium control without illumination. For the dye concentrations without illumination, only the 50μM showed a statistically significant decrease in viability of cells compared to the medium condition (p=0.034). Furthermore, all conditions with dye and illumination showed a significant lower viability than the cells in medium with illumination ($p \leq 0.03$ for all conditions). For the solvent conditions with illumination, only the 50 μM solvent showed a statistically significant decrease in viability compared to the cells with illumination in medium.

For the conditions with 25 and 50 μM dye/solvent without illumination, a lower viability was measured for cells with dye than cells in the corresponding solvent (p=0.016 for 25 μM and p=0.037 for 50 μM). For conditions with illumination, the same holds for cells in 25 μM dye compared to 25 μM solvent (p=0.33).

Uptake of unattached dye

Uptake of unattached Ru-complex depended on the initial dye concentration. The condition without added dye did not show any signal for the RFP-filter. The 10 and 50 μM dye concentrations both showed uptake, which can be seen by the lightly red colouring of the entire cell and the red dots (Figure 11 A and B, with magnification C and D).The red dots are visible within the contours of the nucleus and the cytoplasm of the cells on the 2D image.

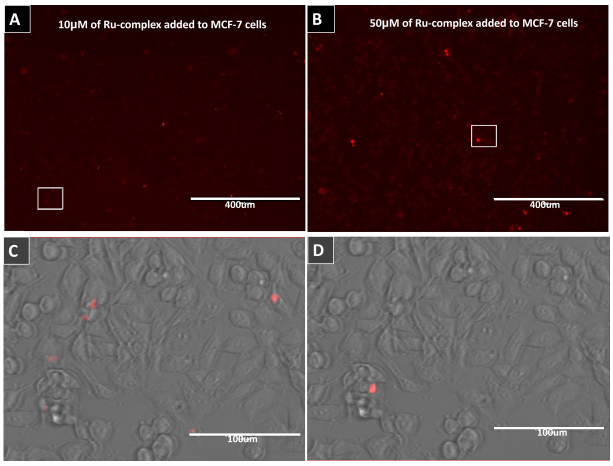

Fig. (11). *Uptake of unattached Ru-complex in MCF-7 cells. Imaged using the EVOS 24h after adding medium with dye to the 96-well plate with attached cells. Red signal represents the Ru-complex. Started seeding density: 16.000 cells/mL.A)Uptake of an initially added 10 μM Ru-complex in DMEM.B)Uptake of an initially added 50μM Ru-complex in DMEM.C)A cropped part B with the trans and RFP filter of the EVOS.D)A cropped part B with the trans and RFP filter of the EVOS. Magnification 10x for A,B and 40x for C,D. RFP intensity set at 40%.*

Correlation between oxygen concentration and luminescence intensity

The atmospheric oxygen concentrations of 3, 6, 9, 21% were converted to oxygen concentration (mol/L) dissolved in the Milli-Q (and a small percentage DMSO) based on the oxygen solubility in water at 37°C with the following formula [16]:

$$[O_2] = \frac{55.56 pO_2}{e^{(3.71814 + \frac{5596.17}{T} - \frac{1049668}{T^2}) - pO_2}} \quad (2a)$$

pO_2=partial pressure above the solution (atm), T= Temperature (K), $[O_2]$= Oxygen concentration dissolved in water (mol/L).

The fluorescence intensity of the Ru-complex (dissolved in Milli-Q and DMSO) quenches linearly with an increasing oxygen concentration (Figure 12). I0 was established for each Ru-complex concentration by extrapolating the

linear fit of correlation between oxygen concentration and fluorescence intensity to 0 mol/L oxygen. The average slope of the quenching for the three measured Ru-complex concentrations is equal to the Stern-Volmer constant (Ksv), in this case 0.11 (± 0.032) L/mol.

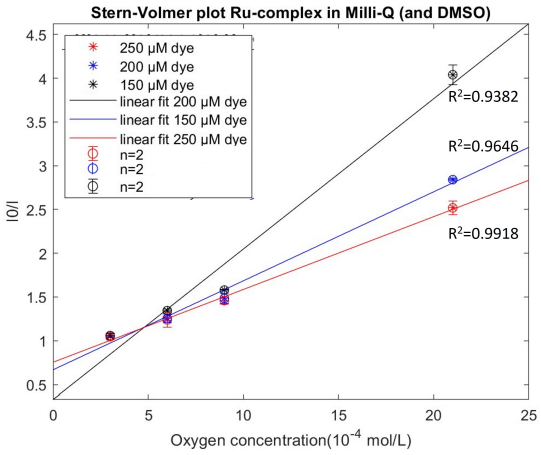

Fig. (12). *Applied Stern-Volmer plot of 150, 200 and 250 µM Ru-complex in Milli-Q (and DMSO from Ru-complex stock) in presence of 3.24, 6.48, 9.73, 22.7 *10^{-5} mol/L oxygen at T=37° C in Milli-Q. Excitation wavelength:450 nm and emission wavelength: 631 nm.*

IV. Discussion

A. Solubility and precipitation forming in presence of the Ru-complex

Preferably compounds added to a cell culture should be dissolved in a isotonic buffer, however the Ru-complex was poorly soluble in aqueous media and was precipitating in commonly used buffers as PBS. Therefore, in this study the dye was first dissolved using DMSO and Milli-Q was added dropwise slowly to avoid precipitation. The concentration of DMSO was kept low in order to limit the effect of DMSO on the permeability of the cell membrane. Controls with DMSO and Milli-Q/collagen were performed in all experiments to correct for effects of the solvent on for example the viability of the cells.

Additionally, the Ru-complex also precipitated with Sulfo-NHS used for the coupling to collagen. Both the phosphate ions and Sulfo-NHS have a negative charge, which can interact with the positively charged Ru-complex and become insoluble. The use of anions in combination with the Ru-complex should be avoided and formation of precipitation should be considered and kept to a minimum if used.

B. Shift in absorbance and fluorescence spectrum

By comparing the absorbance and fluorescence spectra in Milli-Q and collagen a shift in the $\lambda_{maxabsorbance}$ and $\lambda_{maxemission}$ in both spectra of ca. 6 nm was observed. This shift is likely caused by a difference in polarity of the solvent [17]. Milli-Q is a polar solvent and collagen has regionally polar and nonpolar groups, so the polarity is depending on the regionally environment of the collagen. This hypothesis can be confirmed by repeating the experiments in buffers with varying polarity and by comparing the spectra in different concentrations of collagen. The cause of the redshift is not too important, because the same (small) redshift was visible in both the absorbance and fluorescence/emission spectrum. Measurements in (2 mg/mL) collagen have to be performed at a different wavelength compared to Milli-Q if $\lambda_{maxabsorbance}$ and $\lambda_{maxemission}$ are used.

C. Effect temperature on fluorescence intensity

The release of energy after excitation of the Ru-complex increases at higher temperature due to the increased mobility/flexibility of the molecules at higher temperature. In collagen the effect of increasing the temperature is dampened due to the structure of the collagen matrix, which blocks the dye from moving though the gel.

D. Absorptivity

The path length was different for the absorbance measurements of Ru-complex in Milli-Q (with DMSO) compared to collagen (with DMSO) due to for example difference in adhesion to the wells plate or difference in surface tension, causing meniscus forming in the wells with collagen. Relatively small error bars (n=3) of each condition and a R^2 of 0.9868 indicate a consequent level of meniscus forming in the well. The difference in path length was corrected by back calculating the path length in wells by measuring the absorbance in cuvettes (constant and known path length). For more accurate measurements, the absorbance coefficient could be determined in triplet with more different Ru-complex concentrations.

E. linearity of fluorescence intensity

A difference in linear range for Ru-complex concentration and fluorescence intensity was observed by comparing the measurements in Milli-Q and collagen. The Ru-complex shows a linear range until 250 µM in Milli-Q and 50 µM, in which the inner filter effect was observed for higher concentrations [18]. When fluorescence intensity is measured, dye concentration should be used within the linear range.

F. H-NMR & C-NMR analysis of Ru-complex

Two unidentified peaks were measured for H-NMR (3.5 ppm) and C-NMR spectra (60 ppm and 71 ppm), which might be caused by impurities in the solvent. Repetition of the H-NMR and/or c-NMR in a new stock of methanol-d4 could confirm this hypothesis. Both spectra confirmed the presence of the spacer with carboxylic acid, which indicates that the molecule did not degrade.

G. Couplings reaction

The coupling reaction between amines and carboxylic acid groups preferentially is performed by combining activation for the carboxylic groups with EDC, stabilized by addition of NHS/Sulfo-NHS. In this study, the coupling reaction between collagen and the Ru-complex was only performed with EDC, omitting the more stable Sulfo-NHS intermediate. Including Sulfo-NHS in the reaction was not possible due to precipitation forming. Successful covalent

binding using only EDC has been shown in other studies for collagen type I [19].

In this study, 14.67 ($\pm$ 8) % of the initially added 200 μM was covalently attached to the collagen matrix (no statistical difference was measured for the initially added 100 μM condition). To evaluate the coupling protocol, the coupling reaction was performed with 5(6)-Carboxy-X-rhodamine (rhodamine)(21965, Merck). For the same coupling reaction with (initially added 200 μM) rhodamine, a binding efficiency of 11.59 ($\pm$ 6)% was measured, corresponding with the coupling efficiency of the Ru-complex. EDC tends to hydrolyze in aqueous conditions, which decreases the coupling efficiency [20]. Optimization of the used coupling protocol (pH, concentration, reaction time, etc) should be considered to increase the coupling efficiency.

H. Purifying reaction mixture

The Ru-complex dye (and rhodamine as a control) was coupled with EDC chemistry to the collagen matrix. To determine the coupling efficiency and avoid toxicity due to free dye, unattached dye should be removed from the collagen matrix. Before selecting the procedure in section Materials and Methods Ç. several methods have been assessed for purification of the reaction mixture for both the Ru-complex and rhodamine.

First ultrafiltration (UF), by means of Amicon Ultra filter devices, is a fast way of separating molecules with different sizes and is often used to purify collagen. However, centrifuging the collagen and Ru-complex before gelation of the collagen at 14,000 g (recommended g-force, Merck [21]) at 4 °C for 3 minutes, led to formation of aggregates and unsuccessful removal of the Ru-complex. These aggregates might be caused by concentration polarization at the membrane interface [22].

Allowing slow diffusion of the Ru-complex out of the dye was chosen after repetitive fast washing (repetitively replacing Milli-Q on and off the matrix) of a mixture of Ru-complex and collagen was shown to be ineffective (negligible change of absorbance in the Milli-Q).

Observations made during the UF and fast washing indicated that the Ru-complex is likely to interact with the collagen by for example electrostatic and/or hydrophobic interactions, as the dye was hardly separable from the collagen. Above its isoelectric point (pH of 4-5), which will be exceeded during gelation, the collagen will be mainly negatively charged [22]. This can interact with the positively charged Ru-complex. Also, the Ru-complex is hydrophobic, which can create hydrophobic interactions with the nonpolar regions of collagen.

The eventually used purification technique based on diffusion has a large timespan (minimal 2 days), which can only be used for the coupling efficiency measurements. When cells are implemented, they will be in a high concentration of dye during purification, which is toxic to cells as can be seen in Materials and Methods C.

I. Toxicity

Large error bars were shown for multiple conditions in the Presto Blue Assay. A Presto Blue Assay is originally designed for 2D in vitro studies and may be inaccurate in 3D studies [23], due to trapping of the reagents in the (collagen) hydrogel as the (collagen) hydrogel during gelation, even though different studies have shown accurate results for this viability assay in 3D [24]. Also, the difference in fluorescence signal between conditions was relatively low, which can be related to the low seeding density. Still, multiple conditions showed statistically significant differences.

A significant decrease in viability was found in the presence of 50 μM Ru-complex (without illumination) compared to the negative control, which indicates that the dye is toxic to MCF-7 cells. The charge of the double phospholipid layer of the cell membrane is mainly negative, so the positively charged dye can bind/adhere to the cell membrane more easily, causing an increase in interactions [25] [26]. Whenever the dye is used with cells, minimal dye concentration should be used.

Illumination of the cells in presence of the dye showed for all conditions a lower viability compared to the negative control with illumination only, which only holds for the 25 μM of solvent with equal Milli-Q and DMSO concentration as in the Ru-complex stock. This shows that excitation of the dye is likely to contribute to ROS formation in the cell, causing toxicity [27] [5]. A ROS-staining can be performed in future to confirm this hypothesis. Excitation of the dye in presence of cells should be minimized by for example preventing additional excitation from undesired (sun)light.

J. Uptake

Cells will be exposed to unattached dye during purification of the coupling reaction and remodeling of the collagen matrix by cells [28]. Uptake of unattached Ru-complex was seen for concentrations of 10 and 50 μM in MCF-7 cells. In these measurements, 0.14% DMSO (no cytotoxic effect on MCF-7 cells [29]) was present in both conditions, which increases membrane permeability and so uptake. Also the positive charge of the dye increases uptake, as previously described. Reflecting on the viability assay, an increase in uptake and a lower cell viability was observed for an increasing dye concentration. Comparing to literature, uptake of different Ru-complexes (including phen ligands) by HeLa cells (cervical cancer cell lineage) is determined [30].The use of DMSO should be minimized and uptake of unattached dye should be taken into account for effecting cell viability.

K. Correlation between luminescence intensity and oxygen concentration

Fluorescence intensity was measured for 3, 6, 9 and 21% oxygen, because oxygen concentrations between 10 and 20 % was not possible to set due to a defect of the Varioskan. The R^2 decreases when the Ru-complex concentration decreases, possibly as a result of inaccuracy of the used pipettes in combination with the smaller volumes. For the same measurements in collagen, larger stocks of collagen with Ru-complex could be created, so the effect of the (possible) error minimizes.

V. Conclusion

In this work, the oxygen sensing abilities and toxicity towards MCF-7 cells have been validated for the Ru-complex. It has proved that the dye quenches linearly by increasing oxygen concentration. The dye can be covalently coupled to collagen through EDC chemistry with coupling efficiency of 14.67 ($\pm$ 8) % with used parameters (pH, concentration, temperature, time, etc.) described in Materials and Methods B.

It has shown that the dye is unsuitable for cell applications, as it causes cytotoxicity (increased by excitation of dye) and uptake to MCF-7 cells.

Recommendations

To make the Ru-complex more suitable for cell applications, a negatively charged dye could be considered to increase solubility in aqueous media (as PBS buffer) and prevent uptake by cells (repels on negatively charged cell membrane).

Also the use of a different ECM model (than collagen) with amine groups and compatibility with cells could be considered to match optimal conditions for the EDC/(sulfo-)NHS coupling reaction and have a more efficient purification technique. The most efficient pH for binding of (Sulfo-)NHS-activated molecules with primary amines is equal to the pH used for cross linking of the collagen (pH 7-8) [31].

REFERENCES

[1] B. D. Alberts, B. and et al, *essential cell biology*. 5 ed., 2019.

[2] J. C. Bardwell, "Disulfide bond formation, a race between fad and oxygen," *Developmental Cell*, vol. 3, no. 6, pp. 758–760, 2002.

[3] V. Palacio-Castañeda, N. Velthuijs, S. Le Gac, and W. P. R. Verdurmen, "Oxygen control: the often overlooked but essential piece to create better in vitro systems," *Lab Chip*, vol. 22, pp. 1068–1092, 2022.

[4] K. R. Rivera, M. A. Yokus, P. D. Erb, V. A. Pozdin, and M. Daniele, "Measuring and regulating oxygen levels in microphysiological systems: design, material, and sensor considerations," *Analyst*, vol. 144, pp. 3190–3215, 2019.

[5] G.Pizzino and et al, "Oxidative stress: Harms and benefits for human health," *Oxidative medicine and cellular longevity*, 2017.

[6] F. Yu, W. Hunziker, and D. Choudhury, "Engineering microfluidic organoid-on-a-chip platforms," *Micromachines*, vol. 10, no. 3, 2019.

[7] M. Azimzadeh, P. Khashayar, M. Amereh, N. Tasnim, M. Hoorfar, and M. Akbari, "Microfluidic-based oxygen (o2) sensors for on-chip monitoring of cell, tissue and organ metabolism," *Biosensors*, vol. 12, no. 1, 2022.

[8] J. Otero, A. Ulldemolins, R. Farré, and I.Almendros, "Oxygen biosensors and control in 3d physiomimetic experimental models," *Antioxidants(Basel)*, 2021.

[9] A. Al-Ani and et al, "Oxygenation in cell culture: Critical parameters for reproducibility are routinely not reported.," *PluS One*, 2018.

[10] M. D. Brennan, M. L. Rexius-Hall, L. J. Elgass, and D. T. Eddington, "Oxygen control with microfluidics," *Lab Chip*, vol. 14, pp. 4305–4318, 2014.

[11] C.-W. Liu, M. Janyasupab, Y.-H. Lee, and C.-C. Liu, *Electrochemical Oxygen Sensors for Operation at Ambient Temperature*, pp. 528–534. New York, NY: Springer New York, 2014.

[12] A. Kral, F. Aplin, and H. Maier, "Chapter10 - advanced concepts physical chemistry: Electrodes and electrolytes," in *Prostheses for the Brain* (A. Kral, F. Aplin, and H. Maier, eds.), pp. 167–208, Academic Press, 2021.

[13] J. Dornhof, J. Kieninger, H. Muralidharan, J. Maurer, G. A. Urban, and A. Weltin, "Microfluidic organ-on-chip system for multi-analyte monitoring of metabolites in 3d cell cultures," *Lab Chip*, vol. 22, pp. 225–239, 2022.

[14] M. H. Gehlen, "The centenary of the stern-volmer equation of fluorescence quenching: From the single line plot to the sv quenching map," *Journal of Photochemistry and Photobiology C: Photochemistry Reviews*, vol. 42, p. 100338, 2020.

[15] A. Ruggi, F. W. van Leeuwen, and A. H. Velders, "Interaction of dioxygen with the electronic excited state of ir(iii) and ru(ii) complexes: Principles and biomedical applications," *Coordination Chemistry Reviews*, vol. 255, no. 21, pp. 2542–2554, 2011. Controlling photophysical properties of metal complexes: Towards molecular photonics.

[16] W. Xing, M. Yin, Q. Lv, Y. Hu, C. Liu, and J. Zhang, "1 - oxygen solubility, diffusion coefficient, and solution viscosity," in *Rotating Electrode Methods and Oxygen Reduction Electrocatalysts* (W. Xing, G. Yin, and J. Zhang, eds.), pp. 1–31, Amsterdam: Elsevier, 2014.

[17] N. Shanker and S. L. Bane, "Basic aspects of absorption and fluorescence spectroscopy and resonance energy transfer methods," in *Biophysical Tools for Biologists, Volume One: In Vitro Techniques*, vol. 84 of *Methods in Cell Biology*, pp. 213–242, Academic Press, 2008.

[18] J. C. Zwinkels, P. C. DeRose, and J. E. Leland, "Chapter 7 - spectral fluorescence measurements," in *Spectrophotometry* (T. A. Germer, J. C. Zwinkels, and B. K. Tsai, eds.), vol. 46 of *Experimental Methods in the Physical Sciences*, pp. 221–290, Academic Press, 2014.

[19] L. Kalbitzer, K. Franke, S. Möller, M. Schnabelrauch, and T. Pompe, "Glycosaminoglycan functionalization of mechanically and topologically defined collagen i matrices," *J. Mater. Chem. B*, vol. 3, pp. 8902–8910, 2015.

[20] K. Nam, T. Kimura, and A. Kishida, "Controlling coupling reaction of edc and nhs for preparation of collagen gels using ethanol/water co-solvents.," *Macromolecular bioscience*, vol. 8 1, pp. 32–7, 2008.

[21] "Amicron ultra-0.5 centrifugal filter device user guide," Millipore, 2019.

[22] S. Jiang-nan, L. Dan-dan, J. Fei-yan, Q. Jun-hong, and G. Cong-jie, "Purification and concentration of collagen by charged ultrafiltration membrane of hydrophilic polyacrylonitrile blend," *Separation and Purification Technology*, vol. 66, no. 2, pp. 257–262, 2009.

[23] A. Dominijanni, M. Devarasetty, S. D. Forsythe, K. Votanopoulos, and S. Soker, "Cell viability assays in three-dimensional hydrogels: A comparative study of accuracy.," *Tissue engineering. Part C, Methods*, 2021.

[24] M. Gonzalez, I. Cichon, A. Scislowska-Czarnecka, and E. Kolaczkowska, "Challenges in 3D culturing of neutrophils: Assessment of cell viability, journal = Journal of Immunological Methods," vol. 457, pp. 73–77, 2018.

[25] E. Fröhlich, "The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles," *International journal of nanomedicine*, vol. 7, p. 5577—5591, 2012.

[26] S. Behzadi, V. Serpooshan, W. Tao, M. A. Hamaly, M. Y. Alkawareek, E. C. Dreaden, D. Brown, A. M. Alkilany, O. C. Farokhzad, and M. Mahmoudi, "Cellular uptake of nanoparticles: journey inside the cell," *Chem. Soc. Rev.*, vol. 46, pp. 4218–4244, 2017.

[27] G. E. Villalpando-Rodriguez and S. B. Gibson, "Reactive Oxygen Species (ROS) Regulates Different Types of Cell Death by Acting as a Rheostat," 2021.

[28] A. Jagiełło, U. Castillo, and E. Botvinick, "Cell mediated remodeling of stiffness matched collagen and fibrin scaffolds," *Sci Rep*, 2022.

[29] L. Jamalzadeh, H. Ghafoori, R. Sariri, H. Rabuti, J. Nasirzade, H. Hasani, and M. R. Aghamaali, "Cytotoxic Effects of Some Common Organic Solvents on MCF-7, RAW-264.7 and Human Umbilical Vein Endothelial Cells," 2016.

[30] C. A. Puckett and J. K. Barton, "Methods to explore cellular uptake of ruthenium complexes," *Journal of the American Chemical Society*, vol. 129, p. 46—47, January 2007.

[31] T. scientific, "Procedure for two-step coupling of proteins using edc and nhs or sulfo-nhs." https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017125$_{E}DC_{U}G.pdf$.