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FLUORESCENT MATERIALS FOR CHEMICAL SENSING IN 3D HYDROGEL CELL CULTURES

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ABSTRACT

Tara Hämeensaari: Fluorescent materials for chemical sensing in 3D hydrogel cell cultures
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Tracking concentrations of important metabolic substances such as glucose and carbon dioxide gives insights into cell metabolism and cell culture environment. Especially in the more complex 3D cell cultures it is essential to know how the substances flow and if they reach all the cells in the culture. Other environmental factors like temperature and pH should also be monitored since they have an effect on most of the cells' metabolic reactions. This thesis investigates what kind of biosensors and measuring methods have been used so far in 3D hydrogel cell cultures to measure different conditions of the culture environment. The viewpoint is especially on the use of fluorescent materials in the sensing of these factors.

In this thesis the basics of the physical phenomena of fluorescence and phosphorescence are explained to gain sufficient information for the understanding of different detection methods based on these phenomena. Fluorescence and phosphorescence are based on the absorption and re-radiation of energy, usually light photons. The absorbed and emitted light have a different wavelength. Fluorescence Resonance Energy Transfer (FRET) is a fluorescence-based phenomenon where energy is transferred non-radiatively between two fluorescent probes. It is caused by dipole-dipole interactions between the probes and results is a different set of wavelengths than the probes usually have when they are apart. These phenomena can be utilized in biosensors by for example labelling detection molecules with fluorescent dyes. Then the change in the analyte level will cause a change in the fluorescence emission intensity or lifetime, which can be detected to calculate the analyte level.

Although measuring different analytes usually requires different approaches in the sensor design, FRET is a detection method that is widely used in various sensors. Other fluorescence-based detection methods are used too. It was found that a usual problem with the sensors is the lack of testing in 3D conditions and with various cell lines. There were multiple sensors with much potential though. Other disadvantages included small detection ranges and having the sensor genetically encoded in the cells. Various advantages were presented too, for example sensitivity, biocompatibility and tunability of the sensors. It was stated that more studies about different sensors for 3D cell cultures are still needed.

Keywords: Biosensor, 3D cell culture, measurement, fluorescence

TIIVISTELMÄ

Tara Hämeensaari: Fluoresentit materiaalit kemiallisessa tunnistamisessa 3D soluviljelmissä
Kandidaatintyö
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Tärkeiden metaboliatuotteiden kuten glukoosin ja hiilidioksidin konsentraation seuranta antaa arvokasta tietoa solujen metaboliasta ja soluviljelmän kasvuympäristöstä. Varsinkin 2D soluviljelmiä monimutkaisemmissa 3D soluviljelmissä on tärkeää tietää, miten metaboliitit kulkeutuvat ympäri viljelmää ja saavuttavatko ne kaikki solut. Myös muita ympäristötekijöitä kuten lämpötilaa ja pH-tasoa tulee seurata, sillä ne vaikuttavat monien reaktioiden etenemiseen. Tässä työssä kartoitetaan, minkälaisia biosensoreita ja mittaustekniikoita on tähän mennessä käytetty 3D soluviljelmien olosuhteiden seurannassa. Näkökulma kohdistuu erityisesti fluoresenttien materiaalien käyttöön osana mittausmenetelmää.

Tässä kandidaatintyössä selitetään fluoresenssin ja fosforesenssin perusperiaatteet niihin perustuvien tunnistusmetodien ymmärtämistä varten. Fluoresenssi ja fosforesenssi perustuvat energian, yleensä valofotonien, absorptioon ja uudelleensäteilyyn. Absorboidulla ja emittoidulla valolla on eri aallonpituudet. Fluorescence Resonance Energy Transfer eli FRET on fluoresenssiin perustuva ilmiö, jossa energiaa siirtyy ilman säteilyä kahden fluoresentin molekyylin välillä. Sen synnyttää molekyylien väliset dipolivoimat, ja se aikaansaa eri aallonpituista emittoitua säteilyä kuin mitä tavallisesti syntyisi molekyylien ollessa kaukana toisistaan. Näitä ilmiöitä voidaan hyödyntää biosensoreissa esimerkiksi merkitsemällä jotain ainetta tunnistava molekyyli fluoresenteilla väriaineilla. Mitattavan aineen pitoisuuden vaihtelut saavat siten aikaan muutoksen fluoresenssin emission intensiteetissä tai eliniässä, mistä voidaan sitten laskea aineen konsentraatio.

Vaikka eri aineiden mittaaminen monesti vaatii erilaisia lähestymistapoja sensorin suunnittelussa, FRET on tunnistusmetodi, jota käytetään laajasti monissa eri sensoreissa. Muitakin metodeja aineiden tunnistukseen kuitenkin on olemassa. Työssä ilmeni, että yleisin ongelma eri sensoreissa oli se, ettei niitä ollut testattu kunnolla 3D-soluviljelmässä tai eri solulinjojen soluilla. Potentiaalisia sensoreita oli kuitenkin useampia. Muita sensoreiden haittapuolia oli kapea havainnointiväli tai se, että sensoriproteiinit oli geneettisesti koodattu suoraan soluihin. Monia hyviä puolia nousi esiin myös, esimerkiksi mittausherkkyys, bioyhteensopivuus ja mahdollisuus ominaisuuksien säätämiseen. Lopuksi tuotiin ilmi, että lisätutkimus aiheesta on tarpeen.

Avainsanat: Biosensori, 3D soluviljelmä, mittaus, fluoresenssi

PREFACE

This work is a Bachelor's thesis done for Tampere University. I would like to profoundly thank my advisor Mart Kroon for his diverse and extremely helpful comments and advice throughout this writing process. I would also like to show my deep gratitude towards my family and friends who helped me forward on this journey.

Tampere, 10th May 2021

Tara Hämeensaari

CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

C. glutamicum	Corynebacterium glutamicum cells
CDs	Carbon dots
CHO	Chinese hamster ovary cells
DLCDs	Dual-labelled carbon dots
ER	Endoplasmic reticulum
FHGB	Fluorescent Hydrogel Glucose Biosensing
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
HA	Hyaluronic acid
HepG2	Human hepatocellular carcinoma cells
HPLC	High Performance Liquid Chromatography
LCST	Lower critical solution temperature
MglB	Periplasmic glucose/galactose binding protein
NIPA	N-isopropylacrylamide (polymer)
NIPmA	N-isopropylmethacrylamide (polymer)
PAAc	Polyacrylic acid
$Ru(phen)_3$	Ruthenium(II)-tris-1,10-phenanthroline-3-trimethylsilyl-1-propane-sulfonate

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1. INTRODUCTION

Sensing metabolic substances and environmental factors that have an important role in the different biological reactions and functions of living cells provides crucial information about cell biology and the used culture environment. Monitoring how nutrients, gasses and waste are transported in a cell culture gives us better insights on what is happening inside and between the cells instead of simply measuring their size, shape and amount. Tracking different substances may also reveal faults in the used culture method. For example, biosensors can indicate that cells on some specific region have a higher rate of glucose consumption and therefore fast metabolism. On the other hand, abnormal concentrations of some chemical substance may mean that the substance is not diffused or transported homogenously around the whole cell culture.

Taking care of mass transport in a cell culture is essential to make sure that the results acquired from the culture are valid. To find out if the substance flows really cover the whole culture as they should, a variety of different types of sensors and sensor devices have been created. After making sure that the culture conditions are optimal, the same sensors can be used to sense the analyte during the actual study. Awareness around the importance of cell cultures' substance flows is not always at a sufficient level though, or a fitting sensor may be hard to find for certain applications. Optimally the sensing would be done with a non-invasive method that uses a small, selective and user-friendly device and provides continuous monitoring in real time [1]. However, none of the measuring methods are perfect and their requirements vary between the different analytes that are to be measured, which is why the most suitable method is chosen depending on the application. Differences between sensor preferences are also caused by whether the cell culture is in 2D or 3D. Using sensors is especially important for 3D cultures since insufficient spreading of substances is an even more common issue among them than the 2D cultures. For some applications 3D cultures provide more superior features compared to 2D cultures, which is why having sensors suitable for 3D applications is deeply desirable.

For many cell types 3D cultures provide an environment that is more similar to the cells' native tissue compared to the environment provided by a 2D culture. Adding the third dimension has an effect on for example cell proliferation and differentiation, and ultimately cell survival in the culture. 2D cultures are widely used because they are usually simpler and more cost-efficient than 3D cultures. This makes results acquired from 2D culturing more comparable to other 2D studies, whereas 3D culturing is lacking in this area. 2D cultures provide a great tool for simple studies and tests where the complicated structure of a 3D culture would only disturb the process. They are also a reasonable choice when modelling epithelial tissue. However, when a more realistic tissue model is the goal, it is essential to create it in 3D. [2]

In 3D cultures cell aggregation is an even more common problem than in 2D cultures. When cells form clusters the flow of substances inside these aggregates becomes insufficient, which needs to be addressed to get valid results. Usually cell aggregates have necrotic or quiescent cells in the middle of the clusters even though the cells on the surface are surviving just fine. [2] This behaviour is expected since in natural tissues cells are always within 100 to 200 micrometres of a blood vessel that provides nutrients as well as an exit route for metabolic waste and possibility for gas exchange [3]. The cell cultures and the aggregates that form in 3D cultures do not usually contain any vasculature and therefore the cells rely on diffusion for their nutrients. The diffusion distances in cell cultures are however often in the order of millimetres so significantly bigger than the diffusion distance in natural tissues. [4] It was proven by Figueiredo et al. in 2018 that a higher polymer concentration in a hydrogel results in weaker flow of glucose and oxygen in the cell culture. They also found a correlation between reduced mesh size in the polymer hydrogel structure and reduced glucose and oxygen diffusion in the culture. [5] The problem of cell aggregation and poor nutrient flow could be solved with different scaffold materials or mesh sizes or perhaps by creating specific microtopographies. Vascularization or creating artificial perfusion channels could also provide a solution to these problems [6].

The aim of this literary review is to map out what kind of biosensors are or have been used to measure different substances, and to find out the good and bad aspects of these sensors when it comes to applying them to 3D hydrogel cell cultures. This Bachelor's Thesis includes two main chapters. Chapter two will introduce three basic mechanisms that are used in different biosensors: the phenomena of fluorescence and phosphorescence will be explained in subchapter 2.1 and Fluorescence Resonance Energy Transfer

(FRET) will be briefly discussed in subchapter 2.2. Understanding these physical phenomena will help when reading about the different biosensor types in the next main chapter, chapter three. Chapter three will introduce and discuss different biosensors one analyte at a time. It is divided into subchapters based on these analytes that the subchapter's sensors measure. Lastly, chapter four will conclude the contents of the previous chapters to create a summarization of the thesis.

2. THEORY BEHIND THE DIFFERENT DETECTION METHODS

The sensors discussed in this thesis are based on some form of photoluminescence. Mostly the natural phenomena utilized in these sensors is either fluorescence or phosphorescence, both a type of photoluminescence. Based on the interaction between fluorescent molecules, FRET is a phenomenon that is often used as a detection method when the sensor consists of fluorescent materials. All three phenomena will be briefly explained in this chapter.

2.1 Photoluminescence in sensors

Fluorescence and phosphorescence are phenomena that happen naturally among certain chemical substances. They are frequently utilized in different biosensors, which is why their basic theory is good to understand before discussing the actual sensors more thoroughly.

2.1.1 Fluorescence

A widely utilized phenomenon in optical sensing is fluorescence. It was first discovered in 1852 but its use in biological analytics only started in the 1930s. Fluorescence is based on the absorption and re-radiation of energy, usually light photons. As shown in figure 1, when a photon is absorbed into a molecule, the molecule's electrons transition to an exited state and then back to their ground state whilst releasing an emission photon. Some of the electrons' vibrational energy is lost during the relaxation from excited state to ground state. This results in an emission spectrum at longer wavelengths than the original excitation spectrum. The shift in the wavelength spectrum is referred to as the Stokes' shift and it is visualized in figure 2. Because of the different wavelength of the emitted light, the excitation light can be filtered out of the light that is detected. [7, 8]

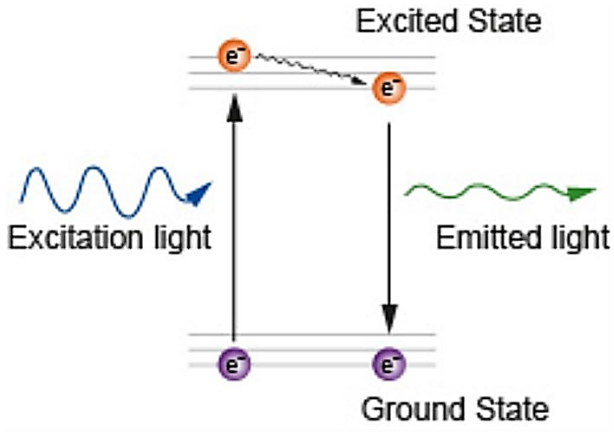

Figure 1 *A schematic presentation of fluorescence.* [8]

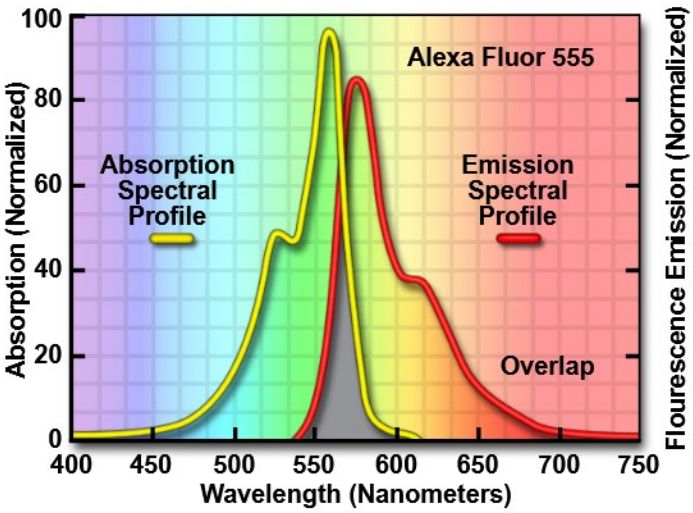

Figure 2 *Absorption and emission profiles of fluorophore.* [7]

Even though fluorescent materials have been around for a long time, they are still desirable due to their ability to sense the analyte without consuming it. Fluorescence can also be detected remotely, which leads to simultaneous detection all around the culture. [9] In many applications the effect of fluorescence is caused via fluorophores that specifically attach to a target compound that is not fluorescent itself [7].

2.1.2 Phosphorescence

Whereas in fluorescence the re-radiation of light happens within 10^{-8} seconds after the absorption of photon, in phosphorescence the reaction happens after a while and lasts longer. This is because in phosphorescence the electron that raised to an excited energy state has fallen to an intermediate metastable level between the usual excited and

ground level. [10–12] The shift from the usual singlet excited energy state to the lower triplet energy state is referred to as intersystem crossing. When an electron relaxes to or from the triplet energy state, its spin is inverted. During phosphorescence the electron loses more energy in non-radiative vibration than it does during fluorescence, which causes a larger Stokes shift. [11] The different energy levels and transitions that occur in fluorescence and phosphorescence are demonstrated in figure 3.

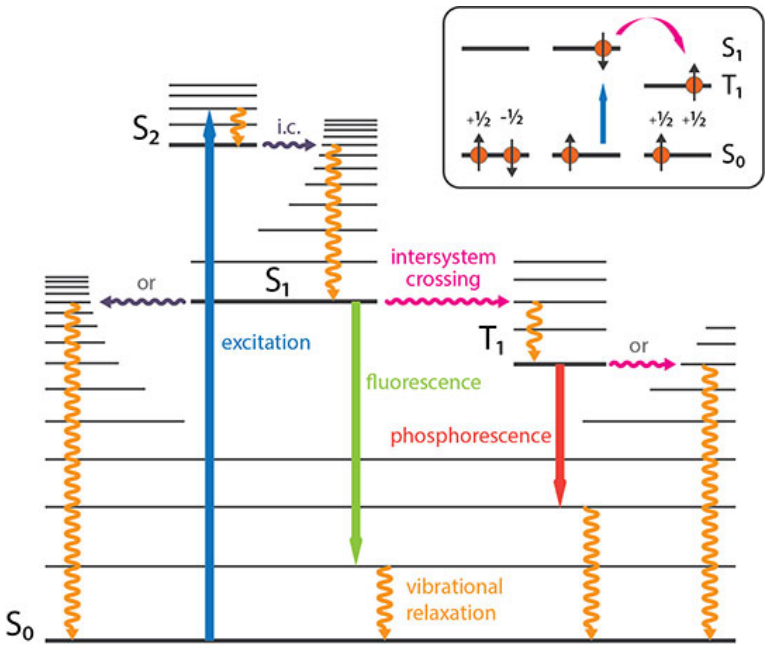

Figure 3 *The electron's energy levels and transitions between them in fluorescence and phosphorescence.* [11]

Because the transition between the triplet excited energy state and the ground energy state is kinetically non-favourable, it takes time for the transition to happen. This is why light emission by phosphorescence happens a while after the original excitation. [12] The lifetime of phosphorescence depends on the time that the electron has spent on the metastable energy level. [10]

2.2 FRET

FRET comes from the words Fluorescence (or Förster) Resonance Energy Transfer and it was discovered in 1946 by Theodor Förster [13]. It is a widely used photophysical detection method that has a variety of applications: It has been used both in vivo and in vitro to study protein conformation, the binding of ligands to receptors and the dynamics of diffusion and cellular membrane. It has also been applied to monitor DNA sequencing

and hybridization. [14] It is also a common element among multiple different biosensors that are discussed in chapter 3.

FRET is caused by the dipole–dipole interactions between a fluorescent donor and acceptor probe. When the probes have a fitting set of spectroscopic properties, there is a non-radiative transfer of energy from the donor probe to the acceptor probe. Since the fluorescent donor molecule usually emits energy at a shorter wavelength than the acceptor molecule, the emitted energy corresponds to the optimal absorption wavelength of the acceptor, which enables the resonant energy transfer between the probes. [13, 14] The final emitted radiational energy has a wavelength that differs from the emission wavelengths of the singular donor and the acceptor probes. Therefore, the emission radiation caused by FRET can be detected by filtering out the wavelengths that are not FRET-related. The process of FRET is presented in figure 4.

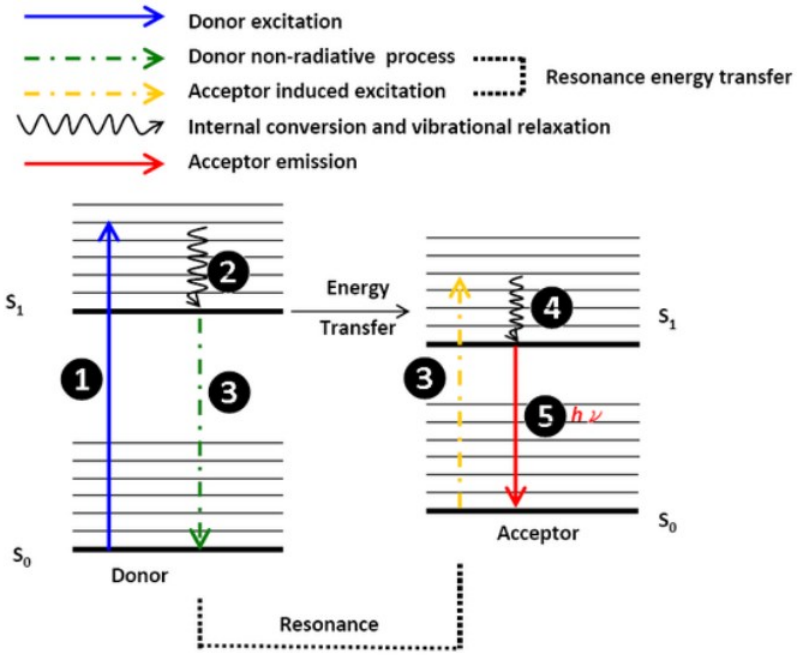

Figure 4 *A schematic presentation of the Fluorescence Resonance Energy Transfer (FRET).* [15]

The efficiency of the energy transfer depends on how much the emission spectrum of the donor probe overlaps with the absorption spectrum of the acceptor probe. It is also dependent on the donor's quantum yield and the relative orientation of the donor and acceptor dipoles. Most importantly the FRET efficiency depends on the sixth power of the distance between the donor and acceptor probes. [14, 16] Because the FRET energy transfer is highly sensitive to the distance between the donor and acceptor molecule, as

precisely as in nanometre-scale, it is a powerful technique to detect even the smallest changes [14].

Because the energy transfer occurs only when the fluorescent molecules are in a close enough proximity of each other and have the correct relative orientation, FRET can be used to detect the distance of two compounds labelled with the FRET molecules. When an analyte is detected with FRET, a detection molecule, that for example undergoes conformational changes when in contact with the analyte, is labelled with one or both of the FRET-responsive molecules. The presence of the analyte causes a change in the detection molecule, which thereafter enables or disables the energy transfer between the fluorescent molecules. This change in the FRET efficiency is detected and the concentration of the analyte is calculated from its FRET response.

3. BIOSENSORS FOR DIFFERENT ANALYTES

The measuring method depends, among other factors, on the analyte that is to be measured. The analyte affects the choice of material, followed by the method used to immobilize the indicator. The following paragraphs discuss the different types of biosensors that have been used for specific analytes. Although some sensor types are used for multiple analytes, it is not the common situation. Since different metabolic substances and environmental factors differ for example by their structure and functionality, also their sensors are required to be different from one another.

3.1 Biosensors for glucose

Glucose is the greatest source of energy in cells' metabolic processes, which makes it also one of the most important substances that need to be monitored. Abnormal glucose levels can be an indication of poor health conditions because too low or high of a glucose concentration causes harmful local and systemic effects both on the cells and the whole human body. Then again, a high glucose consumption rate might be caused by a very active glycolysis, which would in turn be a sign of high cell activity. [17] Because glucose is so important for the cells, it is necessary for cell cultures to have sufficient glucose levels in order to keep the cells alive. Monitoring glucose levels can reveal insufficiencies in its distribution and therefore explain low cell survival rates or other unwanted results.

As stated in chapter 2.2, the use of FRET detection is a desirable method for the sensing of different molecules. Glucose sensing is no different. In 2012 Behjousiar et al. made a study about detecting intracellular glucose levels in Chinese hamster ovary (CHO) cells [18]. They used FRET detection method with protein-based biosensors very similarly to the study performed by Otten et al. in 2019 which is discussed in the next paragraph. Both studies used a protein-based biosensor that consists of periplasmic glucose/galactose binding protein and two fluorescent probes, cyan and yellow fluorescent protein. However, Behjousiar et al. had the biosensor inside the cells by using cells that have been transfected with a protein production encoding plasmid [18]. Having a sensor that requires genetic modification of the monitored cells is not idea, since it might limit the use of that sensor to one specific cell line. Genetically encoding cells is also a troublesome extra step when studying cells since it must be kept in mind all the way from the

beginning when the study is planned. It is much easier to use a sensor that can be applied on any cell line without laborious modification steps.

Otten et al. used FRET as a glucose detection method in 2019 in a study where they created both soluble and immobilized glucose sensors the use of which did not require genetical encoding of the cells. The soluble sensor was made of a periplasmic glucose/galactose binding protein (MglB) and two variants of green fluorescent protein: mTurquoise2 as the donor probe and Venus as the acceptor probe. These variants were chosen because mTurquoise2 is more stable and creates a brighter signal than other donor probes, and Venus is less sensitive to pH and ion concentration changes than other acceptor probes. In the study the FRET ratio of the fluorescent probes was determined as its changes described the occurring glucose concentration. The FRET ratio was determined as the fluorescent intensity of the acceptor probe divided by the fluorescent intensity of the donor, and it got higher values as the glucose concentration rose. The soluble sensor was used in at-line measurements of Corynebacterium glutamicum cells with a sample size of 15 µL. The results were confirmed with HPLC and enzymatic analysis. It was found that the soluble glucose sensor had a very high sensitivity and a detection range of 0,01 mM to 10 mM which could easily be broadened to 0.4-400 mM by dilution to cover the whole glucose concentration spectrum of cell cultures. The measuring process was much faster compared to other methods, which resulted in shorter measurement cycles and therefore increased data density. However, the sensor was found to be unstable in room temperature after 48 hours and it showed vulnerability towards shaking, which prevents its usage in online measurements. [19]

Otten et al. immobilized the soluble glucose sensor to counter the challenges of online analysis. In order to make the sensor tolerate the mechanical stress and temperature of a cell culture long-term, the soluble sensor protein complex had a fusion with HaloTag® and was immobilized onto Sepharose® beads. Because long-term exposure to the CGXII cultivation media of C. glutamicum cells had a strong influence on the sensor's sensitivity due to background and quenching, the use of the immobilized sensors was studied in M9 medium with Escherichia coli cell culture. The sensor performed well but the detection range was only from 0,02 mM to 2 mM, so in order to detect a wide enough spectrum of glucose concentrations there would have to be a mix of sensors with different detection ranges. The immobilized sensor's detection range can be altered by changing its affinity by amino acid substitution in glucose binding proteins, use of different glu-

cose binding proteins altogether, or insertion of linker sequences. The immobilized sensor could of course also be used in a mere at-line measurement, where the detection range could simply be altered by dilution and the strong background of a media would not create a problem. It would still be more stable than the soluble sensor, and it could be made with magnetic particles to create an easily recyclable and washable sensor. [19] This immobilized sensor shows promise to being applicable in 3D hydrogel cell cultures since it can be used in online measurements and it does not require genetic modification of cells. However, its limitations regarding the culture medium will be a problem for some studies.

The detection method of fluorescence resonance energy transfer (FRET) was also utilized in a study performed by Ge et al. in 2018 where they created a glucose sensor with hyaluronic acid (HA) hydrogel microspheres. The microspheres had encapsulated Eu-dextran and Alexa Fluor 647-ConA that are responsible for the FRET effect. The sensing mechanism is based on the reversible attachment of Alexa Fluor 647-ConA to Eu-dextran. The small distance caused by the attachment leads to quenching of the fluorescence signal emitted by Eu-dextran. In the presence of glucose there is competitive binding between glucose and Eu-dextran, which then causes Eu-dextran to get released from Alexa Fluor 647-ConA and begin fluorescence again. This is presented in figure 5. Even though europium is toxic to the human hepatocellular carcinoma (HepG2) cells used in the study, the microsphere structure blocks its effects from causing harm to the cells around it. This results in a biocompatible sensor with a stable fluorescence emission and high sensitivity. The microspheres had a diameter of 60 µm and could detect glucose concentrations from 0,5 mM to 10 mM. [17] Having sensors in hydrogel microspheres that are only permeable to the analyte in question seems like a great fit for hydrogel cell culture since the microspheres would seamlessly fuse into the hydrogel matrix. The signal is also very simple and easy to detect.

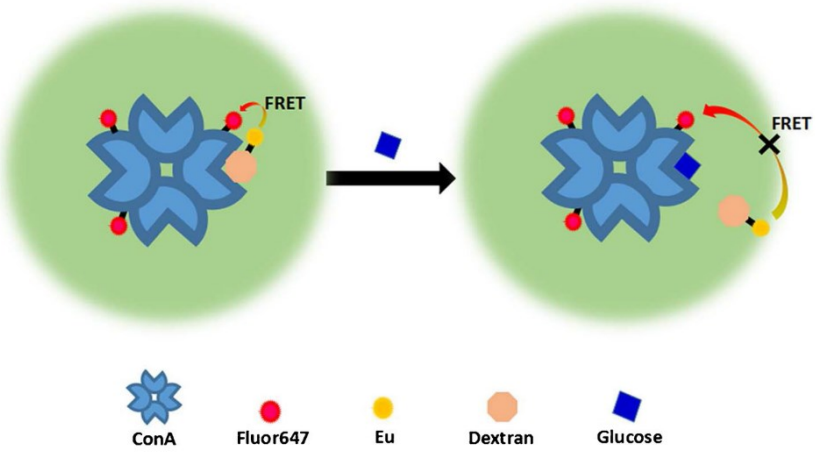

Figure 5 *The principle of FRET effect with the fluorescent probes used in the study by Ge et al. in 2018.* [17]

Fluorescence can also be utilized together with enzymatic detection of molecules. Park et al. combined these two phenomena in a study in 2018 by creating Fluorescent Hydrogel Glucose Biosensing (FHGB) microdroplets with dual-mode detection by fluorescence quenching and size reduction. Carbon dots, glucose oxidase and horseradish peroxidase were immobilized into a polyacrylic acid (PAAc) hydrogel droplet which undergoes a bienzymatic reaction in the presence of glucose. The reaction generates gluconic acid and lowers the pH level. This results in quenching of the sensor's fluorescence and size reduction of the droplet, which are both visible to naked eye. This can be seen in figure 6. The size of the microdroplets in a swollen state is 560 µm and the sensor can detect a glucose concentration up to 30 mM. The sensor is highly sensitive, selective, stable and biocompatible, and it can be stored in a dry environment for at least 4 weeks without losing its detection capabilities. [20] Especially these qualities make the FHGB microdroplets an extremely great option for glucose monitoring in cell cultures. However, since the detection method is based on enzymatic reaction with glucose, using this sensor would spend the culture's glucose that the cells need.

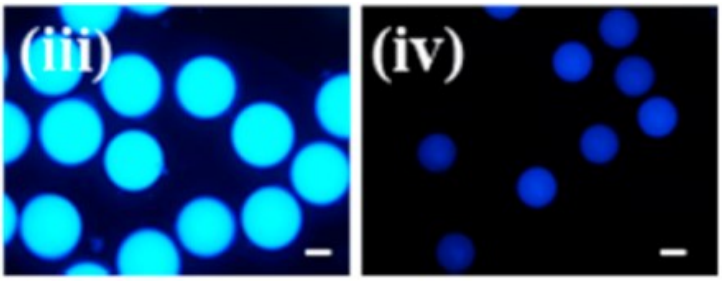

Figure 6 *FHGB microdroplets before (iii) and after (iv) the addition of glucose. The scale bar is 200 μm.* [20]

It seems that there is a variety of fluorescence-based glucose sensors many of which have multiple good qualities when it comes to applying them in 3D cell cultures. Still, many of them have downsides too: having sensors genetically encoded into cells is not ideal and neither is an enzymatic detection method. Even the best sensor types that do not have these disadvantages require further studies about their applicability to different cell lines. However, this can of course be checked for the specific cell line individually before using the sensor. A conclusive chart of the discussed sensors is presented in table 1.

Table 1 *Different glucose sensors.* [17–20]

Analyte	Sensor type	Reference	Year	Cells	Theory	Material	Size	Detection range	Reversible response	Good	Bad
Glucose	Genetically encoded protein-based biosensor with FRET detection	Behjousiar et al.	2012	Chinese hamster ovary (CHO) cells	Transfected plasmid causes production of glucose periplasmic binding protein and fluorescent proteins that detect glucose with FRET	Plasmids, glucose periplasmic binding protein (mglB), enhansed cyan fluorescent protein (ECFP), enhanced yellow fluorescent protein (EYFP, Aphrodite variant)		10 µM-1 M	Yes	Selectivity, in situ calibration curve	Genetically encoded
Glucose	FRET detection with soluble and immobilized sensors	Otten et al.	2019	Soluble: Corynebacterium glutamicum	FRET detection, stabilizing against mechanical stress by immobilizing onto beads	Variants of green fluorescent protein: mTurquoise2 and Venus, periplasmic glucose binding protein (MglB)		Soluble: 0.01-10 mM (0.4-400 mM via dilution)	Yes	Soluble: wide detection range, very good sensitivity, fast measurement process	Soluble: non-stable in room temperature long-term, non-stable under mechanical stress, no online measuring
				Immobilized: Escherichia coli				Immobilized: 0.02-2 mM		Immobilized sensor: easy and simple immobilization straight from crude cell extract, good stability	Immobilized: small detection range, not usable for certain media
Glucose	FRET-based sensor with a lanthanide ion luminophore-based hyaluronic acid hydrogel microspheres	Ge et al.	2018	Human hepatocellular carcinoma (HepG2) cells	FRET for detection, ultrasonic emulsification for creating microspheres	Hyaluronic acid, Eu-dextran, Alexa Fluor 647-ConA	60 µm	0,5-10 mM	Yes	Biocompatibility, fluorescence stability, sensitivity	Probe design, encapsulation
Glucose	Fluorescent hydrogel glucose biosensor (FHGB) microdroplets with dual-mode fluorescence quenching and size reduction	Park et al.	2018		Bienzymatic reaction between glucose, glucose oxidase and horseradishperoxidase cause fluorescence quenching and FHGB droplet size reduction	Polyacrylic acid (PAAc), carbon dots (CDs), glucose oxidase (GOx), horseradish peroxidase (HRP)	363-571 µm	<30 mM	No	Biocompatibility, sensitivity, selectivity, stability, visibility to naked eye, long shelf life	No testing with cells

3.2 Biosensors for oxygen

Molecular oxygen is an important metabolite and vital for most types of cells because it has a key role in the respirational energy metabolism [21, 22]. During the electron transport chain in oxidative phosphorylation, oxygen takes part in producing ATP for cells to use in other metabolic reactions. It also works as a substrate in many enzymatic reactions. [22] An interesting feature of molecular oxygen that is often utilized in oxygen biosensors is its ability to naturally quench molecular photoluminescence. When oxygen molecule collides with a photoluminescent indicator molecule, some of the indicator's energy is transferred to oxygen and therefore the indictor's emission signal has a lower intensity and lifetime. The amount that the intensity and lifetime are reduced depends on the oxygen concentration around the indicator molecule. It is theoretically a linear dependence but any microheterogeneities in the biosensor probes can cause non-linear behaviour. The reduced lifetime is less dependent on the luminophore concentration and is therefore more often used to calculate the actual oxygen concentration. Oxygen's tendency to quench photoluminescence can be partly explained by the fact that it has a special triplet energy ground state. This makes energy transfer between other triplet state molecules very efficient and the measured quenching effect highly specific with only minor cross-reactions. However, quenching results in singlet state oxygen molecules, which may cause damage to the cells if they are not protected against this influence. The process of phosphorescence quenching by molecular oxygen is presented in a diagram in figure 7. [21]

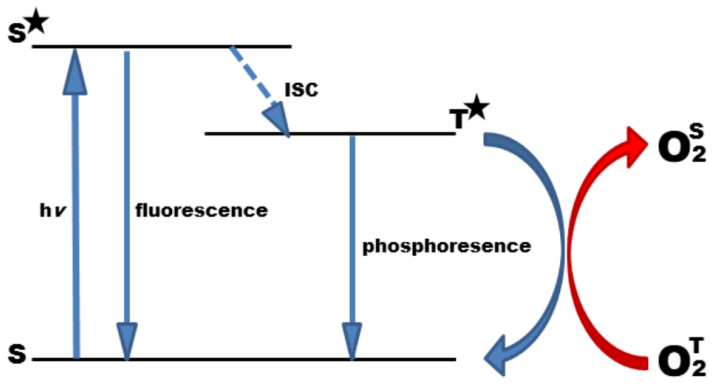

Figure 7 *Diagram of possible energy states of the indicator molecule: ground state (S) before absorption of radiation (hv), and excited singlet state (S*) and excited triplet state (T*) after the absorption. Phosphorescence quenching is caused by triplet oxygen (O_2^T) and results in singlet state oxygen (O_2^S).* [21]

Oxygen sensors usually consist of an indicator dye that is immobilized into some sort of a probe. The indicator dyes are organometallic materials that can be categorized into metalloporphyrins and transition metal complexes with pyridine derivatives [21]. A great benefit of metalloporphyrins is the capability to tailor their qualities and therefore create variations. This can be achieved by using different metal ions as luminophores or adding complexing agents with special chemical structure. Tailoring can also be done by creating a specific matrix around the luminophore, which is usually connected to the immobilization method. The most commonly used metalloporphyrins are platinum(II)- and palladium(II)-porphyrins. Pt(II)-porphyrins have a shorter luminescence emission lifetime and a preferred oxygen range of 0-200 µM, whereas Pd(II)-porphyrins have a longer lifetime and are better for oxygen ranges below 50 µM. [21, 22] Both porphyrins have distinct absorption spectra, high signal intensity and good photostability. They can be used in room temperature and aqueous solutions. [21] Also, they rarely show signs of phototoxicity, which makes them applicable in biological applications [22]. Most common transition metal complexes with pyridine derivatives are ones with ruthenium or iridium. Compared to organometallic materials, ruthenium complexes have shorter lifetimes, which results to lower oxygen sensitivity but also faster image acquisition. Cyclometallated complexes of iridium have high brightness and medium long lifetimes, but face a problem with poorer photostability when used continuously for longer times. [21, 22]

To immobilize these sensor molecules, they are encapsulated within a matrix that is permeable to oxygen molecules. This both increases the dye's signal intensity and shields from any undesired interference with other molecules. The matrix materials used for this encapsulation can be for example polystyrene, polymethylmethacrylate or any glass-like materials. Often soluble sensor probes are wanted, which is why the hydrophobic aggregate forming Pt(II)- and Pd(II)-porphyrins must be conjugated to hydrophilic molecules. [21] The sensor structures can also be produced and expressed inside living cells by genetically modifying the cells to produce them themselves [21, 22].

Ruthenium dibipyridine 4-(1-pyrenyl)-2,2-bipyridine chloride [$Ru(bpy\text{-}pyr)(bpy)_2$] was tested for measuring molecular oxygen in J774 murinae macrophages in a study by Ji et al. in 2002. The indicator dye passively permeated into the cells and were sensitive towards oxygen for as long as 5 hours. When there was a concentration of over 80 µM of the dye there was a self-quenching effect, which is why the cells were incubated with a dye concentration of only 50 µM. The ruthenium complex showed strong absorption visible for the naked eye, efficient fluorescence and long lifetimes of the excited state. The

response of the complex to different oxygen concentrations is shown in figure 8. Also, the complex's absorption and emission spectra had a wide Stokes shift which made the signal detection much easier. In addition, the complex had a high level of chemical and photostability: the fluorescence intensity of the complex had dropped to half after as long as 30 minutes of continuous exposure to light. Because the detection of oxygen was a result of reversible attachment of oxygen, it did not take oxygen away from the use of the cells. This was a great quality for online measuring of oxygen in a cell culture, as well as the fact that mechanical movement of the cell culture medium did not affect the accuracy of the measurement. However, there was some undesired interaction between the indicator molecule and the cells' cytoplasm that caused extra fluorescence and so affected the accuracy of the result. Also, the indicator dye could be distributed with heterogeneity which would then result in variations between the cells. [23]

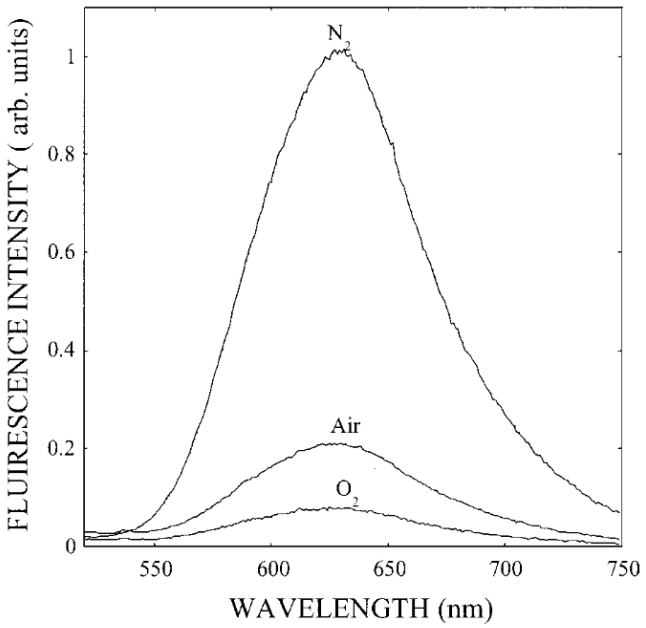

Figure 8 *Emitted fluorescence intensity of Ru(bpy-pyr)(bpy)$_2$ complex in different oxygen concentrations in aqueous solution.* [23]

Koren et al. used iridium in a porphyrin complex in a study in 2011. They found that the synthesis and chemical structure of different variations of Ir(III)-porphyrins was more difficult than those of corresponding platinum and palladium structures. Koren et al. experimented with the addition of different axial ligands and so induced solubility, polarity or binding to wanted molecules. The chemical structures of the four different Ir-complexes that were studied are shown in figure 9. The improved solubility to organic solvents made it possible to incorporate the sensor molecules into a polymer like polystyrene. The addition of binding groups enabled coupling to silica gel without the modification of the

porphyrin macromolecule itself. The addition of polar groups like imidazole ligands with a carboxyl group makes the complex soluble to polar solvents like alcohols and water, but also enables coupling to other biomolecules, which disables oxygen from reaching the dye and so decreases the efficiency of the quenching effect. The tested Ir(III)-porphyrins had very linear changes in their phosphorescence intensity and lifetime when the surrounding oxygen concentration increased. The linearity was better than the linearity of the widely used platinum-porphyrin complexes. Ir(III)-porphyrins also had strong emission in room temperature and a tailorable sensitivity to small oxygen concentrations. All this makes them highly ideal for biological measuring applications. [24]

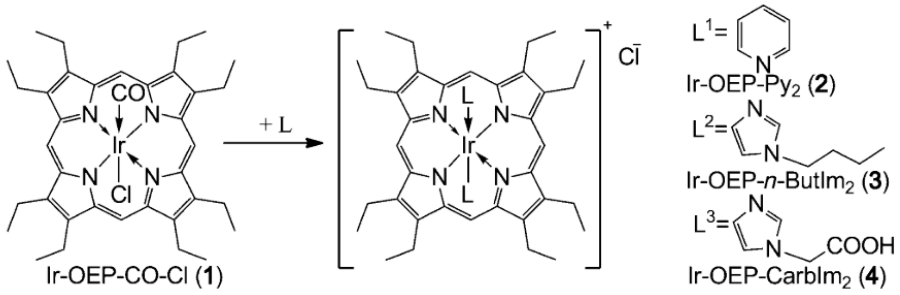

Figure 9 *Chemical structures of the four Ir-complexes that Koren et al. used in their study.* [24]

As a conclusion, oxygen sensors are more often based on molecular oxygen's own ability to quench photoluminescence rather than FRET or some other method. The sensors are based on luminescent metal ion complexes that can be chosen to fit a specific need. A table of the discussed sensor study articles was made to summarize the sensors' features. The summary is presented in table 2.

Table 2 *Different oxygen sensors.* [23, 24]

Analyte	Sensor type	Reference	Year	Cells	Theory	Material	Reversible response	Good	Bad
Oxygen	Ruthenium (II) diamine-pyrene complex used as a solution	Ji et al.	2002	J774 murinae macrophages	Fluorescent quenching caused by molecular oxygen	Ruthenium dibipyridine 4-(1-pyrenyl)-2,2-bipyridine chloride [Ru(bpy-pyr)(bpy)2]	Yes	Strong visible absorption, efficient fluorescence, long excited state lifetime, large Stokes shift, high stability, online measuring	Undesired interactions between indicator and complex cytoplasm may affect fluorescence, heterogenic dye distribution causes variation between cells
Oxygen	Iridium(III)-porphyrins	Koren et al.	2011		Phosphorescence quenching when in contact with molecular oxygen	Variants of Ir(III)-porphyrins with different axial ligands	Yes	Tailoring, linear changes in phosphorescence, coupling to biomolecules and other wanted surfaces, strong emission in room temperature, sensitive to small oxygen concentrations	Difficult chemical structure and synthesis, no testing on cells

3.3 Biosensors for Ca^{2+} ions

Calcium ions are important signalling molecules since they function as vital second messengers for various environmental stimuli in cells. Their levels affect for example many catabolic reactions and how cells move. [25, 26] Calcium also regulates many different physiological phenomena in cells for example cell development, differentiation and apoptosis [26].

FRET was utilized in calcium sensors too. Already in 1997 Miyawaki et al. created a new class of indicators called cameleons. The four-part indicator complexes showed FRET signal when in contact with calcium ions. Their mainly used indicator complex formed of calmodulin (CaM), calmodulin-binding peptide M13, and cyan and yellow mutants of green fluorescent protein (GFP). Surrounding calcium ions would bind to calmodulin which would then bind to M13. Because the GFP probes were located one at calmodulin and one at M13, their binding would bring the donor and acceptor probe into such a close proximity of each other that it resulted in FRET signal. The protein complex was expressed and folded efficiently in HeLa cells and showed a strong FRET signal. The indicator also had high specificity towards calcium ions and was not affected by other molecules surrounding it. The sensor's detection range, from 10^{-8} to 10^{-2} M, was rather wide and located at the smaller calcium concentrations. The indicator complex could also be tailored: using mutations of calmodulin calcium ion affinities could be tuned, and fusion to localized host proteins or specific targeting signals would direct the sensor to a different location like nucleus or endoplasmic reticulum (ER). However, the sensor had a pH limitation since pH levels below 7 affected their functionality. Too strong of an illumination created a problem too, since it caused the acceptor probe's reversible photochromism. [27]

In 2001 Truong et al. made a new version of the cameleon that Miyawaki et al. created. Since the old sensor had a rather small change in FRET emission between calcium's bound and un-bound state, also known as small dynamic range of FRET, it was difficult to detect the smaller changes in calcium concentration. In order to make the dynamic range of FRET higher, they changed the calmodulin-binding peptide to CKKp. CKKp was also fused with glycine rich bonding sequences at a linker region between the N- and C-terminal of calmodulin instead of being linked to calmodulin's C-terminal like in the old sensor protein complex. The protein structure of the new cameleon variant is shown in figure 10. In the presence of calcium the CKK peptide forms a tight structure with α-helix

and a hairpin loop, which brings the mutants of the GFP in the calmodulin's N- and C-terminus even closer together than the old structure could bring. This effect of calcium ions is presented in figure 11. In the old cameleon the fluorescent probes were about 50-60 Å apart from each other in the calcium-bound state whereas the corresponding number for the new cameleon was well under 40 Å. The calcium-free distance was assumed to be fairly the same between the two variants. This change in distance gives the new sensor a larger dynamic range of FRET efficiency that is distributed to a smaller detection range altogether, making the new sensor more sensitive to physiologically important calcium concentrations. [26] Although this sensor is clearly an improved version of the sensor by Miyawaki et al., both are produced inside the cells as genetically encoded proteins. That remains a downside for this sensor.

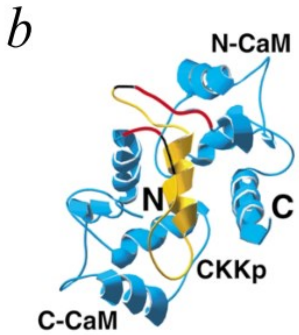

Figure 10 *The protein structure of (N-CaM)-CKKp-(C-CaM) sensor protein complex. Calmodulin-binding CKK peptide is fused at a linker region (red) of calmodulin protein (CaM) with glycine rich binding sequences (black). The fusion site is located between the N-terminus of calmodulin (N-CaM) and C-terminus of calmodulin (C-CaM).* [26]

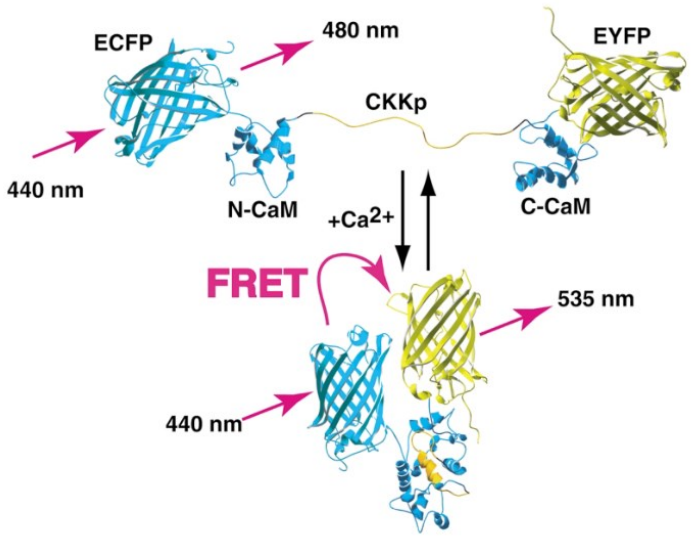

Figure 11 *A schematic showing Treong et al.'s sensor's response to calcium ions. Enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) are bound to opposite ends of calmodulin (CaM). In the presence of calcium the calmodulin-binding peptide CKK folds into a tight structure bringing the two fluorescent proteins in close proximity from each other, which results to FRET.* [26]

Another widely used set of proteins for measuring intracellular calcium levels is GCaMP proteins but they have multiple limitations when measuring calcium ion concentration in the endoplasmic reticulum. Using GCaMP proteins is based on measuring their fluorescence intensity, which may cause conflicting results since fluorescence intensity is dependent on the expression level and density of the fluorescent probes. The expression levels and densities are very difficult to control and as an addition the ER has local microdomains and a dynamic structure that makes it even more challenging. A ratiometric sensor would not have these problems but many previously used ratiometric sensors had their own problems with phototoxicity, aggregation or distracting FRET among other things. Luo et al. however created a ratiometric sensor in 2019 that overcomes those difficulties too. The genetically encoded calcium sensor fused a calcium detecting protein with a SNAP-tag that was also fluorescent itself. This way the fluorescent signal could be calibrated using the SNAP-tag's signal, which enables accurate measuring of calcium levels even in the dynamic ER. [25] Unfortunately this sensor too is genetically encoded into the cells, which makes the sensor more difficult to apply on different cell lines.

It appears that there are many forms of genetically encoded calcium sensors, but the availability of independent calcium sensors is non-existent. But perhaps the genetically encoded protein complexes could be produced separately and afterwards injected into

the cells. This of course damages the cells more than when the proteins are produced inside the cells. It seems that independent calcium sensors that would be usable in 3D cell cultures is still a subject that requires more studies about it. A conclusion of the different aspects of the calcium sensors discussed in this chapter is presented in table 3.

Table 3 *Different calcium sensors.* [25–27]

Analyte	Sensor type	Reference	Year	Cells	Theory	Material	Detection range	Reversible response	Good	Bad
Calcium	Genetically encoded protein complex with calmodulin, calmodulin-binding peptide M13 and cyan and yellow mutants of GFP	Miyawaki et al.	1997	HeLA cells	Binding of calcium to calmodulin makes calmodulin wrap around M13 resulting in close proximity between GFPs and FRET	Calmodulin, calmodulin-binding peptide M13, enhansed cyan and yellow mutants of green fluorescent protein	10 nM - 10 mM	Yes	Strong FRET signal, targetability	Small dynamic range of FRET, influenced by pH lower than 7, reversible photochromism of acceptor probe if illuminated too strongly, genetically encoded
Calcium	Genetically encoded protein complex with calmodulin, calmodulin-binding peptide CKK in the middle of calmodulin, and cyan and yellow mutants of GFP	Truong et al.	2001	HeLA cells, rat hippocampal neurons	Binding of calcium to calmodulin makes CKKp fold and bring the fluorescent proteins close to enable FRET	Calmodulin, calmodulin-binding peptide CKK, enhansed cyan and yellow mutants of green fluorescent protein	0.05-1 µM	Yes	Large dynamic range of FRET, linear response, high sensitivity in physiologically important calcium concentrations	Smaller detection range, genetically encoded
Calcium	Genetically encoded ratiometric calcium sensor for ER calcium	Luo et al.	2019	COS7 cells, MEF cells	Fluorescent calcium sensor protein has higher fluorescent intensity when in contact with calcium. Fusion with a fluorescent tag enables calibration.	GCEPIA1 protein, SNAP-tag		Yes	Accuracy, direct and easy real-time calibration, ability to measure dynamic ER calcium in live cells, multicolour imaging	Genetically encoded

3.4 Biosensors for pH

Having the correct pH level around cells is crucial for numerous chemical reactions and biological processes. It has been shown that pH levels also differ between healthy cells and tumor cells: Tumor cells have more acidic surroundings, which has an effect on their migration and adhesion abilities as well as drug resistance. [28, 29] Intracellular pH affects the cells' basic functions like proliferation, metabolism and apoptosis, but also ion transportation, muscle contraction and endocytosis. [29]

Shi et al. used carbon dots (CDs) to immobilize their pH sensor in 2012. Their CDs were coated with 4,7,10-trioxa-1,13-tridecanediamine (TTDDA) and then treated with a pH-sensitive fluoresceinisothiocyanate (FITC) dye and pH-insensitive rhodamine B isothiocyanate (RBITC) dye to form dual-labelled CDs (DLCDs). After purification the red solution of DLCDs could be used directly or lyophilized for later use. The DLCDs have a diameter of 7 nm in water and had a clear fluorescence response to change in pH. Since the increased pH increased the fluorescence intensity of RBITC only a little whereas the fluorescence intensity of FITC increased significantly, their fluorescence emission intensity ratio could be used to calculate the pH level ratiometrically. The change in the dyes' fluorescence intensity is shown in figure 12. [30]

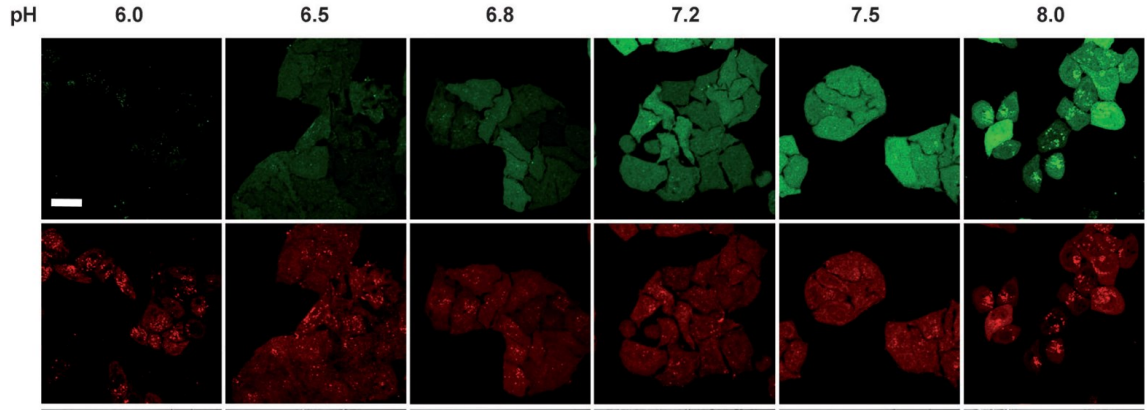

Figure 12 *The fluorescence emission of FITC dye (green) and RBITC (red) at different pH levels. The scale bar is 20 µm.* [30]

The DLCD sensor has a reversible response to pH and a detection range from 5.2 to 8.5. This could be affected by changing the dye ratio, but too much of RBITC made the sensor less sensitive, so the optimal FITC/RBITC ratio that resulted to said detection range without affecting the sensitivity of the sensor was 1:10. The change in sensor sensitivity can be seen in figure 13 where the fluorescence intensity ratio is plotted against

the different pH values. The ratiometric carbon nanodot sensor of Shi et al. was applied with HeLa cells and MCF-7 cells. Since the cells' viability practically did not change in 24 hours of measuring, it is safe to say that the sensor is non-cytotoxic and biocompatible. The sensor also proved to be un-influenced by any intracellular molecules or ions. It was shown that the DLCDs were distributed in the whole cells instead of just certain organelles, which was one of the goals of the study. The great dispersibility of the DLCDs was explained by their excellent biocompatibility and small size. [30] As a whole, these dual-labelled carbon dot sensors seem to be a great option for online pH measurement. However, their applicability to other cell lines should me studied more since Shi et al. used only two cell lines both of which were cancer cells.

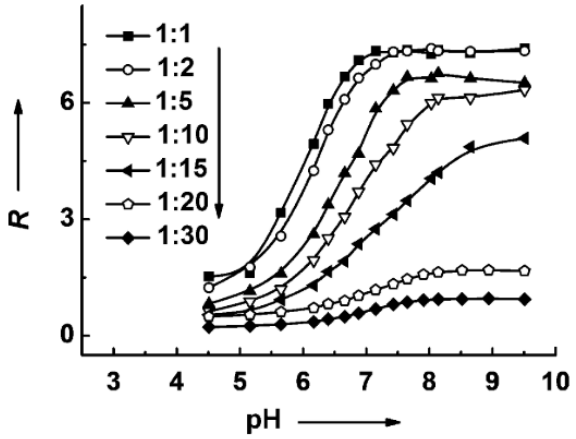

Figure 13 *The fluorescence intensity ratio (R) at different pH levels with different FITC/RBITC dye ratios.* [30]

Another very promising ratiometric pH sensor was created by Ke et al. in 2014 based on a previously made similar sensor that was genetically encoded into the cells. Ke et al. wanted to overcome the disadvantages of a genetically encoded sensor including the cells' complex manipulation and the sensor's big size. The new lipid-DNA-based fluorescent sensor that anchors to the cell surface proved to have many great qualities that make it applicable to 3D cell cultures. The sensor is an amphiphilic lipid-DNA molecule that consists of a hydrophobic diacyllipid tail, a hydrophilic DNA strand, a green-emission pH-sensitive dye as an indicator and an orange-emission pH-insensitive dye as a reference. The structure is presented in figure 14A. Because of the hydrophobic interaction between the hydrophobic lipid of the sensor and the phospholipid bilayer of the cell membrane, the sensor can anchor itself onto the cell surface. The DNA strand and the fluorescent dye at its end reach outwards from the cell, making sure to measure extracellular pH specifically. The pH-insensitive dye has a constant fluorescence emission intensity

regardless of the surrounding pH level, which enables the sensor's ratiometric properties. The pH-sensitive dye, however, has an increase of fluorescence intensity as the surrounding pH level rises. This effect can be seen in figure 14B. The difference in the pH-sensitive dye's fluorescence intensity is caused by a change in its chemical structure: The dye is either a cation, dianion, monoanion or a neutral molecule depending on the pH level around it. By calculating the fluorescence emission intensity ratio between the two types of dyes, it is possible to find out the existing pH level around the cells. The intensity ratio is the same in a specific pH level even if the dye concentrations change. [28]

The cell-surface-anchored ratiometric sensor was tested with flow cytometry and laser confocal imaging and it was assured that the sensor indeed localizes on the cell surface regardless of the cell type: both adherent and suspension cells as well as healthy and cancer cells were studied. The sensor was deemed biocompatible since there was no cytotoxic effect when used at its working concentration of 1 µM. It is also stable and does not lose its fluorescence intensity even after 12 hours. The sensor probe's response is reversible, which makes it reusable and applicable for long-term monitoring and conditions with repeating pH cycling. The sensor was also proven to function properly when applied in 3D collagen gel. The range of pH levels that this sensor can measure is from 5.0 to 8.0. [28] As a whole, this ratiometric fluorescent sensor has almost all the qualities necessary for a great pH sensor. It is small and stable and can be used for a variety of different cells. The only downside to this sensor is the detection range since the sensor can not be used to measure any massively unusual pH levels. However, this might be fairly easily solved by using a different fluorescent dye.

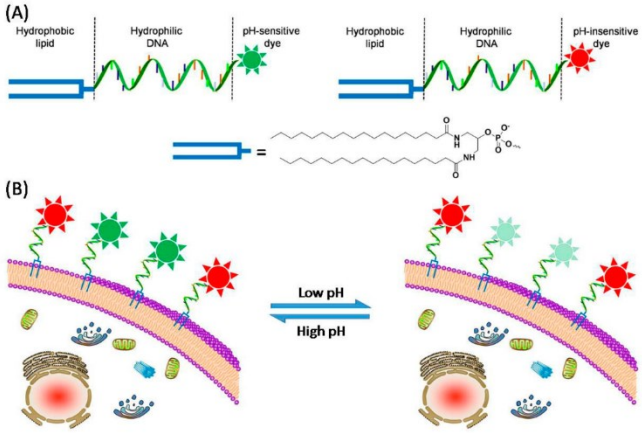

Figure 14 *A) The structure of a cell-surface-ancored ratiometric pH sensor. B) The working principle of the sensor.* [28]

This last pH sensor discussed in this chapter offers a different approach in pH monitoring. In 2020 Yang et al. combined fibre-optics and fluorescent detection and created a pH sensor with a response time around 20 s and resolution of 0.02 pH units. Their sensor has a detection range from 6.17 to 8.11 and it proved to be efficient even in cells with cytotoxic conditions. Yang et al. wanted to avoid intracellular labelling of cells, because the agglomeration of non-native substances may cause damage to the cells or affect the measured results. Therefore their optical nanoprobe was used at-line to acquire data at certain time points by inserting the probe into a cell and extracting it after the measuring. [29] This is why they studied only one cell, and why the method is not applicable to 3D hydrogel cell cultures as is. However, the used 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) pH dye is highly photostable and the sensor had a high spatial resolution and efficient emission collecting. So perhaps this sensor or an improved version of it could be used on the side of the actual measurement method to double-check any abnormalities.

It seems that there are multiple great options for monitoring pH via a fluorescent detection method. A summary of the discussed sensors is presented in table 4.

Table 4 *Different pH sensors.* [28–30]

Analyte	Sensor type	Reference	Year	Cells	Theory	Material	Size	Detection range	Reversible response	Good	Bad
pH	Tunable ratiometric intracellular pH sensor with carbon dots	Shi et al.	2012	HeLa cells, MCF-7 cells	Carbon dots have pH-sensitive and -insensitive dye that react to changes in pH levels causing change in fluorescence intensity ratio.	Amino-coated carbon dots, fluoresceinisothiocyanate (FITC), rhodamine B isothiocyanate (RBITC), 4,7,10-trioxa-1,13-tridecanediamine (TTDDA)	5–7 nm	5.2-8.5	Yes	Biocompatible, small size, sensitive, stable, easy preparation, ratiometric, tunable	
pH	Cell-surface-anchored ratiometric fluorescent probe for extracellular pH sensing	Ke et al.	2014	HeLa cells, human liver cancer cells QGY-7703, human liver normal cells QSG-7701, human acute T cell leukemia cells Jurkat	Fluorescent dye's chemical structure is changed by pH causing difference in fluorescence intensity. Lipid-DNA molecules anchor onto cell surface due to hydrophobic interactions.	Lipid, DNA strand, pH-sensitive and -insensitive fluorescent dye		5.0-8.0	Yes	Biocompatible, stable, reusable, studied in 3D hydrogels, usable for a variety of cell lines	Not too wide of a detection range or sensitivity
pH	Fibre-optic fluorescent nanoprobe to detect intracellular pH	Yang et al.	2020	Human bronchoalveolar carcinoma -derived A549 cells	Optical nanoprobe with small tip of fluorescent dye is injected and extracted from a single cell to aquire data at time points.	Nanoprobe, gold/palladium, sol-gel coating, 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) dye		6.17-8.11	Yes	Excellent resolution, efficient emission collection, no accumulation of exogenous particles	Data at time points, measuring only a single cell

3.5 Biosensors for temperature

Temperature is an important variable to measure since it plays a part in various biological functions and biochemical reactions. It has an affect on for example cell metabolism and division, enzymatic reactions and expression of genes. It is also shown that a higher temperature is displayed in cells that suffer from unusual medical conditions, like cancer. [31]

Temperature sensors are often used together with oxygen sensors since oxygen sensors have usually a temperature-dependent response to different oxygen levels. The temperature sensor part of these dual sensors can be used separately too if needed. A dual fluorescence sensor made in 2008 by Baleizão et al. is an example of this kind of an oxygen–temperature dual sensor. It was designed to sense concentrations of trace oxygen in a wide range of temperatures. It consists of two photoluminescent dyes that are immobilized into separate polymer films. The films are located on top of each other and on the bottom is a reflective layer of silicone or TiO_2 that increases the light collection. The layers of the dye–polymer mixtures are from 6 to 12 µm thick. The temperature sensitive dye used in the sensor is Ruthenium(II)-tris-1,10-phenanthroline-3-trimethylsilyl-1-propanesulfonate [$Ru(phen)_3$] which has a strong temperature dependence. The fluorescence lifetime and brightness of the dye decreases when the temperature increases, and the response is sensitive. The $Ru(phen)_3$ dye has high photostability but oxygen quenches its luminescence which is why the dye must be incorporated into a polymer that is not permeable towards oxygen. Another advantage of this temperature sensor is that its response to temperature changes is fully reversible. Also, the detection range is as wide as from 0 to 120 °C. [32] A disadvantage of this sensor regarding its use in cell cultures is that it has not been tested with any cell line, only in a flow chamber without cells.

Independent temperature sensors have been developed as well. One that uses FRET was created by Ding et al. in 2018. Their ratiometric temperature sensor is composed of two polymers that have a different response to temperature. The two polymers, N-isopropylacrylamide (NIPA) and N-isopropylmethacrylamide (NIPmA), have different lower critical solution temperatures (LCSTs) of 31.1 °C and 48.6 °C, respectively. NIPA was fused with blue emitting 7-[4-(Trifluoromethyl)coumarin]methacrylamide molecule with a fluorescent emission peak at 436 nm and NIPmA was fused with red emitting BOBPBYX molecule with an emission peak at 628 nm. The polymer solutions were mixed into one

solution with NIPA-based polymer/NIPmA-based polymer ratio of 20:1. The mixture solution presents a linear response to temperature between 30-50 °C as the fluorescence intensity ratio increases when temperature is increased. This is caused by an increase of FRET between the two polymers. The FRET is a result of a smaller proximity between the fluorescent molecules, which is caused by a different shrinking speed of the two temperature-responsive polymers. The fluorescence change of the different polymer variations is shown in figure 15. The change is visible to the naked eye. [31]

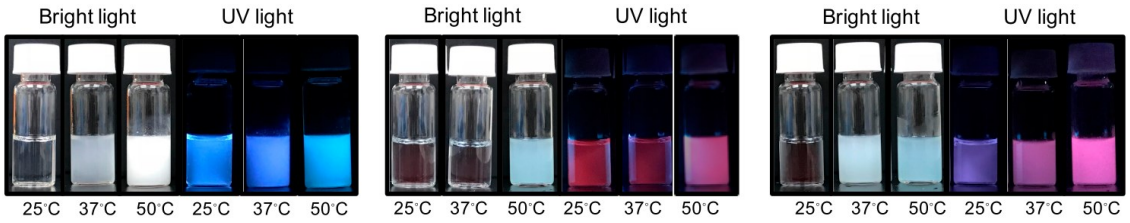

Figure 15 *The change of fluorescence in aqueous solutions of the NIPA-based polymer, the NIPmA-based polymer and the polymer mixture, respectively, at different temperatures. This is the fluorescence change as seen by the naked eye.* [31]

The sensor made by Ding et al was tested simply as an aqueous solution and with HeLa cells that were grown adherently on 2D culture dishes. Since the sensor polymers diffused into the cells when the HeLa cells were incubated with the polymer solution, the temperature readings described the cells' intracellular temperature levels. The sensor turned out to be biocompatible and stable and it performs well both in aqueous solutions and cell cultures. [31] This FRET-based ratiometric sensor seems to be a good fit for detecting intracellular temperature since it is applied as an aqueous solution and its detection range covers the physiologically relevant temperature range of human cells. However, the cells were incubated with the polymer solution and then washed to make sure the polymers were located only inside the cells, which is difficult to do in 3D hydrogels. So, in 3D cell cultures the sensor polymer would remain all around the culture and therefore show both the intra- and extracellular temperature. Also, since the sensor was only applied to a 2D cell culture, its use in 3D cultures should be further studied to make sure it really is applicable for 3D cell cultures as well.

Both of the discussed temperature sensing methods have great potential for application in 3D cell cultures. They require further testing though to make sure that they function the same in a 3D environment. It would also be ideal to test them on various cell lines. A conclusive chart has been made of these sensors and it is presented in table 5.

Table 5 *Different temperature sensors.* [31, 32]

Analyte	Sensor type	Reference	Year	Cells	Theory	Material	Size	Detection range	Reversible response	Good	Bad
Temperature and oxygen	Dual fluorescence sensor for trace oxygen and temperature	Baleizão et al.	2008		Temperature sensitive dye has change in fluorescence lifetime, oxygen sensitive dye has fluorescence quenching	Fullerene C70, Ruthenium(II)-tris-1,10-phenanthroline 3-trimethylsilyl-1-propanesulfonate [Ru(phen)3], polyacrylonitrile (PAN), ethyl cellulose (EC), organosilica (OS)	20 µm	Temperature: 0-120 °C, oxygen: 0-50 ppmv	Yes	Full reversibility, high photostability, high sensitivity, no interference, large temperature range	Coating spreading must be done twice, no testing with cells
Temperature	Thermo-responsive fluorescent polymers for ratiometric temperature sensing through FRET	Ding et al	2018	HeLa cells	Polymers get into a closer proximity in higher temperatures. This causes FRET and an increased fluorescent intensity ratio.	N-isopropylacrylamide (NIPA) polymer, N-isopropylmethacrylamide (NIPmA) polymer, 7-[4-(Trifluoromethyl)coumarin]methacrylamide (TCMA) blue fluorescent dye, BOBPYBX red fluorescent dye		25-50 °C	Yes	Stability, biocompatibility, low toxicity, linear response to temperature, soluble to water, ratiometric	

4. CONCLUSIONS

Monitoring different metabolic substances and environmental factors of a cell culture gives important information about the cells' activity and functionality. It also reveals whether the used cell culture is functioning as it should. For example, abnormal glucose levels may indicate an accelerated or slowed glucose metabolism among the cells, or it may be a sign that glucose is not dispersed around the cell culture as evenly as aimed for. Sufficient level of measuring is not always succeeded, either because of a lacking awareness of its importance or the non-existent availability for the needed sensor type. Often a sensor is wanted for online measuring, and it should be biocompatible, stable and sensitive. Small size and clear results are also desired. In addition, it is often optimal to have a detection method that does not consume analytes. The use of fluorescent materials provides detecting techniques that usually fulfil many of those needs. Fluorescence can be detected remotely, which enables the creation of non-invasive sensors.

Using these sensors to track different substances is a necessity especially when it comes to 3D cell cultures. 3D cultures are more complex than 2D cultures and create a tissue model more similar to natural tissue. Their complex structures however create problems with the diffusion of nutrients, waste and gasses, which almost inevitably affects the validity of the acquired results. In this thesis different fluorescence-based biosensors for a variety of analytes were discussed from a point of view of applying them in 3D hydrogel cell cultures.

Firstly, the physical phenomena of fluorescence, phosphorescence and FRET as in Fluorescence Resonance Energy Transfer were explained, since they are the basis of the sensors discussed in this thesis. Fluorescence and phosphorescence are based on the absorption and re-radiation of light. Fluorescent molecules absorb a photon that excites the molecule's electrons. As the electrons relax to their ground state, a new photon is emitted but with a longer wavelength. In phosphorescence the molecule's electrons do not relax straight back to the ground state but transition to a triplet state in between. The emitted radiation caused by phosphorescence is not emitted right after the excitation like it is in fluorescence, but instead the initiation of the reaction takes a while. Also, the phosphorescence emission lasts longer and has an even longer wavelength. FRET is caused by dipole-dipole interactions between fluorescent molecules and depends mainly

on the separation distance of the two fluorescent probes as well as their orientation. Because of FRET the fluorescent probes emit energy at a different wavelength than they would if they were separated.

Biosensors can utilize these phenomena in different ways. For example, labelling detection molecules with FRET-responsive molecules will result in different FRET efficiencies depending on the concentration of the analyte. Molecular oxygen is also able to function as a phosphorescence quencher, which is taken advantage of in oxygen sensors. There are differences in the availabilities of fluorescent sensors for different analytes. Biosensors for glucose, oxygen, calcium, pH and temperature are discussed. With all of these analytes, further testing is required or preferred to ensure their use in 3D cultures and with various cell lines. Having sensor proteins produces inside the cells by genetically encoding them was also a usual approach to creating these sensors. It however is not a preferred method because of the specificity and laborious procedures required to apply them in a study. Especially calcium sensors turned out to be mostly genetically encoded instead of being a separate sensor. All in all, there is a need for more studies to further develop and test the available sensors, as well as to create new sensors and detection methods for a variety of analytes.

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