

Universidade do Minho
Escola de Engenharia

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Real-Time Imaging Of Cell Mineralization

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Real-Time Imaging Of Cell Mineralization

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"I was taught that the way of progress is neither swift nor easy."

- *Marie Curie*

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RESUMO

Imagem em tempo real de Calcificação Celular

A calcificação ectópica tem-se revelado um problema emergente na área da saúde devido à sua correlação com várias doenças, como a doença renal crónica (DRC), hipertensão, *diabetes mellitus* (DM), doença cardiovascular aterotrombótica e envelhecimento. Este tipo de calcificação está associado principalmente à deposição anormal de complexos de sais de cálcio e fosfato – hidroxiapatite – em células do músculo liso nos vasos sanguíneos. Estudos atuais têm revelado o papel de proteínas plasmáticas que poderão inibir a calcificação tal como a fetuína-A.

A presente dissertação visa estudar o papel da proteína plasmática – Fetuína-A – na calcificação ectópica, utilizando o dispositivo de aquisição de imagens em tempo real –CELLCYTE – para seguir e avaliar a sequência de eventos biológicos e a cinética celular. Desta forma, uma variante recombinante de fetuína-A com propriedade de fluorescência, mFa-mRuby, foi produzida e estudada, uma vez que esta proteína é caracterizada por se ligar a depósitos minerais intrínsecos da calcificação ectópica. Protocolos como expressão proteica em células de ovário de hamster chinês (CHO), purificação, transfecção de alto rendimento, Western Dot Blot e SDS-PAGE foram realizados para produzir as amostras de fetuína-A em estudo. Para mimetizar a calcificação patológica no corpo humano, cultura de duas linhas celulares de células do músculo liso vasculares (VSMC) – IM1 e IM3 - foram cultivadas assim como células de osteossarcoma (SaOs-2) e cementoblastos (OCCM), para mimetizar mineralização fisiológica. Todas as linhas celulares foram incubadas em meio de cultura ou de calcificação, para avaliar a proliferação celular e a mineralização ao longo de 7 dias, com ou sem adição de fetuína-A ao meio. Os depósitos minerais foram revelados utilizando o protocolo de coloração de fetuína-A e posterior coloração Alizarin Red. Os resultados observados revelaram uma maior taxa de calcificação das VSMC em comparação com os mineralizadores profissionais (SaOs-2 e OCCM) e uma ligeira inibição da calcificação e mineralização das quatro linhas celulares com adição de fetuína-A em ambos os meios de cultura.

Em suma o presente trabalho, proporcionou uma base robusta para pesquisas aprofundadas sobre a calcificação ectópica e o papel da fetuína-A, assim como expandir as aplicações médicas desta proteína plasmática promissora.

Palavras-chave: Calcificação ectópica; CELLCYTE; Fetuína-A; SDS-PAGE; VSMC

ABSTRACT

Real-Time Imaging of Cell Mineralization

Ectopic calcification has become an emerging problem in the health field due to its correlation to various diseases such as chronic kidney disease (CKD), hypertension, diabetes mellitus (DM), atherothrombotic cardiovascular disease, and aging. This type of calcification is associated mainly with the abnormal deposition of calcium and phosphate complexes– hydroxyapatite – onto smooth muscle cells in blood vessels. Current studies rely on the comprehension of the role of proteins that can play a role in inhibiting calcification, such as mineral chaperones like fetuin-A.

Therefore, this thesis aims to study the role of plasma protein - fetuin-A - in ectopic calcification, by using the real-time acquisition imaging device CELLCYTE, to follow and evaluate the sequence of biological events and cell kinetics. Therefore, recombinant fluorescent Fetuin-A probe, mFa-mRuby, was produced and studied, as Fetuin-A is known to bind to calcium phosphates. Protocols like protein expression in Chinese hamster ovary cells (CHO), purification, high-yield transfection, Western Dot Blot and SDS-PAGE were performed to produce the fetuin-A probes. To mimic the pathologic calcification in the human body, two cell lines of vascular smooth muscle cells (VSMC) were cultured (IM1 and IM3). To mimic physiological mineralization osteosarcoma cells (SaOs-2) and cementoblasts (OCCM) were cultured. All cell lines were incubated in a culture or calcification medium to assess cell proliferation and mineralization throughout a 7-day study, with or without fetuin-A addition to the medium. Mineral deposits were revealed by using fetuin-A staining protocol and Alizarin red staining. Those results revealed a higher calcification rate of the VSMC in comparison to the professional mineralizers (SaOs-2 and OCCM) and a slight inhibition of calcification and mineralization of the four cell lines with both mediums with the addition of Fetuin-A.

In conclusion the present work, provided a robust foundation for in-depth research on ectopic calcification and the role of fetuin-A, as well as expand the medical application of these promising plasma protein.

Keywords: CELLCYTE; Ectopic Calcification; Fetuin-A; SDS-PAGE; VSMC

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LIST OF ABBREVIATIONS

VSMCs	Vascular smooth muscle cells
SaOs-2	Osteosarcoma cells
OCCM	Immortalized murine cementoblasts
VC	Vascular Calcification
CKD	Chronic Kidney Disease
DM	<i>Diabetes Mellitus</i>
CVDs	Cardiovascular Diseases
Ca	Calcium
P	Phosphate
ACP	Amorphous calcium phosphate
HAP	Hydroxyapatite
CaP	Calcium-Phosphate
DMSO	Dimethyl Sulfoxide
PBST	Phosphate Buffered Saline with Tween 20
PBS	Phosphate Buffered Saline
FBS	Fetal Bovine Serum
His-tag	Histidine tag
HRP	Horseradish Peroxidase
MW	Molecular Weight

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LIST OF SYMBOLS

°C	Degrees Celsius
μm	Micrometer
g/L	Grams per Liter
%	Percentage
w/v	Weight per volume
mM	Micromolar
M	Molar
Rpm	Rounds per minute
mL	Milliliter
μL	Microliter
ng	Nanograms
g	Gram
mA	Milliampere
kDa	Kilo Dalton

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1. MOTIVATION AND GOALS

Ectopic calcification has become an emerging alert in the health field as its treatment is complex. This pathologic condition is characterized by the inappropriate deposit and precipitation of calcium phosphate in the soft tissues along the human body that leads to calcification of the smooth muscle cells (Hutcheson et al., 2015; Leopold, 2015; Snijders et al., 2023). Various disorders such as hypertension, chronic kidney disease (CKD), *diabetes mellitus* (DM), atherothrombotic cardiovascular disease and aging can be associated with ectopic calcification (Hutcheson et al., 2015; Leopold, 2015; Millán et al., 2021; Snijders et al., 2023). This type of calcification is associated mainly with the final form of phosphocalcic crystals known as hydroxyapatite in smooth muscle cells (Giachelli, 1999; Kempf et al., 2021).

Smooth muscle cells are responsible for the contraction and relaxation process of the vessels along the body to control blood flow and tissue oxygenation (Webb, 2003). This type of cell is mostly affected by unwanted calcification, as described previously (Hutcheson et al., 2015; Millán et al., 2021; Snijders et al., 2023).

However, biological processes such as the molecular pathogenesis of ectopic calcification are poorly understood and are still largely unknown (Snijders et al., 2023). Therefore, an early diagnosis is possible through the quantification of amorphous calcium phosphate (ACP), as this is the main precursor of calcification. The Ca^{2+} (calcium ions) and PO_4^{3-} (phosphate ions) from ACP are converted in hydroxyapatite (HAP) crystals associated to calcification (Millán et al., 2021).

Currently, the treatments available are based on surgical removal of the calcified tissues or breaking the calcification apart with sound waves. Independently of the treatment chosen, both are extremely invasive for the patient and can be dangerous to the point of putting the patient's life at risk. Nevertheless, the heterogeneity of the disease also hinders the establishment of efficient treatment and the irreversibility of calcified tissues (Dzhanaev et al., 2023; Snijders et al., 2023).

Ectopic vascular calcification has been reported to be an extremely complex biological process that implicates cell regulation processes like activation of cellular signalling pathways, presence of calcification inhibitors, genetic interference and hormonal activity (Leopold, 2015).

Current studies rely on the comprehension of the role of proteins that play a role in inhibiting calcification, such as mineral chaperones, which are important to stabilize minerals in solution in the case of supersaturated solutions (Dzhanaev et al., 2023).

Therefore, an emerging mineral chaperone protein has been studied and reported to be an active factor in the inhibition of cell calcification (Dzhanaev et al., 2023). The plasma protein fetuin-A/ α_2 -HS-glycoprotein has been found to be a key element in the inhibition of ectopic calcification by preventing undesirable biomineralization in the plasma on the blood and in smooth tissue fluids, due to its early state calcification inhibition activity and its capacity to bind and stabilize precursor of calcium phosphate deposits, by forming colloidal calciprotein particles (CPPs) (Dzhanaev et al., 2023; Jahnhen-Dechent et al., 2008; Schinke et al., 1996; Westenfeld et al., 2007).

This thesis aims to study the role of the plasma protein fetuin-A in cell calcification and mineralization and the production of recombinant protein with fluorescence proprieties entitled murine Ruby Fetuin-A, also referred to as mFa-mRuby. Furthermore, the real-time imaging of the differences between mineralization and calcification, by using osteosarcoma cells (SaOs-2) and cementoblast (OCCM) for mimicking professional mineralizers and two cell lines of smooth muscle cells (IM1 and IM3, same cell type, but from different patients) to mimic calcifying cells. A recombinant chicken Fetuin-A was equally produced to be delivered to Berlin BioGenes for Antibodies production, therefore not included in the subsequent methods.

Thus, the study had the following aims:

- Amplification of Fetuin-A plasmids - mFa-mRuby and Chicken Fetuin-A - in Escherichia Coli cells;
- Transformation and expression of Fetuin-A recombinant proteins: mFa-mRuby and Chicken Fetuin-A - in Chinese Hamster Ovary (CHO) cells;
- Purification and quantification of Fetuin-A protein variants produced;
- Calcification assays on vascular smooth muscle cells, cementoblasts, and osteosarcoma cells under culture or calcification conditions and with or without the addition of the mFa-mRuby, the recombinant protein produced previously;
- Live cell imaging with the fluorescence Fetuin-A probe – mFa-mRuby;
- Cellular calcification and mineralization assessment.

2. STATE OF ART

2.1 Physiologic Mineralization

The human blood is a complex, dynamic fluid composed of various elements including minerals that allow it to function properly. Therefore, minerals like calcium and phosphate stand out as being one of the most vital ones, due to their role in different biological processes, like maintaining the normal cardiovascular and neuro-muscular function, enzyme-mediated and cellular signalling processes, and ensuring structural integrity to the skeleton, which serves as the primary deposition site of mineral ions in the body (Evenepoel & Wolf, 2013; Gharibzahedi & Jafari, 2017). These minerals can be found mainly in teeth and bones, where they play a critical role in human physiology and skeletal mineralization (Evenepoel & Wolf, 2013; Gharibzahedi & Jafari, 2017; Schäfer et al., 2003). For example, the bones contain around 99% and 80% of the body's calcium and phosphate concentration, respectively (Bonjour, 2011). The balance of these ions regulated at renal and intestinal levels leads to the maintenance of bone health (osteoporosis management) and influences the bone-forming and bone-resorbing cells (Bonjour, 2011).

Hard tissue biomineralization can be described as the process where ions, like calcium and phosphate, accumulate in a coordinated way to form bone and teeth tissue. This biological process is reported to be a correlation between extracellular matrix suitable for mineralization, high concentration of extracellular inorganic phosphate and calcium and levels of mineralization inhibitors that may be expressed locally or systemically (Brylka & Jahnen-Dechent, 2013). Calcium and phosphate deposits in and outside the collagenous fibrous network, a significant component of the extracellular matrix, in the cells, and form hard tissue structures. In the process of biomineralization, one of the main purposes is the formation of hydroxyapatite – a mixture of inorganic phosphate and calcium ions – one of the principal components of fully developed hard tissues. For example, particularly in bones, osteoblast secrete an extracellular matrix and its subsequent mineralization by crystalline hydroxyapatite formation that forms in a coordinated way along with the organic protein molecules, strengthening the organic matrix due to Ca and Pi influx in the cells in hard tissues (Bonjour, 2011; Sharma et al., 2021; Snijders et al., 2023). This mineral homeostasis is one of the most important prerequisites in the biomineralization process since maintaining the correct concentration of calcium and phosphate ions allows physiologic mineralization.

Also, this dynamic equilibrium of intake and uptake of ions is reported to be highly controlled by several regulatory processes – such as feedback inhibitions - and to be responsible for preventing the formation of calcific deposits in soft tissues (Sharma et al., 2021; Snijders et al., 2023).

However, a disturbance in these biologic processes influencing the Ca and P concentration balance and its regulatory processes may lead to calcification of soft tissues, also called pathological ectopic calcification. This study aims to comprehend the mechanisms behind the process of biomineralization and furthermore analyze the biological differences, cellular behaviour and responses associated with this process and calcification.

2.2 Vascular Calcification – Ectopic Calcification

Cellular mineralization is one of the most vital physiologic processes in the human body, as it is integral in the development and maintenance of bone and teeth tissues. However, not all forms of mineralization are beneficial for the organism, such as in the case of “soft” tissue mineralization entitled vascular calcification (Kempf et al., 2021; Schäfer et al., 2003).

Vascular calcification is a pathological mineralization widely described as the inappropriate deposition of calcium and phosphate in soft tissues such as blood vessels, joints, or tumours (Giachelli, 1999; T. Li et al., 2022; Persy & D’Haese, 2009; Snijders et al., 2023). The mineral deposits within the soft tissues tend to disrupt the biomechanical function of these tissues, restricting the patient's mobility and possibly contributing to mortality (Hutcheson et al., 2015). This complex biological process involves elaborate pathophysiological mechanisms, that can induce metabolic alterations due to calcium and phosphate dysfunction, absence of calcification and mineralization inhibitors, osteogenic transdifferentiation of vascular smooth muscle cells (VSMCs) to calcifying vascular cells, the release of apoptotic bodies or necrotic debris, deposits calcium phosphate hydroxyapatite due to modifications in mineral homeostasis or establishment and development of ectopic mineralization and nucleation complexes due to bone remodelling (Pan et al., 2023). Also, local and systemic changes within the human body can trigger or favor ectopic calcification by modifying the supply of calcium and phosphate, ectopic activation of osteogenic signalling, or by downregulating inhibitory mechanisms (Schäfer et al., 2003). Hence, a continuous, in-depth study of the available inhibitors in these biological processes, along with an understanding of their role and contribution, could be the basis for developing and establishing therapy to prevent disease-associated calcification.

This pathology is classified broadly, according to the part of the body and soft tissue it affects, such as kidney, skin, or tendons, as illustrated in Figure 1 (Giachelli, 1999). Some literature even describes the existence of a genetic component associated with ectopic calcification correlated with a main disease like atherosclerosis, cancer or chronic renal failure. In this case, the inappropriate deposition of minerals can be broadly distributed and affect multiple organs (Schäfer et al., 2003). Vascular calcification can be correlated to cardiovascular disease, by affecting arteries and heart valves. Although valves are predisposed to calcify with apatitic mineral deposits, calcification in the arteries is followed by atherosclerotic plaque burden, increased risk of myocardial infarction, increased ischemic episodes in peripheral vascular disease, and increased risk of dissection, following angioplasty. All these repercussions are the basis of some of the most common cardiovascular diseases: hypertension, atherosclerosis, coronary artery disease, aortic valve stenosis and atherothrombotic cardiovascular disease as outlined in Figure 1 (Leopold, 2015; Pan et al., 2023; Snijders et al., 2023). Due to the deposits, these tissues become stiffer, harder and more susceptible to general failure, which can lead to a significant disease burden. On the other hand, ectopic calcification is strongly associated with the pathological mechanism of conditions like chronic diseases, such as atherosclerosis, hypercholesterolemia, diabetes mellitus (DM), hypertension, chronic kidney disease (CKD) and coronary artery disease (Figure 1). It may also manifest in the skin, as seen in rare conditions like Pseudoxanthoma Elasticum (PXE), systemic calcification, where skin plaque might arise or develop in the basal ganglia like the Primary Familial Brain Calcification (PFBC) condition (Snijders et al., 2023).

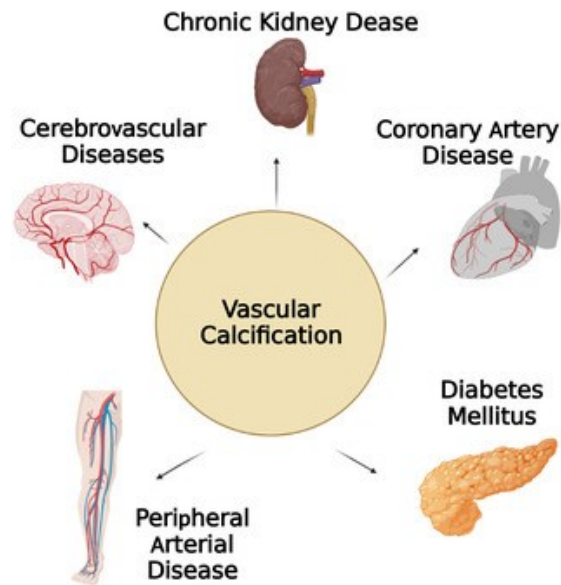


Figure 1: Example of different diseases correlated to vascular calcification (VC). VC plays a key role in the pathological process of some diseases, like cerebrovascular diseases, peripheral arterial diseases, and coronary artery disease (Pan et al., 2023).

Vascular calcification can be classified into different types based on the layer/area of the blood vessels where the calcification begins to appear: intimal calcification, media calcification, or cardiac valve calcification as presented in Figure 2. Intimal calcification is typically associated with atherosclerotic diseases and the development of atherosclerotic plaques, which may eventually lead to microcalcifications. These microcalcifications tend to scatter along the inner wall of the blood vessels in the form of small spots (Figure 2, Li et al., 2022; Nakahara et al., 2017). On the other hand, medial calcification, as the name suggests, occurs in the media layer of blood vessels and is more common in small and medium arteries. This type of calcification is associated with aging, diabetes, and chronic kidney disease (Zazzeroni et al., 2018). Cardiac valve calcification is normally correlated to metabolic diseases like diabetes or uremia, resulting from mechanical stress and inflammation (Shekar & Budoff, 2018).

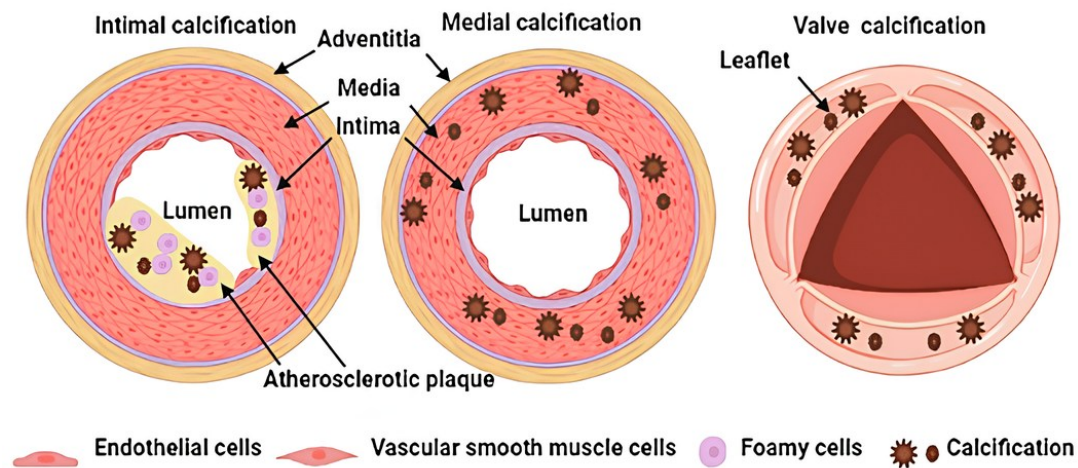


Figure 2: Schematic representation of the different calcification and constitution of ectopic calcification in the blood vessels and valves within the human body (Millán et al., 2021).

Moreover, vascular calcification is followed by a complex biological process involving the formation of precipitates (Johnson et al., 2006; Persy & D’Haese, 2009; Shekar & Budoff, 2018; Zazzeroni et al., 2018). Those precipitates are typically composed of calcium-phosphate complexes, specifically hydroxyapatite (HAP) crystals. In the early stages of calcification, these deposits are described as consisting of amorphous calcium phosphate (ACP), which gradually transforms into HAP crystals over time (Figure 3, Millán et al., 2021; Persy & D’Haese, 2009). Therefore, when the calcium and phosphate ions level rise and exceed the solubility product, it tends to create a supersaturated environment that initiates the calcification process and promotes the nucleation of calcium-phosphate deposits. As illustrated in Figure 3, initially the small ACP clusters are unstable and disordered, but with the constant aggregation and organization of these, more stable and crystalline structures start to appear, such as HAP crystals. As depicted in Figure 3, the transformation of small clusters into HAP crystals tends to be an irreversible process, even with the normalization of the calcium and phosphate concentration levels in the vascular layers (Millán et al., 2021).

The conversion of an unstable, amorphous form into a crystalline one, is the basis of pathological ectopic calcification.

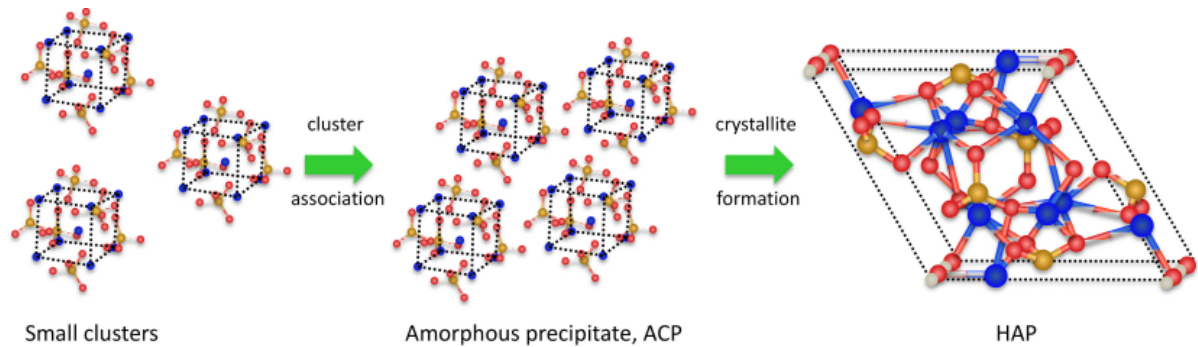


Figure 3: Scheme illustration of the evolution and transformation of small clusters of calcium-phosphate complexes into HAP crystals during the calcification process in smooth muscle cells. Figure adapted from (Millán et al., 2021). The process requires structural and organizational modifications from an amorphous precipitate into a crystalline form.

Therefore, further investigation on this topic is imperative to accurately diagnose patients and develop specific and targeted therapies to prevent, inhibit, mitigate, or even reverse the calcification process in smooth muscle cells.

2.3 Fetuin-A

Fetuin-A also known as α_2 - Heremans-Schmid glycoprotein (AHSG), is a negatively charged multifunctional glycoprotein, that can be found in plasma and belongs to the group of cystatin protease that inhibits cysteine proteinases like papain, calpain, cathepsin, and caspases families (Chekol Abebe et al., 2022). This cystatin family is known to be divided into three main groups: type 1, associated with mostly intracellular proteins; type 2, mostly extracellular proteins and type 3, plasma proteins, where Fetuin-A belongs (Brylka & Jahnen-Dechent, 2013; Schäfer et al., 2003). Fetuin-A is reported to be part of this particular superfamily of cystatins due to mutations in the relevant sequence motifs in its two cystatin-like domains that lead to the loss of its cystatin protease inhibition function. Its molecular weight can vary, depending on the degree of glycosylation, between 51 to 67 kDa (Chekol Abebe et al., 2022).

Human Fetuin-A is ciphered by a gene in the 3q27 chromosome and principally expressed by hepatocytes, major parenchymal cells that can be found in the liver that are responsible for protein metabolism; around 95% of Fetuin-A in the human body is produced and released by the adult liver. Additionally, this protein is entitled as a plasma carrier due to its role in transporting and ensuring the availability of various substances in the bloodstream (Chekol Abebe et al., 2022; Gong et al., 2023). Fetuin-A concentration in the bloodstream can vary from 0.4 to 0.8 mg/mL in the human body and can be found in the extracellular space in almost every vascularized tissue, and prominently absent in cartilage without vascularization. Human Fetuin-A is classified as a heterodimeric three-dimensional protein, as it is formed by a heavy A-chain (282 amino acids) and a light B-chain (182 amino acids) bound by a connecting peptide (40 amino acids) (Kovářová et al., 2021).

As previously mentioned, Fetuin-A is produced in the liver and afterward released in the bloodstream, based on physiological factors such as high blood glucose or free fatty acid (FFA). These factors tend to increase the secretion of this plasma protein due to stimulation of extracellular signal-regulated kinase by the blood glucose and increase of NF- κ B activity by the FFA (Pal et al., 2012). Also, high Fetuin-A levels are normally associated with metabolic diseases as well as chronic kidney disease.

Hence, this protein is documented as a polypeptide with various roles in the human body - protease inhibitor, inflammatory mediator, anti-inflammatory partner, atherogenic factor and adipogenic factor, alongside several others (Chekol Abebe et al., 2022; Sardana et al., 2021). Based on the literature, Fetuin-A has been reported as an important protein in biological processes such as bone and calcium metabolism, insulin signalling, protease inhibitor, osteogenesis, anti-inflammatory partner or inflammation (Chekol Abebe et al., 2022; Jahnen-Dechent et al., 2011).

Thus, various health conditions have been associated with Fetuin-A activity, such as *diabetes mellitus*, cardiovascular diseases (CVDs), tumours and chronic kidney disease (CKD) (Chekol Abebe et al., 2022; Westenfeld et al., 2009). Therefore, Fetuin-A has been studied and documented to be able to modulate vascular calcification, by operating through two approaches: local and systemic (figure 4). Locally, Fetuin-A may inhibit calcification in the wall of the blood vessels, by being incorporated into intracellular vesicles in VSMCs. Afterward, the plasma protein is released within apoptotic bodies and matrix vesicles that leads to inhibition of mineral nucleation, meaning it prevents the mineral ions (like calcium and phosphate) to aggregate and evolve into calcification (Reynolds et al., 2005; Sedor, 2009).

Systemically, Fetuin-A is reported to be a key element in buffering the mineral ion supersaturation, by forming fetuin-mineral complexes (Figure 4), also entitled calciprotein particles. This approach prevents the potential deposition of minerals in the blood vessels, thus the development and formation of calcification deposits, since the excess accumulation of ions like calcium can lead to severe complications such as arteriosclerosis (Brylka & Jahnke-Dechent, 2013; Chekol Abebe et al., 2022; Price et al., 2002; Sedor, 2009).

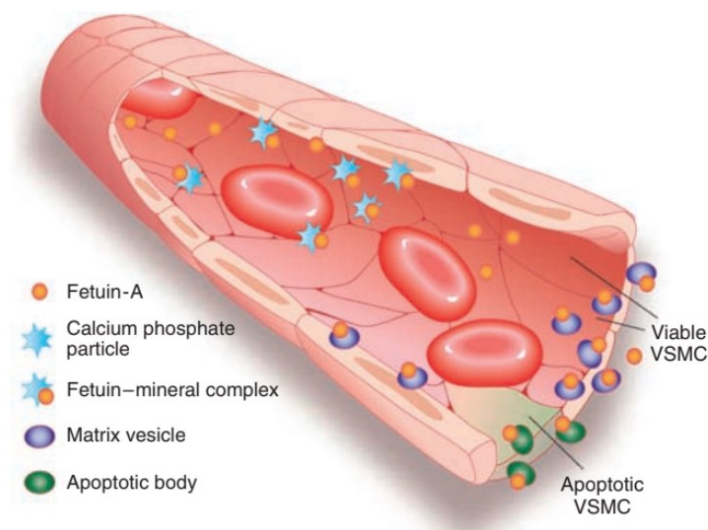


Figure 4:Representation of the hypothesis of the inhibition mechanisms of vascular calcification of Fetuin-A. The literature reports two approaches through Fetuin-A inhibit and modulate vascular calcification in a local and a systemic manner within the human body blood vessels (Sedor, 2009).

Research within the field of Fetuin-A is of great interest due to the multifaceted functionality that has been widely reported and documented, from its binding properties to its anti-inflammatory activity. Furthermore, this protein could be studied for therapeutic applications, serving as a diagnostic tool, as a biomarker for the detection of early-stage calcified lesions in tissues. This perspective paves the way for new therapeutic strategies in the development of treatments for conditions like ectopic calcification, CKD, or atherosclerosis (Brylka & Jahnen-Dechent, 2013; Johnson et al., 2006).

3. MATERIALS AND METHODS

3.1 Design of the Chicken Fetuin-A Plasmid

As the chicken Fetuin-A Plasmid was not available in the laboratory, it was crucial to design the chicken Fetuin-A plasmid for a future effective and successful production of the recombinant protein. For the selection of chicken Fetuin-A plasmid, it was necessary a study about the DNA sequences available and decide which one was the most suitable for the aims of the study. So, by using the Uniprot database software, two different chains of Chicken Fetuin-A were found, a longer one entitled AOA8VOYWF8_CHICK, with about 344 amino acids and a shorter one designated AOA8VOYUT8_CHICK with 317 amino acids.

For the selection of the most appropriate chain, a protein chain alignment was processed to compare the different chains commonly used in current studies, like the cotja (Japanese quail) and mouse (also reported as murine) Fetuin-A chains, as outlined in Attachment 7.1. Based on the percentage identity matrix and the blast alignment, the longest protein chain of chicken fetuin-A (AOA8VOYWF8_CHICK) was selected as it showed the most similarity with the fetuin-A protein chains from murine and cotja, in comparison with the shorter fetuin-A protein chain (AOA8VOYUT8_CHICK) (Attachments 7.1). Additionally, the use of a longer amino acid chains enhanced protein stability by becoming more resilient to denaturation or degradation during expression and purification methods. Afterward, the chicken fetuin-A sequence chosen was modelled into a 3D structure using the CHIMERA software to provide a clearer perception of the distribution and layout of the amino acids (Attachment 7.2).

For the design of the chicken Fetuin-A construct, pcDNA™3.4TOPO expression vector was used. The amino acid sequence (AOA8VOYUT8_CHICK) was inserted into the vector, a start codon (ATG) was added in the beginning to initiate protein production, followed by the addition of a six-histidine chain (his-tag) for an efficient future purification through IMAC chromatography (Chapter 3.6.5). Finally, a stop codon TAG was added.

3.2 Culture of *Escherichia Coli* cells

The organism chosen for the plasmid amplification was the bacteria *Escherichia Coli*. This organism is widely used for investigations on microbiology and biotechnology projects because of its simple well known genome with around 4.400 genes and fast reproduction rate. Therefore, it was the organism of choice for this project (Rosano & Ceccarelli, 2014).

Three different mediums were necessary for the culture of the *E. coli* cells (Table 1). So, the LB medium (lysogeny broth) has a rich composition of nutrients that are important for bacteria growth and crucial for cultivating *E. coli* cells in suspension. It is subsequently used in protein production methods (chapter 3.6.3). LB agar was used for cell culture growth in a solid medium, such as Petri plates (chapters 3.6.1 and 3.6.2). Super optimal 25 medium with catabolic repressor (SOC) was used for glucose supplement and as the last step for maximum transformation efficiency of *E. coli* cells (chapter 3.6.1). LB Medium and LB agar were autoclaved for 20 minutes at 135°C and 3 bar. The sterilization process for the SOC medium required filtration of the medium through a 0.22 µm bottle-top filter (Corning INC. Corning, USA) due to a heat-sensitive glucose composition.

Table 1: Description of the medium composition of the 3 media necessary for culture, growth and proliferation of *E. Coli*.

Medium	Concentration
LB Medium	Trypton 10g/L
	NaCl 10g/L
	Yeast Extract 5g/L
LB Agar	Trypton 10g/L
	NaCl 10g/L
	Yeast Extract 5g/L
	Agar 15g/L
SOC Medium	Trypton 2%(w/v)
	NaCl 10mM
	Yeast Extract 0.5%(w/v)
	MgSO₄*7H₂O 10mM
	MgCL₂ 10mM
	Glucose 20mM

Since it was only necessary for the growth of *E. coli* cells it was crucial the use of antibiotics to create a selective bacterial medium. Therefore, a concentration of 100 µg/mL of ampicillin was adjusted and spiked into the culture medium. The ampicillin used was provided from a previously prepared stock solution sterile filtered using a 0.22 µm syringe filter (VWR, Radnor, Pennsylvania, USA) and stored at 20°C. Sterile *Petri* dishes with the selective bacteria conditions were stored at room temperature until further use (chapter 3.6.1).

E. Coli suspension cultures were let to grow overnight in autoclaved Erlenmeyer flasks at 37°C and 250 rpm in a shaking incubator. For each suspension culture volume, 1/5 of the cell suspension was filled with LB medium to ensure adequate gas exchange.

The transformation of *E. Coli* cells was afterward performed as detailed in chapters 3.6.1 and 3.6.2.

3.3 Chinese Hamster Ovary Cells – ExpiCHO-S™

Chinese Hamster Ovary cells (Gibco, Thermo Scientific, Waltham, USA) were used for protein expression due to their high-density suspension growth properties, resembling human post-translational modification patterns and their ability to produce satisfactory yields and high-quality protein expression (Z. M. Li et al., 2022).

Firstly, ExpiCHO-S™ cells were taken and defrosted from a liquid nitrogen tank (-196 °C). The cryovial was warmed in the water bath (37°C) and added to a previously prepared Erlenmeyer flask with 30 mL of prewarmed ExpiCHO™ Expression medium (Gibco, TermoFisher Scientific, USA). The cell culture rested in an incubator with a shaker (110 rpm) at 37°C, 5% CO₂.

After 3.5 days the cell culture density was checked with the Neubauer Chamber (Attachment 7.3) to verify if the first cell splitting could be performed (density < 4 × 10⁶ cells/mL). The cell counting process was possible with a Neubauer Chamber (Attachment 7.3). The next two subculture densities should be around 4 × 10⁶ – 6 × 10⁶ cells/mL, and the new culture was adjusted to a cell density 0.2 × 10⁶ cells/mL as well as fresh ExpiCHO™ Expression medium in an Erlenmeyer flask.

Before the day of the transfection, a cell density of 3.5 × 10⁶ viable cells/mL was prepared with a fresh ExpiCHO expression medium. The cells used for transfection were at least, split three times.

3.4 Thawing of OCCM, SAOS, IM1 and IM3 Cells

For the cell growth curves and calcification assays (chapters 3.7 and 3.8), professional mineralizing cells such as SaOs and OCCM and calcifying cells like IM1 and IM3 were thawed. SaOs-2 cell lines were derived from osteosarcoma, while the OCCM cell line originated from cementoblasts found in dental cementum. Additionally, the IM1 and IM3 cell lines were derived from vascular smooth muscle cells located in the blood vessels of two different patients. Thus, two cell lines of smooth muscle cells – IM1 and IM3, one cell line of osteosarcoma cells – SaOS-2 and one line of cementoblasts (OCCM) were taken from the liquid nitrogen at -196°C. Low glucose DMEM medium with 10% FBS and 1% PSGL was prepared and pre-warmed in the water bath at 37°C. The cryovials were placed in the water bath for some seconds to one minute to allow the cells to thaw but not completely as the presence of DMSO, a very toxic cryopreservant, decreases the cell viability. So, after the first layer of ice has melted, the cells are resuspended in 10mL of the medium previously prepared in T75 flasks. The flasks were placed in an incubator at 37°C, 5% CO₂.

Since the medium had a red-color pH indicator, the cell culture was carefully checked daily to verify any color changes on the plates, indicating cell activity and proliferation of the cell lines in the study. The shift to an orange color indicated the production of cell activity residues and oxygen consumption inside the flask. Therefore, the medium was changed after around 2-3 days.

3.5 Cryopreservation of Eukaryotic cell lines

Cryopreservation was performed for all the cell lines used during the present study at the end of each assay (Chapters 3.7 and 3.8), ensuring sufficient cell viability for future use. Cells were distributed in Falcon tubes and centrifuged at 300 × g for five minutes, at room temperature. The supernatant was discarded, and the cell pellet was resuspended in a culture medium previously described, containing 10% DMSO. The cell concentration was adjusted to 1×10⁷ cells/mL, in 1mL volume cryovial. The cryovials were put in a freezing container (Mr. Frosty, NALGENE, Thermo Scientific, Waltham, USA) filled with isopropanol. The freezing container was stored for one day at -80°C and afterward the cells were transferred into the liquid nitrogen tank, at -196°C.

3.6 Protein Production and Purification

3.6.1 Transformation of NEB®Turbo Competent *E. coli* cells

The fetuin-A expression vector (Chapter 3.1) was received lyophilized, so it was necessary the addition of a liquid for use. The plasmid powder was first centrifuged for 10 seconds to allow the powder to settle down. 50µL of highly pure nuclease-free water was added to prevent DNA sample loss, since this type of water is free of any kind of nucleases (DNase and RNase). The plasmid was incubated for 10 minutes at 37°C and 800 rpm in the ThermoMixer®.

The introduction of Fetuin-A plasmid into *Escherichia Coli* cells (transformation) was based on a heat shock protocol. A NEB®Turbo Competent *E. Coli* cells cryovial was taken from a -80°C freezer and thawed, until all ice crystals defrosted. 50µL of cells suspension was removed into a new transformation eppendorf, previously resting in an ice bath, and 1.5µL containing 0.9ng of fetuin-A plasmid was added to the cell mixture. The tube was flicked carefully 4-5 times and put in ice for 2 minutes, so the cells and the DNA could mix without using the vortex. The heat shock was processed by prewarming the ThermoMixer® at 42°C, without any type of shaking, quickly putting the tube in for precisely 30 seconds, and putting it back on ice. Lastly, 600 µL of room temperature SOC medium (Table 1) previously prepared was pipetted into the tube and left to incubate for one hour at 37°C and 300 rpm in the ThermoMixer®.

With a pre-prepared sterile LB agar plate with ampicillin (Table 1 and chapter 3.2), 620 µL of cell mixture was added and spread evenly on the plate and incubated for 8-12 hours at 37°C.

3.6.2 Amplification of fetuin-A plasmid in *Escherichia Coli* cells

For the amplification of the plasmid in bacteria, a previously prepared *Petri* dish with a selective medium was used (chapter 3.2). Therefore, the transformed *E. Coli* cells with the fetuin-A plasmid were added to the *Petri* dish for plasmid amplification. A colony in the incubated plate (Chapter 3.6.1) was taken with a sterile pipette and dropped in a 100 mL sterile Erlenmeyer filled with 20 mL of sterile LB medium. Ampicillin was added to adjust a final concentration of 100µL/mL of the antibiotic. The culture was incubated overnight at 37°C and 250 rpm.

On the next day, the plasmid was amplified and purified with the kit “Pure Yield Plasmid Midiprep System” from Promega (Madison,USA) as described in attachment 7.4.

For storage, 660 μL of transfected *E. Coli* cell culture was added to 660 μL of sterile glycerol to stabilize the cells during the frozen state. Therefore, the cells were stored for future use in the freezer at -80°C .

3.6.3 Transfection of ExpiCHO-S^T cells

Transfection is the process that requires the intentional introduction of genetic material like DNA in cells to overexpress a gene of interest in CHO cell line. So, for the study, it was necessary to transfect ExpiCHO-S^T cells (Gibco, Thermo Scientific, Waltham, USA) using fetuin-A plasmid.

The transfection protocol requires two days to achieve the cell density required for an efficient process. Thus, a density of ExpiCHO-S^T cell culture (chapter 3.3) of 3.5×10^6 viable cells/mL was prepared on day -1 from an ExpiCHO-S^T cell culture, with a range of $4 \times 10^6 - 6 \times 10^6$ viable cells/mL. On day 0 (transfection day), the cell density and percent viability were determined. Subsequently, a final density of 6×10^6 viable cells/mL was prepared with fresh ExpiCHOTM Expression medium pre-warmed to 37°C , until a cell culture volume of 25 mL was reached.

Since the fetuin-A plasmid concentration in stock was $423\mu\text{g}/\mu\text{L}$ and the goal was to have a concentration of $0.9\mu\text{g}/\mu\text{L}$ for the transfection, a volume of 53 μL of the stock concentration was used. The transfection required a kit constituted by ExpiFectamineTM CHO reagent, OptiPROTM medium, ExpiCHOTM Feed and ExpiCHOTM Enhancer. So, 80 μL of ExpiFectamineTM CHO reagent was added to 920 μL of OptiPROTM medium in a 1mL Eppendorf and inverted 4-5 times to mix the compounds. The 53 μL of fetuin-A plasmid was added to 947 μL of OptiPROTM medium in a 2mL Eppendorf. The Eppendorf was added to the 25mL Erlenmeyer with the CHO cell suspension cell culture, and the flask was left to rest in an orbital shaker in the incubator, at 125 rpm 37°C and 5% CO_2 . During cell division of CHO cells, the plasmid present on the medium reaches the nucleus and the gene of interest is transcribed. A time of 18-22 hours *posteriori* to transfection is required for optimal expression of the transfected genes.

On day 1, 150 μL of ExpiCHOTM Enhancer and 6mL of ExpiCHOTM Feed were added to the suspension cell culture, and the incubator temperature was shifted to 32°C to slow down the growth of the cells and thereby enhance the expression of the protein of interest.

In the next days, a 1mL sample of the CHO cell culture was taken, to verify and determine the cell percentage viability of the cell culture suspension by using the Neubauer chamber method (Attachment 7.3). The harvesting of the protein was only performed when the cell viability was above 60%, after a couple of days.

All the cell culture was divided into 50mL Falcon and centrifuged at 300 rpm and 4°C for 15 minutes. The pellet and the supernatants were separated on different Falcons and protease inhibitor was added to the supernatant. To preserve the samples, the Falcons were dropped into a liquid nitrogen bucket to shock freeze them. Afterward, the samples were put in the -20°C freezer until purification (chapter 3.6.5).

3.6.4 Western Dot Blot

The Western Dot Blot protocol was used for the immunodetection of protein, utilizing a specific antibody, as described below, for the detection of protein in the nitrocellulose membrane.

During protein production (chapter 3.6.3) 1 mL samples were taken every day to check the efficiency of expression. Each sample was vortexed and slightly centrifuged (15 minutes, 4°C, 300g) to allow the separation of the cells and cell debris (pellet) from the supernatant. The supernatant was removed and transferred to a new eppendorf. Both components were then rapidly frozen using liquid nitrogen (-196°C) and stored in the freezer. Since cell viability on day 5 was around 67.9%, the cell culture was harvested, and all the suspension culture was distributed in 50 mL volume falcons. Each falcon was then vortexed and centrifuged (15 minutes, 4°, 300g) to separate the cell pallet from the supernatant. Both compounds were separated, shock freeze with liquid nitrogen (-196°C), and stored in the freezer.

After the end of the cell culture, each supernatant eppendorf of the five days samples were taken from the freezer and pre-warmed in the water bath at 37°C. A droplet of around 3µL of supernatant each day was pipetted onto a nitrocellulose membrane and let to air dry. In a 50 mL volume falcon a 20mL blocking buffer was prepared (Table 2). The membrane was then rolled and incubated for one hour inside a falcon with 10mL of the blocking solution prepared.

Then, the membrane was removed from the falcon, put into the 50mL-falcon with the remaining blocking buffer with 10µL of a His-blot (antibody used for immunodetection of full-length murine Fetuin-A), and incubated for another hour at 37°C. The membrane was washed three times with PBST (0.05% Tween) at intervals of five minutes transferred into the chemiluminescent buffer (Table 2) and incubated for one minute. Immunodetection fundamentally relies on the principle of antigen-antibody binding. Primarily the His-blot (antibody) binds to His-tag attached to the protein intended for detection. The antibody used is directly coupled with HRP (horseradish peroxidase). This arrangement allows the His-tag to associate with a single primary antibody, thereby serving a dual function as both a signal amplifier and a visualizer.

So, the reaction of luminol and hydrogen peroxide, H_2O_2 (both from the chemiluminescent buffer) with the HRP (from His-tag) emits light and its intensity is proportional to the concentration of the target protein in the samples (chapter 4.2).

After the washing steps, the membrane was taken to the chemiluminescence signal detection, named fluorescence imager (Fuji LAS Mini 4000, GE Healthcare, Boston, USA), to verify the presence of protein in the membrane.

Table 2: Reagents required for the western Dot Blot protocol.

Blocking Buffer		Chemiluminescent Buffer	
Reagent	Volume	Reagent	Volume
PBST (0.05% Tween)	20 mL	0.1M TRIS/HCl pH 8.5	20mL
Albumin	0.5g	90 mM p-Coumaric acid in DMSO	100 μ L
Block buffer	25%	250 mM Luminol IN DMSO	100 μ L
-	-	30% H_2O_2	10 μ L

3.6.5 Immobilized Metal Chelate Affinity Chromatography (IMAC)

The purification of Fetuin-A was crucial to extract all the desired protein from the remaining supernatant of the CHO cell suspension culture (Chapter 3.6.3). Therefore, since all proteins produced were expressed with a 6x-Histidine-tag, purification was performed using immobilized metal chelate chromatography (IMAC). The column material of Ni^{2+} -nitrilo-acetic acid (NTA) column (HisTrap HP 5 mL, GE Healthcare, Boston, USA) consists of Ni^{2+} ions that are bound to sepharose. Then, the his-tagged proteins specifically bind to the Ni^{2+} ions and form high-affinity complexes (Figure 6). Thus, IMAC has continued to be a widely used protein purification protocol since its foundation in the mid-1970s (Moore et al., 2021).

Firstly, the frozen falcons from chapter 3.6.3 containing the supernatant were defrosted in the 37.5 °C water bath and the centrifuge at 20.000 g, 10 minutes, 4°C. During centrifugation, a column was attached to the pump with flow rate of 1 mL/min.

All the buffers and solutions required (Table 3) were filtered and degassed to prevent the entrance of large particles or the formation of air bubbles during the process.

The column was attached on the end of the tubing using a wet-to-wet method with 20% ethanol (Table 3). The ethanol was left to rinse tubing and His-trap column for around 10 minutes and a waste goblet was placed under the exit structure to collect the reagents.

After the period of centrifugation, the supernatant was poured into a 500 mL bottle built with a 0.22 μm filter (Corning Inc., Corning, USA) that was previously placed inside an ice recipient. As the supernatant was being poured and sterile filtered, a volume of equal parts of low-imidazole buffer was added simultaneously (Table 3). All procedures were done on ice, to avoid protein denaturation.

After the ethanol wash, MilliQ and the low imidazole buffer (at a flowrate of 2mL/min) were used for washing the tubing, with each wash lasting for 10 minutes. After the supernatant was connected to the affinity chromatography tube, the waste goblet was exchanged for another recipient to collect the flow-through during the process. The supernatant was then filtered through the affinity chromatography tube at a flowrate of 4mL/min until only around 5mL of solution remained, as the tube cannot be completely dry. The supernatant goblet was exchanged for the low imidazole buffer and a falcon with a volume of 15mL was taken – wash elution (Figure 6). So, the unbound proteins were flushed from the column by this washing step, while the His-tagged proteins were still bound specifically to the Ni^{2+} ions of the column.

To extract the protein of interest that was bound to the affinity chromatography column, the supernatant goblet was exchanged for the high imidazole buffer with a flowrate of 3mL/min. Since the chemical structures of the imidazole on the buffer and the histidine (on the His-tag) are similar, the imidazole binds to the Ni^{2+} ions in the column allowing the protein with the His-tag to detach and be collect in a first Eppendorf, with a volume of 5mL (dead volume elution), followed by 10 consecutive eppendorfs, with a volume of 1mL each (10 elution fractions) (Figure 6).

Finally, the supernatant, flow through, wash elution, dead volume and the 10 elution fractions were analyzed spectrophotometrically to verify the presence of fetuin-A and its concentration (chapter 3.6.6). To verify the presence of Fetuin-A and other proteins in the previously mentioned solutions, an SDS-Page gel was conducted (chapter 3.6.7). To preserve the column for future use, it was firstly buffered in Milli-Q and stored in 20% ethanol.

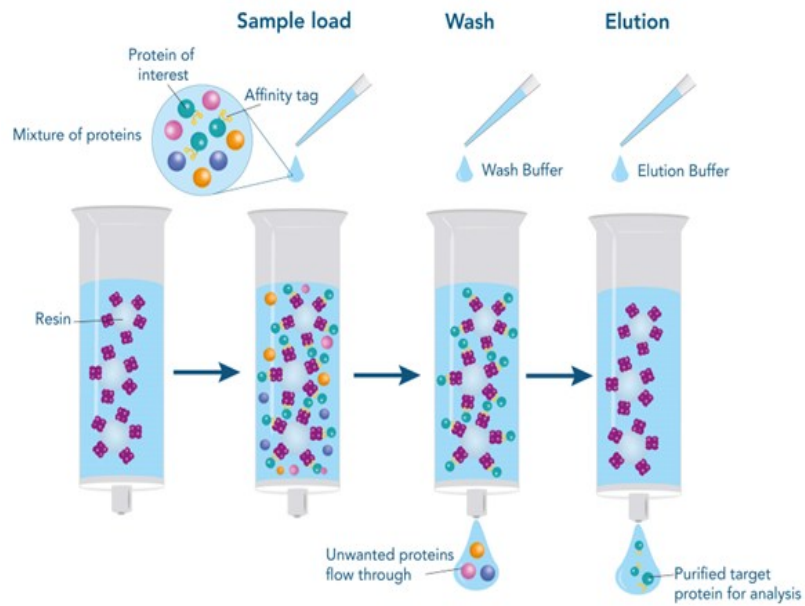


Figure 5: Scheme of the overall purification steps required on the IMAC chromatography process to purify a His-tagged protein sample.

Table 3: List of buffers and solutions used in the affinity chromatography process as well as each reagent concentration needed.

Reagent	Volume
20% Ethanol	20 mL 100% Ethanol
	80 mL MiliQ
Milli-Q	50 mL
Low Imidazole Buffer	20mM Na_3PO_4
	500mM NaCl
	40mM Imidazole
	MilliQ
High Imidazole Buffer	20mM Na_3PO_4
	500mM NaCl
	500mM Imidazole
	MilliQ

3.6.6 Spectrophotometer – Protein Concentration

The concentration of protein was determined by spectrophotometry. This procedure is based on ultraviolet (UV) light absorbance, to determine the protein concentration in a sample. In general, proteins absorb UV light around the 280 nm wavelength, as amino acids like the tryptophan or tyrosine have aromatic side chains. Thus, a UV-Vis-Spectrophotometer (Eppendorf, Hamburg, Germany) with UV cuvettes were used in this work as each sample from chapter 3.6.5 was analyzed. The blank used was sodium chloride (NaCl) to minimize interference in the determination of protein concentration.

3.6.7 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis – SDS-PAGE

SDS-PAGE (sodium dodecyl-sulfate polyacrylamide gel electrophoresis) is a technique widely used to separate proteins of a sample according to their molecular weight in an electric field. The main procedure is the denaturation of the proteins with an anionic surfactant, that attributes the proteins a negative charge based on their molecular weight. The proteins are then separated by their weight with electrophoresis in an acrylamide gel. (Nowakowski et al., 2014).

The protocol required the preparation of a separating and the stacking gel as described in Table 4 and briefly illustrated in Figure 6.

Table 4: List of reagents for the production of the gels required for the SDS-PAGE method. This table represents the compounds necessary to produce 2 gels.

Reagent	Separating Gel (10%)	Stacking Gel
Milli-Q (mL)	4.2	3
Separating Gel Buffer (mL)	2.5	-
Stacking Gel Buffer (mL)	-	1.25
Acrylamide (30%, mL)	3.3	0.75
TEMED (μL)	8	10
APS (10%, μL)	50	50
End Volume (mL)	10	5

The buffers used were prepared in a 4-times stock solution: 1.8 M TRIS, 0.4% w/v SDS, pH 8.8 as separating gel, and 0.5M TRIS, 0.4% w/v SDS, pH 6.8 as a stacking gel. The gels were prepared with the Mini-PROTEAN[®] Tetra Cell Casting Stand from Bio Rad (Hercules, California, USA).

First, the separating gel was poured between the glass plates and left to polymerize, and a thin layer of isopropanol was added on top of the gel for straight gel edges, for 30 minutes and 37 °C.

After the time established, the separating gel polymerized, and the layer of isopropanol was removed with the help of an absorbent paper. The stacking gel was added on top of the previous gel. A comb with the desired number of columns was placed inside the stacking gel and let to polymerize for 30 minutes at the same temperature. Laemmli buffer (25 mM TRIS pH 8.3, 192 mM glycine, 0.1% SDS) was added as the running buffer inside the gel caster until the guidelines were reached.

The samples for the procedure were provided by the previous method: IMAC chromatography (chapter 3.6.5). The samples were prepared with a 1:2 dilution with protein solution and 2× SDS sample buffer (0.125 M TRIS/HCl, pH 6.8, 5% SDS, 10% glycerol, +/- 10% β-mercaptoethanol, 0.01% bromophenolblue) and cooked on the ThermoMixer® (Eppendorf) for 5 minutes at 95 °C to allow the proteins to denaturize. The increase of temperature disrupts the hydrogen bonds of the proteins and as a result the amino acid-chains lose the secondary and tertiary structure. The proteins acquire a negative charge proportional to their length.

Afterwards, the comb was removed and all volume of 15µL of each sample was added to each column. 15µL of low molecular weight marker (GE Healthcare, Boston, USA) was added to the first column to provide as a guideline of the molecular weight distribution. In each pocket created by the comb was added 15µL of low molecular weight marker as well as of each sample. The gel was run at 33 mA for an hour until the samples reached a close point to the end of the casket. As the proteins within the sample are negatively charged, these migrate towards the positive electrode (anode, on the bottom of the casket). As smaller proteins migrate quicker through the gel matrix and stop closer to the end of the casket, larger proteins encounter more resistance, move slower, and tend to remain closer to the starting point. This differentiation in mobility allows for an effective protein separation based on molecular weight.

The vertical gel apparatus Mini-PROTEAN ® Tetra Cell from Biorad (Hercules, California, USA) was used.

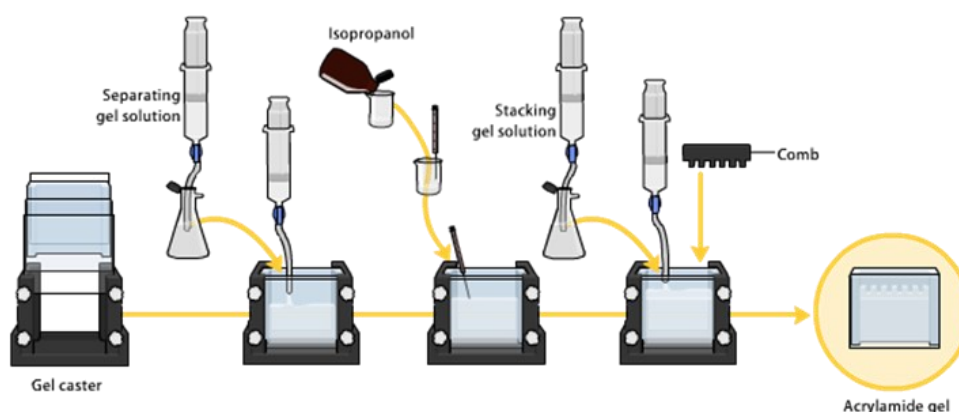


Figure 6: Illustration of the SDS-PAGE protocol performed, and the materials used (Kozak et al., n.d.)

3.6.8 Coomassie staining of SDS-Gels

After the SDS-PAGE the gel (chapter 3.6.7) was carefully submersed in water and let to rest for five minutes on a shaking plate. The water was drained and the gel was stained with Coomassie (Imperial Protein Stain, Thermo Scientific, Waltham, USA) for one hour in the shaking plate at room temperature. Coomassie Brilliant Blue is a triphenyl methane dye that is commonly used to stain proteins as the gels from the SDS-PAGE are inherently transparent. This stain binds to the proteins revealing the protein bands and its distribution along the acrylamide gel. The intensity of staining revealed in each band was directly proportional to each protein concentration in each sample tested. The gel was rinsed in water to allow the adhering unspecific colour to be removed. Representative pictures of the SDS-PAGE gel were captured.

3.6.9 Calcium-Phosphate Precipitation Inhibition Assay

The Calcium-Phosphate Precipitation Inhibition Assay is an experimental method used to evaluate the inhibition activity of a compound, in this study a protein, to prevent the formation of calcium-phosphate precipitates. The assay is based on the observation that proteins with the ability to bind calcium-phosphate, stabilize the mineral in the supernatant, thus inhibiting precipitation. On the other hand, for proteins that don't have mineral binding characteristics, the process of crystallization begins and undergoes crystal growth, ultimately leading to precipitation that can be observed by the naked eye. The amount of calcium in the supernatant and the pellet (precipitate) is determined by the photometric measurement of calcium to evaluate the protein's inhibition properties. More calcium in the precipitate would indicate a lower inhibition activity and the absence of a Ca-P (calcium-phosphate) precipitate would reveal a high concentration of calcium in the supernatant and consequently a higher inhibition.

So, all the buffers needed for the assay are enumerated in Table 5. These solutions were previously prepared under sterile conditions and additionally sterile-filtered with a 0.22 μm filter before usage.

Table 5: Constitution of each solution and concentrations of the reagents utilized in the calcium-phosphate precipitation inhibition assay.

Solution	Reagents	Concentration
Tris Buffer	NaCl	140 mM
	Tris/HCl pH 7.4	50 mM
Protein Solution (Fetuin-A)	NaCl	140 mM
	Tris/HCl pH 7.4	50 mM
	Protein	0 - 8 μM
Calcium Solution	NaCl	140 mM
	Tris/HCl pH 7.4	50 mM
	$\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	5 mM
Phosphate Solution	NaCl	140 mM
	Tris/HCl pH 7.4	50 mM
	$\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	3 mM
Ammonium Buffer	NH_4Cl pH 10.5	45 mM
	NH_4OH	5% v/v

Solutions with a concentration range of 0 - 8 μM of Fetuin-A eppendorfs (Table 6) were filled up to a final volume of 150 μL with the Tris buffer prepared (Table 5). Following, 25 μL of the phosphate solution (Table 5) was added to each Eppendorf, gently vortexed and centrifuged using a benchtop centrifuge (VWR® Galaxy Mini, USA). Additionally, 25 μL of the calcium solution (Table 5) was added, equally vortexed and briefly centrifuged. With a final volume of 200 μL each all the eppendorfs were incubated for one hour at 37°C within the water bath. After the time established, the samples were centrifuged at 20000g for 10 minutes at 4°C. This approach is illustrated in Figure 7.

Table 6: Range of concentration of protein used in the calcium phosphate precipitation inhibition assay.

Sample	Protein Concentration (μM)
1	8.0
2	4.0
3	2.0
4	1.0
5	0.8
6	0.4
7	0.2
8	0.05
9	0

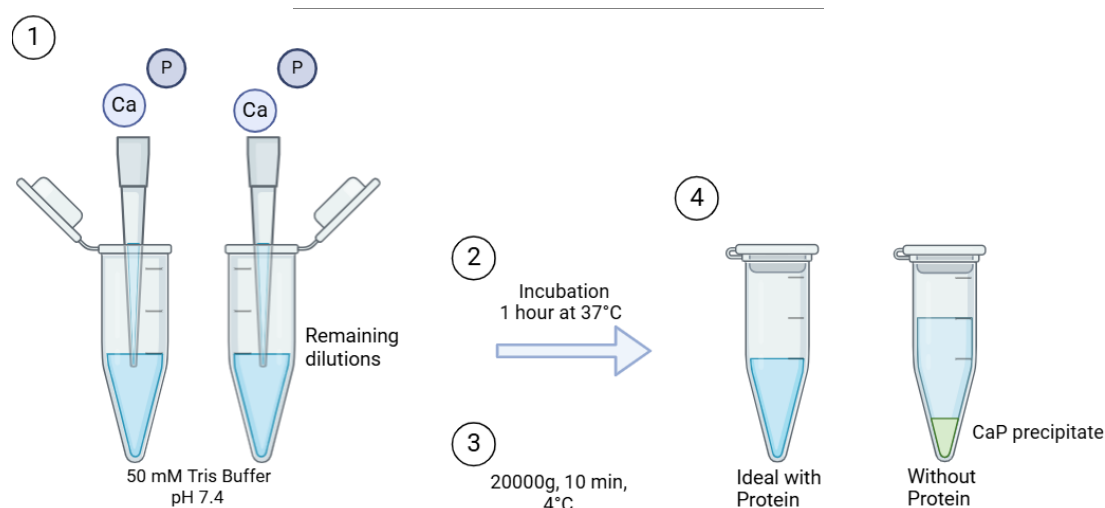


Figure 7: Illustration of the initial steps (1 to 4) of calcium-phosphate precipitation inhibition assay prior to supernatant and precipitate analysis. The samples were mixed with calcium (Ca) and phosphate (P) in a Tris-buffered system. With an inhibitor such as Fetuin-A is expected that the crystal growth is inhibited (like the ideal with protein eppendorf in step 4), so without the development of a pellet.

The scheme was designed using the BioRender software.

Subsequently, it was necessary to perform a supernatant analysis to evaluate the soluble amount of calcium. So, 100 μL of the supernatant of each sample was taken and mixed with 50 μL of 0.6 M HCl and 50 μL ammonium buffer. into a new Eppendorf. The residual 100 μL of supernatant was carefully removed using a gel loading tip and avoiding touching the pellet.

The pellet remaining in each sample was stored on ice for the precipitate analysis. This analysis aimed to determine the calcium amount within the precipitate formed. Each pellet was resuspended 100 μL 0.9% NaCl, 50 μL 0.6 M HCl and 50 μL ammonium buffer. For the supernatant and the precipitate analysis, the hydrochloric acid (HCl) was used to dissolve the precipitate, and the ammonium buffer is used to neutralize the pH of the solution.

In due course, it was required to assess the amount of calcium. So, a *Randox Calcium reagent* assay (Randox Laboratories, Crumlin, UK) was conducted according to the manufacture's specification as it is used for the quantitative *in vitro* determination of calcium concentration. This assay is based on the formation of a violet complex by calcium ions with O-cresophthalein complexone (OCPC) in an alkaline medium. The violet complex can be measured photometrically at 570nm. The protocol consists of two reagents provided in the the assay kit: Reagent 1 (Buffer – 2-amino-2-methyl-propan-1-ol); Reagent 2 (Chromogen, O-cresophthalein complexone, 8-Hydroxyquinoline and HCl). Firstly, the working solution was prepared by mixing reagents 1 and 2 at a rate of 1:2 and incubated for at least 10 minutes at room temperature. A range of 5 to 0.039 mM CaCl_2 (from the calcium solution in Table 5) was prepared, and 5 μL of each dilution was pipetted into a 96-well plate. Each sample was measured in duplicates. 200 μL of the working solution previously prepared was added to each well and the plate was placed in a shaker (Titramax 100, Heidolph Instruments GmbH & CO. KG, Schwabach, Germany) for five minutes at 1050 rpm. Afterward, the plate was put in a plate reader and was measured photometrically at 570 nm.

To determine the calcium in the sample the generation of a standard curve was necessary. This standard curve was prepared from the 40 mM stock calcium solution by first using a 1:4 dilution and then a serial 1:2 dilution, which was used from 5-0.039 mM in the assay as the standard curve. As calcium forms a violet complex with O-cresophthalein complexone in an alkaline medium, the calcium content of each sample can be determined by photometric measurement and comparisons with the standard curve. In the case of proteins with mineral-binding properties, such as Fetuin-A, the calcium is stabilized in the supernatant and therefore does not precipitate. In contrast, in proteins without mineral-binding properties, the calcium-phosphate is found only in the pellet and not in the supernatant, because it precipitates. Thus, the assay provides information about the properties of the protein such as some insights regarding its inhibition activity.

3.7 Cell Culture Proliferation Assay

The main goal of this assay was to check how fast the cell lines grow, quantify the growth rate of the cells and to verify if they could be used for future assays. SaOs-2, OCCM, IM1 and IM3 cell lines (chapter 3.4) were put to the test. The cells were cultured in a basal medium composed of low glucose DMEM (Dulbecco 's Modified Eagle Medium, Thermo Fisher Scientific, 11054-020) supplemented with 10% fetal bovine serum (FBS, Pan, Bio, p-30-3306 p190304, 1% Penicillin-Streptomycin (PS, Thermo Fisher Scientific, 15140122) and 2% of CTS™ GlutaMAX™ (Thermo Fisher Scientific, A12860-01) to allow cell proliferation. Two different densities were tested – 1 million cells/per flask and 2 million cells/per flask. Cells were seeded onto a T75 flask, which was put inside a real-time imaging device CELLCYTE in an incubator at 37°C and 5% CO₂. The mediums of each flask were exchanged three days after the initiation of the proliferation assay.

The study was conducted on the CELLCYTE over 7 days and with an interval of each image taken every 3 hours in bright field, that produces a dark image against a bright background. The employment of this device allowed real-time live cell imaging of four spots of each T75 flasks within the incubator. Also, it allows the capacity to rewind and replay the images acquired, at every single time point established, to diligently follow the sequence of biological events. Thus, the cell kinetics were verified every day, during the time of the experiment, by accessing through the CELLCYTE CYTENA software to analysis the pictures taken.

3.8 Calcification Assay

3.8.1 Cell Culture

The four cell lines in the study – IM1, IM3, OCCM, SaOs-2 (chapter 3.4) – were cultured in a basal medium composed of low glucose DMEM (Dulbecco 's Modified Eagle Medium, Thermo Fisher Scientific, 11054-020) supplemented with 10% fetal bovine serum (FBS, Pan, Bio, p-30-3306 p190304, 1% Penicillin-Streptomycin (PS, Thermo Fisher Scientific, 15140122) and 2% of CTS™ GlutaMAX™ (Thermo Fisher Scientific, A12860-01). The medium was prepared by adding each compound to a 500 mL low-glucose DMEM bottle (Table 7). The cells were cultured in T-75 cell culture flasks (Greiner, 658175) with a volume of cell culture medium of 10mL/per flask. The medium of each flask was exchanged every two to three days and the cells were split when 90-95% confluency was reached. After reaching the confluency needed, the cells were split and cultured in 24-well plates (Falcon, 353047) as described in attachment 7.6. The cells were seeded at a density of $4.5 \times 10^4/\text{cm}^2$ SaOs-2, $1.5 \times 10^4/\text{cm}^2$ OCCM and $2 \times 10^4/\text{cm}^2$ IM1 and IM3 with a 1mL volume of medium per well. The 24-well plates were incubated in the CELLCYTE device at 37°C and 5% CO₂ to study the behavior of the cells during the assay period. Representative pictures of each well were taken every 3 hours in the CELLCYTE device within the incubator. For three days the cells were allowed to attach and grow before the addition of calcification conditions.

Since one of the aims of the present thesis was the study of the four cell-lines in culture and calcification conditions, two 24-well-plate were replicated. Therefore, the mediums were exchanged with culture and calcification conditions applied to different columns within the same plate, as described in attachment 7.6. Additionally, the impact of the presence of Fetuin-A was investigated through two methods: in the first plate, a concentration of 20µg/mL of m-Fetuin-A-mRuby was added to the medium for the entire duration of the experiment, according to each medium exchange, and as an end-point staining (chapter 3.8.2); in the second plate, Fetuin-A was only added at the experiment 's conclusion as an end-point staining. In both methods, the fluorescence fetuin-A – mFa-Ruby - previously produced (chapters 3.6.3 and 3.6.6) with an initial concentration of 9.1mg/mL was adjusted to a final concentration of 100µg/mL and added to each well. At a confluency of around 80-85% per well, the first medium exchanged was performed by adding the calcification medium, culture medium, and mFa-Ruby to the respective columns according to each plate condition (attachment 7.6). The calcification medium was prepared by adding 3 mM phosphate and 4.3 mM calcium to the medium (Table 7).

The cells were cultured for seven days. After seven days, the cells were taken out of the CELLCYTE device, and parameters such as cell viability and calcification lesions were evaluated through Fetuin-A staining (chapter 3.8.2), FDA/PI staining (chapter 3.8.3), and Alizarin Red staining (chapter 3.8.4). The plate configuration incorporated duplicate samples.

Table 7: List of reagents and each volume used during the preparation of both mediums necessary for the different phases of the calcification assay. The culture medium was prepared, stored on the fridge, and used whenever necessary during the assay. The calcification medium was prepared fresh and used on the same day.

Reagent (mL)	Culture Medium	Calcification Medium
Low-Glucose DMEM	500	10
Fetal Bovine Serum	50	1
Penicillin-Streptomycin (PS)	5	0.1
GlutaMAX	10	0.2
Calcium	-	4.3 mM
Phosphate	-	3 mM

3.8.2 Fetuin-A Live Staining

All the 24-well plates (chapter 3.8.1) with and without Fetuin-A on the media were stained with mFa-mRuby. This protein probe is a murine Fetuin-A probe with fluorescent properties, due to the Ruby chain, since it binds to mineral deposits allowing the visualization of early-stage cellular calcification. Therefore, the mFa-mRuby variant was produced as described in chapter 3.6.3 – with an initial concentration of 9.1mg/mL (based on the protocol in chapter 3.6.6), was adjusted to a final concentration of 100µg/mL and added to each well for 20 minutes at 37°C.

The cells were washed with PBS three times for five minutes and representative pictures were taken at a LEICA 6000 microscope.

3.8.3 Fluorescein/propidium iodide Staining

Fluorescein diacetate/Propidium iodide staining or FDA/PI is used for live/dead staining. Therefore, fluorescence microscopy was required, as this staining protocol leans on the excitation of fluorophores by a specific wavelength of light and the subsequent emission of light at a longer wavelength (Shihan et al., 2021). By the selection of the appropriate fluorescence channel during microscopy, the cells take up the FDA (Fluorescein diacetate, Sigma, F7378) and convert the non-fluorescent FDA into a green fluorescent metabolite fluorescein, which can be observed in real-time simultaneously on both microscopy and the computer displaying the images. The intensity and signal measured provide an indicator of viable cells. On the other hand, PI (Propidium iodide, Sigma, 81845) is used for nuclei staining of cells with disordered areas on the membrane and binds to the cell deoxyribonucleic acid (DNA).

Both PI and Fetuin-A (implemented in chapter 3.8.2) emit a red signal under fluorescence microscopy, posing difficulties in differentiation between them. Therefore, PI was not used during this staining protocol. The main objective of this assay was to visualize the cell outlines, hence FDA staining was employed for this purpose.

A working solution was previously prepared by diluting the FDA stock solution (5 g/L in acetone) at a rate of 1:100 in sterile PBS. The cells on the 24-well plates (chapter 3.8.1) were washed with PBS and subsequently stained with the FDA working solution for 30 seconds. Cells were washed with PBS. 1 mL of FluoroBrite™ DMEM (Thermo Fisher Scientific, A1896701) was added to each well, and representative pictures were taken with a LEICA 6000 microscope. The red and green fluorescence channels of the microscopy were used to detect signals emitted by the fetuin-A and FDA, respectively.

3.8.4 Alizarin Red Staining

The alizarin Red staining is used to colour calcium deposits red. Before the staining steps, it was necessary to fix the cells (from chapter 3.8.3) by causing their death by adding to each well a volume of 250 μ L of 4% PFA (paraformaldehyde, ApppliChem, A3813,1000) for ten minutes at room temperature.

After the time established, the cells were washed three times with 500 μ L PBS and stained with 750 μ L of filtered (10-20 μ m, VWR,516-0308) Alizarin Red working solution (40mM, pH4.1, Sigma, A5533) for ten minutes at room temperature. During this period the red colour stain binds to the calcification lesions. After the staining, the cells were washed around 5-6 times with PBS until the supernatant became clear. Representative pictures of each well and condition were taken with a Leica DMI 600 microscope with a bright field channel, that produces a dark image against a bright background.

4. RESULTS

4.1 Construction of the Chicken Fetuin-A Plasmid

The Chicken Fetuin-A plasmid was acquired from the Thermo Fisher Scientific website, but firstly it was necessary the construction of the desired plasmid, according to the needs of the study. For the design of the chicken Fetuin-A construct, pcDNA™3.4TOPO (A14697, Thermo Fisher Scientific) expression vector was used. This expression vector is a high-efficiency cloning plasmid for amplification protocols in *Escherichia Coli* cells (Chapter 3.6.2) and subsequently for production for recombinant protein in mammalian systems, such as CHO cells (Chapter 3.6.3). This vector enables a high yield of plasmid DNA as it contains an ampicillin resistance gene, which exclusively permits the growth of *E. coli* cells that have taken up the plasmid. This method enhances plasmid amplification and protein production ensuring reliable expression studies and high-quality protein variants.

Therefore, for the design of the chicken Fetuin-A was crucial the initial addition of a start codon (ATG), to initiate protein production, followed by the addition of a six-histidine chain (his-tag). Subsequently, the amino acid chain chosen (A0A8V0YUT8_CHICK, illustrated in Figure 8) was inserted into the vector. Ultimately, a stop codon TAG was added to signal the termination of protein synthesis.

Length 344		Checksum ⁱ D0252526A15F893F	
Mass (Da) 37,172			
10	20	30	40
MKALVAFILL	VQLPIHRAAP	AAPPPPLGCD	DPEIEAAEF
50	60	70	80
AVIYINGHSH	HGYRFALNRI	EQVRVLFQGP	NNEILFLELD
90	100	110	
LLETTCHILN	PTPLVNCSVR	TFAEHAVEGD	
120	130	140	150
CDVKLQKVNQ	QFSVLASKCH	SHADSAEDIR	EVCPCNPPLA
160	170	180	190
NLNDTDVLAA	VSSALNDYNS	KNPDVYLMML	EIGRAVKQYH
200	210	220	
PVQMVSVEFA	VAATNCTSQQ	AKDNLAACQL	
230	240	250	260
LPEDQSNFGF	CTAKMVTGPS	QDLIAECQLY	GHQPGVTYPH
270	280	290	300
PGQDTSAGLV	PSAGQGFTNH	NLKISHNNPV	ASESSSSSEFP
310	320	330	
SLLSAKSVAK	RAAAEVAQHD	KVPHPVGFPV	
340			
PPPLCPGKIR	HFKI		

Figure 8: Sequence of amino acids of the A0A8V0YUT8_CHICK (chicken Fetuin-A) protein available in the UniProt Software.

The addition of a histidine tag (His-tag) constituted by six histidine amino acids – HHHHHH – was crucial to facilitate some methods used for protein detection and purification. The addition of this polyhistidine affinity tag to the sequence of Chicken Fetuin-A makes an impactful difference, as it facilitates the detection of the target protein in the Western Dot Blot (Chapter 3.6.4), by using anti-His antibodies for an effective track of protein production, and to perform a high yield purification through IMAC chromatography (Chapter 3.6.5), as the his-tag has a high affinity to Ni ions present in the column of the purification system, allowing an optimal recovery of the desired protein. This specific affinity tag was chosen due to its versatility in several expression systems from bacteria to mammalian cells, a small size chain of around 0.84 kDa, which reduces any potential alterations on the proteins' structure and function, enables one-step purification of the desired proteins from complex samples and the commercial availability of the anti-His-Tag antibodies.

4.2 Analysis of the Western Dot Blot

The Western Dot Blot method is a protocol used to detect and analyze the presence of protein. This method allowed a quick verification of the quality of the expression recombinant proteins in CHO cells (chapter 3.6.3) and to perceive the presence of fetuin-A protein and its production during the five-day suspension cell culture. The recombinant proteins (chicken Fetuin-A and mFa-Ruby) presence and expression were confirmed by chemiluminescence detection.

The nitrocellulose membrane produced as described in chapter 3.6.4 was taken and processed to the fluorescence imager (Fuji LAS Mini 4000, GE Healthcare, Boston, USA) for the development of a signal to perform qualitative and quantitative analysis (Figure 10). This signal originated from the reaction of luminol, hydrogen peroxide and the HRP linked to the Hist-tag (chapter 3.6.4) and can provide crucial information about the concentration of the target protein. Each sample illustrates a droplet of supernatant collected daily during recombinant protein expression. The samples were labelled according to the harvest day, ranging from day 1 (D1) to day 5 (D5). So, if Fetuin-A was present in the supernatants, a positive signal would appear as a visible shade of grey with the shape of the pipetted droplet providing important insight into the recombinant protein expression and production.

Figure 9 illustrates the nitrocellulose membrane after the chemiluminescence detection protocol. The visualization of the shape and colour of each droplet indicated the presence of Fetuin-A.

Therefore, it is possible to say that the production of fetuin-A protein was constant and increased during the 5 days, as evidenced by the darkening of the droplets - therefore an increasing signal of the His Probe (six histidine residues in the Fetuin-A amino acid chains). Also, the droplets do not look perfectly round as it looks more like there are more than one droplet, indicating certain inaccuracies during the protocol.

As the presence of fetuin-A was verified as desired, an IMAC chromatography was then performed to purify the supernatant obtained at the fifth day, stored in the freezer after the period of protein production and expression (chapter 3.6.6).

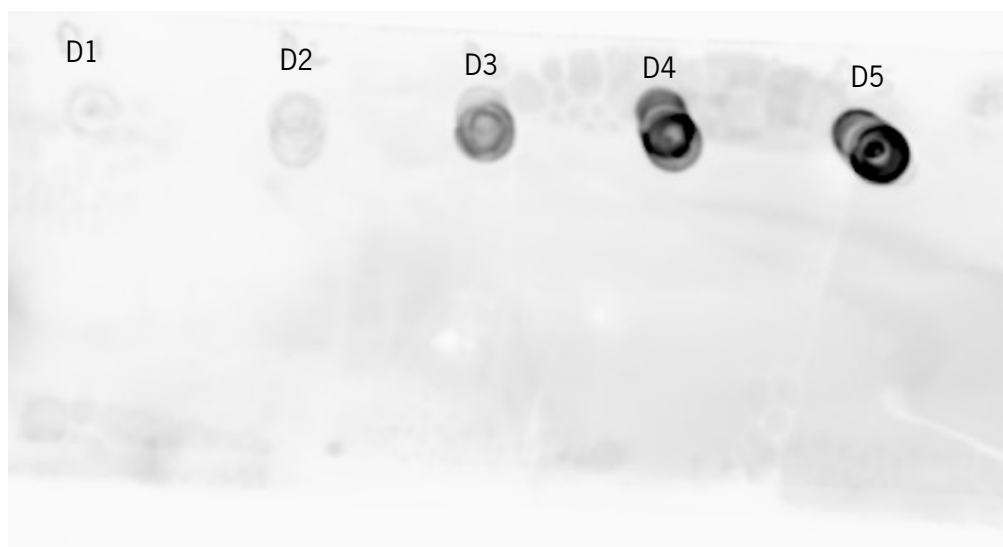


Figure 9: Western dot blot membrane result of days 1, 2, 3, 4 and 5 of fetuin-A protein expression and production on a suspension of CHO cell culture. The droplets of each day are derived from the supernatant of the sample that was taken once a day.

4.3 Expression and Purification of Recombinant Fetuin-A Probes and Determination of Protein Concentration

Recombinant fetuin proteins – chicken and mRuby – were expressed in chinese ovary hamster cells, entitled ExpiCHO-S^T cells (Gibco, Thermo Scientific, Waltham, USA). The proteins were produced based on the protocol described in Chapter 3.6.3 and purified via IMAC chromatography (chapter 3.6.5). Thereafter, IMAC-purification fractions were analyzed by SDS-PAGE (chapter 3.6.7) pursued by Coomassie staining (chapter 3.6.8). Figure 11 displays the results of the purification of chicken fetuin-A and the mFA-mRuby. Thus, three rounds of purification were conducted two of them for chicken Fetuin-A and the last to produce m-Fetuin-A-mRuby for subsequent use in the calcification assay (chapter 3.8.1) and microscopy experiments (chapter 3.8.2).

In all the SDS-PAGE gels in Figure 10, the first column on the left was used as a reference with a low molecular weight marker that indicates a range of molecular weights. This endorses the identification of Fetuin-A in the samples through its molecular weight, which is around 51-67 kDa. Since the ruby is a protein itself the mFetuin-A-mRuby molecular weight can go up to 90kDa. Therefore, in Figure 11A it was possible to verify the presence of fetuin-A in SDS-PAGE gel especially on elution 3 and strongly on elution 4 (from the IMAC – chapter 3.6.5), which indicated a high concentration of fetuin-A due to the thick band detected by Coomassie staining. Although elution 5 revealed a very narrow band on the fetuin-A molecular weight range, it was not preserved since the protein concentration was not significant. The absence of a band on the fetuin-A MW range on the flow-through, wash, and dead volume indicated that the purification method was well performed. Since the main goal of the method is to ensure that the protein appears solely in the elution fractions, the buffer is exchanged for one with a higher concentration of imidazole *prior* to dead volume eppendorf and the ten elutions. This is achieved because histidine binds the column through its imidazole ring, which enables imidazole to bind to the Ni²⁺ ions in the column, releasing the His-tagged Fetuin-A and allowing its elution.

Afterward, elution 3 and 4 were rebuffed and taken to the spectrophotometer to determine the protein concentration of each sample (chapter 3.6.6). Due to the low yield of chicken fetuin-A with a final concentration of 1,55 mg/mL after rebuffing (Figure 10A), a second round of production and purification was conducted (Figure 10B). On the second round of production of chicken Fetuin-A, elutions 1, 2, and 3 were subjected to spectrophotometric analysis to reveal the concentration of protein produced, due to a higher presence of the target protein, as indicated by the prominent bands in the Fetuin-A molecular weight.

The protein concentration obtained was 2,56 mg/mL, indicating a higher yield compared to the previous round. This result was expected, as more prominent bands were observed in the second round (Figure 10B).

A third round of protein production was carried out, during which the Fetuin-A mRuby supernatant was purified to assess the effectiveness of the production method and to evaluate the resulting production concentration. Thin bands with distinct molecular weights were identified in the supernatant, revealing unspecific protein on the sample and therefore a less effective purification. Also, dense layers in the elutions fractions 3, 4, and 5, spanning a high-spectrum molecular weight were spotted. However, these fractions displayed prominent bands on the Fetuin-A molecular weight, indicating a significant concentration of the target protein. So, the elutions 3,4 and 5 were subjected to spectrophotometric analysis, revealing a final concentration of 9.10 mg/mL of m-Fetuin-A-mRuby (Figure 10C).

All the elution fractions previously analysed with the spectrophotometer were rebuffed, aliquoted, labelled and shock frozen in liquid nitrogen at -196°C, and stored for future use in a -20 °C freezer. The necessary aliquots were subsequently used for the calcification assay (chapter 3.8.1) and fetuin-A staining (chapter 3.8.2).

The chicken Fetuin-A produced initially was sent to Berlin BioGenes for Antibody production.

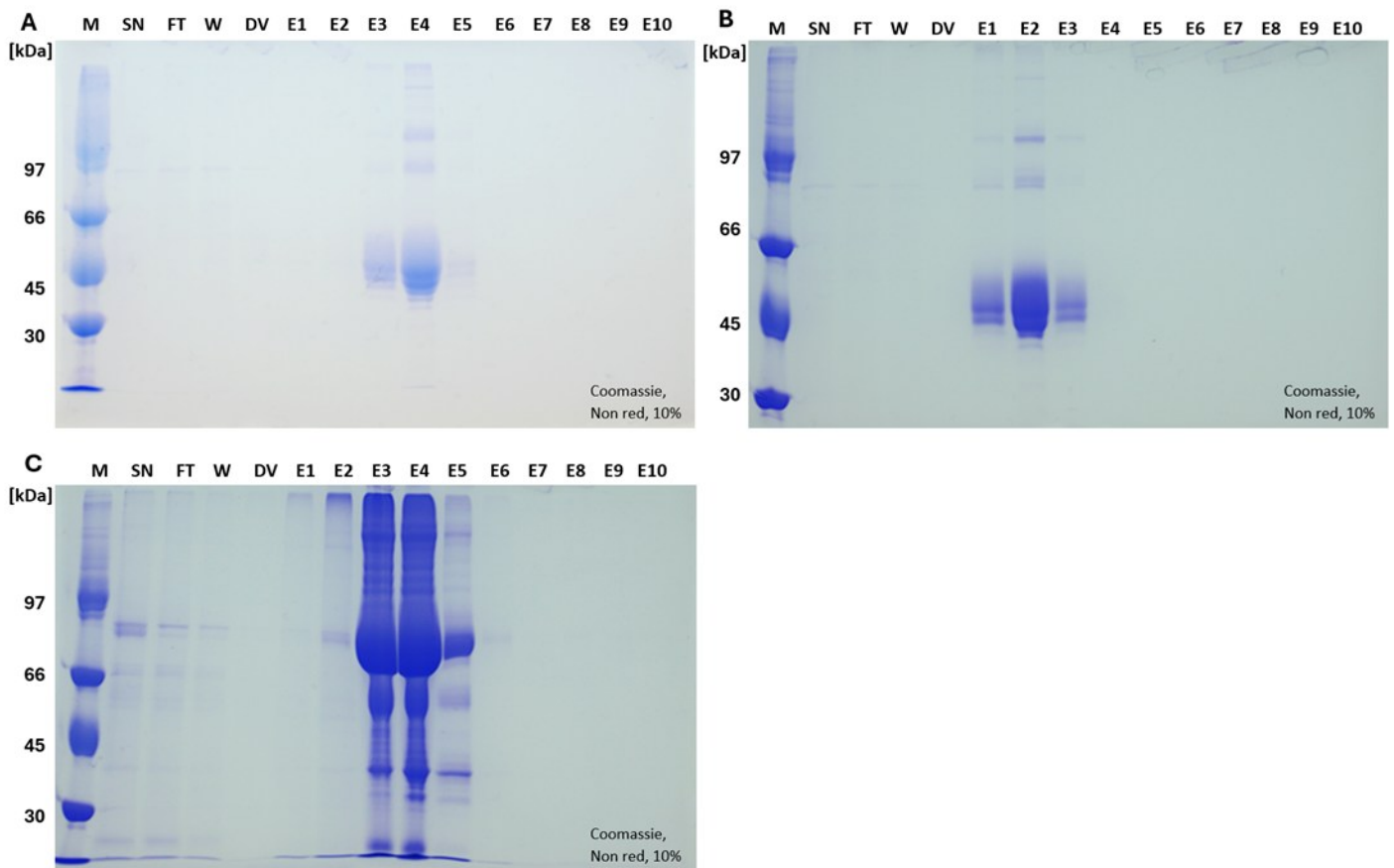


Figure 10: SDS-Page of expression and purification of recombinant proteins. Two rounds of chicken Fetuin-A (10A and 10B) and one round of Fetuin-A-mRuby (Figure 10C) were purified by IMAC chromatography (chapter 3.6.5).

From each purification process, 14 fractions were analyzed to verify the presence of the protein in the study: Fetuin-A. The culture supernatant (CM) was applied onto to the second column since the first one was used as a marker for the KDa scale. 15 μ L volume of culture supernatant (CM), flow-through (FT), wash fraction (W), dead volume (DV), and 10 consecutive elution fractions (1-10) solutions were prepared and were added to each column as described in chapter 3.5.8. On the second round of protein production (figure 7B) the 6 $\times$ histine sequence (his-tag) on the protein chain produced was digested and sent to Berlin BioGenes for antibody production. On the mFA-mRuby production due to a high concentration of the protein of interest, the elution fractions 3, 4, and 5 were rebuffed, aliquoted, and stored. E=elution.

4.4 Calcium-Phosphate Precipitation Inhibition Assay

The Calcium-Phosphate Precipitation Inhibition Assay is a method used extensively to understand how a variety of substances stabilizes calcium and phosphate in a supernatant and consequently inhibit the precipitation of these minerals. Having expressed and produced Fetuin-A probes (chapter 3.6), it was imperative to evaluate the protein inhibition activity through this particular assay. As fetuin-A is widely reported in the literature as an inhibitor of calcification within the human body, ensuring and certifying the functionality of the protein probes was fundamental for accurate experimental outcomes in the forthcoming assays (Brylka & Jahnen-Dechent, 2013; Chekol Abebe et al., 2022; Jahnen-Dechent et al., 2008, 2011; Kovářová et al., 2021).

The data obtained from the spectrophotometrically measured samples provided insight into an inhibitory potential of the Fetuin-A. The results obtained, with the establishment of the protocol described in chapter 3.6.9, were analysed in the GraphPad Software and the obtained graphs are shown in Figure 11. The commercial bovine Fetuin-A from Sigma was used as a control. The chicken Fetuin-A (Figure 11B) and m-Fetuin-A-mRuby (Figure 11C) were tested.

The graph illustrates that the precipitate curve (red) in the bovine Fetuin-A (Figure 11A) started at 3.4 mM calcium and dropped close to zero after the turning point. The supernatant (blue) starting at 1.3 mM, moved up to around 4.0 mM calcium after the turning point. For the chicken Fetuin-A (Figure 11B) the precipitate curve (red) started at 3.2 mM calcium, remained relatively stable at this concentration, and dropped sharply until around 1.0 mM. The supernatant in blue starting at 1.9 mM, moved up to around 2.1 mM calcium. Additionally, in the murine Fetuin-A-mRuby (Figure 11C) the precipitate curve (red) started at 3.3 mM calcium and dropped close to 1 mM after the turning point. The supernatant (blue) started at 2.0 mM and moved up to around 4.9 mM calcium.

In the three samples, the calcium content in the supernatant and the precipitate inverts their direction creating a turning point. The turning point is a parameter that marks the protein concentration at which its inhibition activity modifies the rate of precipitation. This parameter can also be entitled IC_{50} (half maximal inhibitory concentration). The IC_{50} of each approach was determined and documented in Figure 11.

The nomenclature, IC_{50} , refers to the concentration of protein necessary to inhibit the formation of a precipitate, so a lower IC_{50} value indicates a higher potency of inhibition with a lower concentration of protein. As displayed in Figure 11, the chicken Fetuin-A revealed a higher IC_{50} value of $3.88 \mu M$ compared to the m-Fetuin-A-mRuby with a IC_{50} value of $0.49 \mu M$.

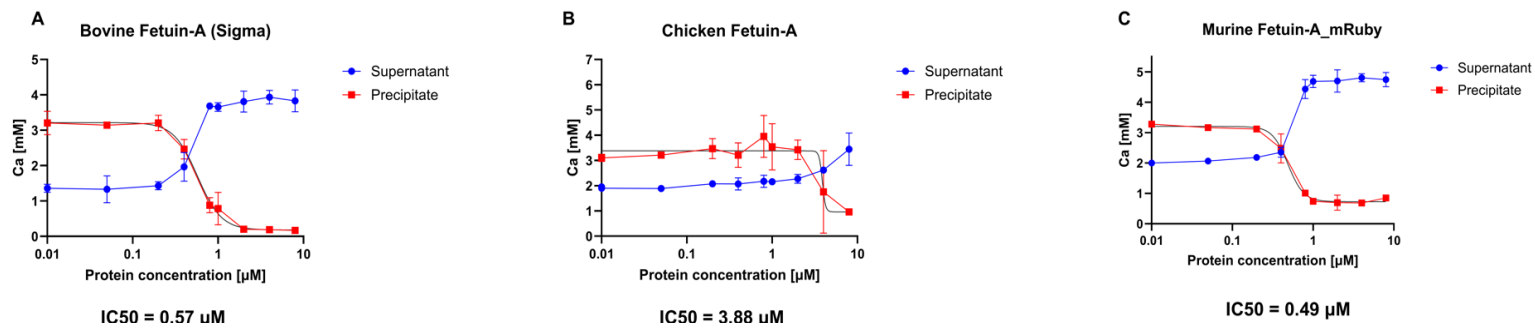


Figure 11: Calcium phosphate precipitation inhibition assay results for the Fetuin-A probes produced. Figure 11A indicates the results of the bovine fetuin-A (bF). Figure 11B shows the chicken Fetuin-A probe and Figure 11C shows the murine Fetuin-A-mRuby.

Bovine fetuin-A in Figure 11A was purchased by Sigma and used as a control. The used concentration of recombinant proteins was plotted against the measured calcium concentration in precipitate (red) and the calcium content found in the supernatant (blue). The IC_{50} values were determined using a non-linear function. Error bars indicate standard deviations. In Figure 11A the IC_{50} value of $0.57 \mu M$ was determined. In Figure 11B the IC_{50} was $3.88 \mu M$. In Figure 11C the IC_{50} value was $0.49 \mu M$.

4.5 Analysis of Cell Culture-based Proliferation Assay

To mimic the biological conditions of the human body it was crucial to establish the most accurate in vitro biological scenario possible. Therefore, the adoption of the same cell type involved in the calcification and mineralization processes was imperative. Since calcification is a pathological process that often involves vascular smooth muscle cells (VSMCs), which play a critical role in ectopic calcification, these cells were taken part in the study with two variants from different patients acquired from Uniklinik RWTH Aachen. On the other hand, cementoblast are often involved in bone-forming and mineralization processes as well as human osteosarcoma cells (SaOs-2). These cell lines were purchased (Chapter 3.4). Given these cell lines have distinct functions in the human body and anatomies, these differences might reflect on their proliferation rate. Thus, to plan the calcification experiments, firstly it was important to study and characterize each cell line proliferation, replication rate, and assess whether the medium conditions and compositions were optimal.

The 7-day growth experience described in chapter 3.7 culminated in the results illustrated in Figure 12. CELLCYTE enabled the photographing of different points within the T-75 flasks, to more accurately and precisely characterize the proliferation. A 4-image field of view was selected. The graphs, generated by the CELLCYTE software, were based on the confluence of each four fields of view of the T-75 flasks throughout the experiment. Each final graph line was plotted to incorporate the mean data with the corresponding standard deviation included at all points.

In the graphs in Figure 12, it is possible to confirm that all reached high confluency (80-100%) at a certain time, independently of the cell line and of its initial cell density. The T-75 flask with a seeding density of 2 million cells revealed a higher initial confluency in comparison to the seeding density of 1 million cells, as expected. In most of the conditions, nearly 100% confluency was reached within the time course of the 7-day period of the trial. Therefore, the graphs showed the time point where each flask reached the desired confluency.

The SaOs-2 cells (Figure 12A and 12B) behaved close to linear growth, reaching a nearly 100% confluency at around 96 hours (4 days) for the 1 million cells/flask and at around 122 hours (around 5 days) for the 2 million cells/flask density.

The OOCM cells (Figure 12C and 12D) resemble the literature cell growth curve (Attachment 7.5), reaching full confluency at around 60 hours (2 and a half days) for the 1 million cells/per flask and around hour 54 (around 2 days) for the 2 million cells/flask density.

The IM1 (Figure 12E and 12F) and IM3 (Figure 12G and 12H) cells behaved in quite similar ways as expected, reaching a nearly 100% confluency around 120 hours (5 days) for the 1 million cells/flask and around hour 60 (2 and a half days) for the IM1 cells and hour 96 (4 days) for the IM3 for the 2 million cells/flask density.

Like the OCCM cells, the IM1 cells demonstrated a great example of a cell growth curve with the log phase marked by the increased cell proliferation and a stationary phase where 100% confluency was achieved.

Due to some focus problems with the CELLCYTE camera at some time points during the study, the graphs are not as linear as anticipated. These focus problems were due to some instability/movement of the device, or to cells floating around, altering the focal plane of the camera and resulting in blurry pictures. Such pictures are challenging to quantify for confluency, so the software counts their confluency as 0%.

Despite this setback, the results obtained revealed a highly satisfactory proliferation rate for all the cells within a 2-5-day interval. The rapid proliferation facilitated the continuation and repetition of subsequent experiments, as needed to achieve optimal results, due to the cells' swift growth. It was also confirmed that the assessment of the medium conditions and compositions was optimal by promoting effective and proactive cell growth.

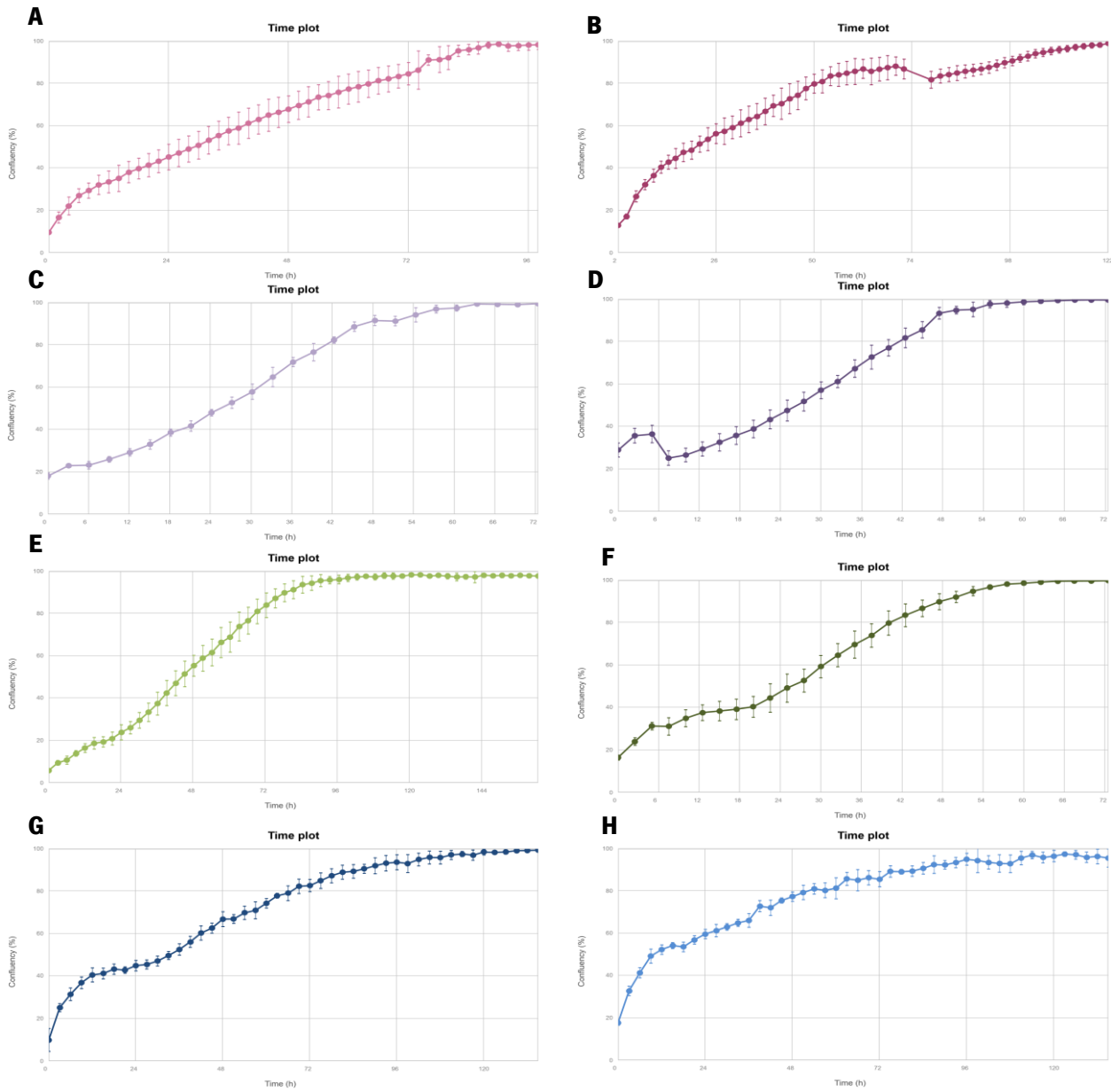


Figure 12: Growth curves of four cell line types: SaOs-2 (figure 12A and 12B), OCCM (figure 12C and 12D), IM1 (figure 12E and 12F) and IM3 (figure 12G and 12H).

The seeding density of 1 million cells/per flask results are depicted on figure 12A, 12C, 12E and 12G. The seeding density of 2 million cells/per flask results are depicted on figure 12B, 12D, 12F and 12H.

4.6 Cellular Calcification Assessment and Alizarin Red Staining

The calcification assays can provide important insights into the mechanisms of pathologic conditions like cardiovascular diseases, *diabetes mellitus*, chronic kidney disease and physiological events like bone formation, all related to mineral depositions. Either quantification or observation of calcified deposits can implicate a deeper comprehension of the interplay between cellular activity, calcification and mineralization. Therefore, the assay focused essentially on the study and verification of significant differences between professional mineralizing cells – SaOs-2 and OCCM - and calcifying cells – IM1 and IM3.

To assess the cellular calcification, it was crucial to create an optimal environment to allow the cell lines in study to calcify (or not). Consequently, a cell growth analysis was performed to evaluate the viability of the four cell lines (IM1, IM3, OCCM and SaOs-2) for the subsequent tests, as described in chapter 3.7. A healthy culture showing a good proliferation rate, of each cell line, was split and subjected either to calcification or regular culture conditions. The study was conducted with two replicates of each Fetuin-A methodologies, to ensure the reliability and reproducibility of the methods and consistency of the results. The study was carried out in 24-well plates, as illustrated in Attachment 7.6, with duplicate samples. Half of the 24-well plate were incubated with mFa-mRuby, the fluorescent Fetuin-A variant (chapter 3.8.1). The Fetuin-A staining protocol was applied to all the plates, in which the mFa-mRuby variant was used as an end-point stain (chapter 3.8.2) for the capture of representative microscopy pictures.

A calcification assay was thus performed to evaluate the cell behaviour and activity under regular cell culture and calcification conditions, as described in chapter 3.8.1. As outlined in chapter 3.8, FDA/PI staining was performed afterward to assess the cell confluency, cell viability and presence of Fetuin-A in each condition and cell line. These parameters were evaluated through fluorescence microscopy, where the FDA staining produced a green signal, while the Fetuin-A variant, mFa-mRuby, emitted a red signal. These dual-colour staining protocols enabled an easy and simple distinction between cells (FDA) and Fetuin-A deposits due to the emission of visually contrasting colours. Thereupon, the establishment of the Alizarin Red staining protocol (chapter 3.8.4) was crucial to evaluate and confirm the presence or absence of mineral deposits linked to the Fetuin-A in all cell lines conditions according to the bright-field microscopy channel results. This channel was exclusively dedicated to the Alizarin Red staining method, since the red pigment was visible to the naked eye.

IM1, IM3, OCCM and SaOs-2 cell lines were studied under calcification and regular proliferation conditions, where the concentration of calcium and phosphate differ (chapter 3.8.1). A period of seven days was settled and the four cell lines in study were left growing in T75-flasks within the CELLCYTE device inside the incubator. Representative microscopy pictures of each condition were taken with the green and red fluorescence channels combined with the bright field channel.

The results of the calcification assay and the staining protocols are presented below in figures 13 and 14. In both figures, the first two columns on the left portray the implementation of the fetuin-A as an end-point staining protocol aligned with the FDA staining, on the green and red fluorescence channels combined, in regular culture and calcification mediums. The remaining two columns present the Alizarin Red staining results of each cell line, under regular culture and calcification conditions. In Figure 13A – 13D, all the cell lines in DMEM culture medium conditions seemed to proliferate healthily as it is possible to confirm with the bright green signal, verifying a high number of FDA-positive cells. The IM1 and IM3 revealed an elongated morphology, the SaOs-2 cells a polygonal elongated shape and the OCCM a more rounded shape. The same results can be portrayed in the calcification conditions in IM1, IM3 and OCCM cells (Figure 13E, F and H), apart from the SaOs-2 cells (Figure 13G) where the visible green signal decreased drastically in comparison to the culture medium condition. In parallel, no Alizarin Red stain was observed in the four cell lines, under DMEM culture conditions (Figure 13I – 13L). In DMEM calcification medium, red staining was found in IM1 and IM3 cell lines (Figure 13M and 13N). No Alizarin Red Staining was detected in the SaOs-2 and OCCM cell lines, under calcification conditions (Figure 13O – 13P).

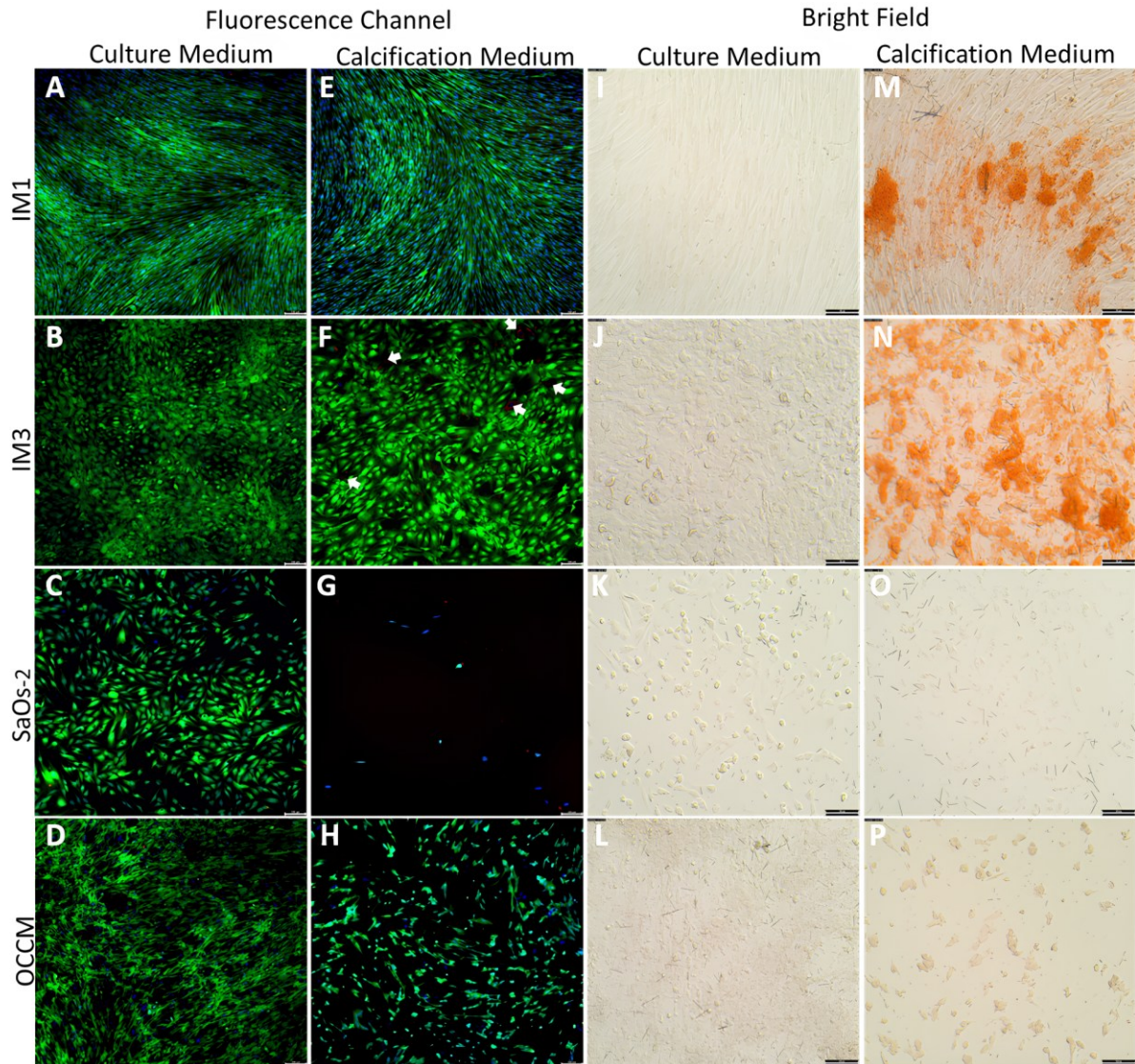


Figure 13: Live staining of IM1, IM3, SaOs-2, and OCCM cells over a period of seven days.

FDA, Hoesht and Fetuin-A staining of the 4 cell lines incubated in culture medium (A-D, I-L) and calcification medium (E-H, M-P). Live cells were stained with the colour green (FDA), cell nuclei with blue (Hoechst), and red for fetuin-A signal, and both medium were studied using the fluorescence channels (overlap of the green, blue, and red channels) available on the microscope. Significant calcification lesions were highlighted with white arrows. Calcification lesions were observed in the alizarin red staining protocol, from the fetuin-A staining with the bright field. The fetuin-A staining was used as an end-point staining protocol.

The last results (figure 14) portray the implementation of Fetuin-A as a continuous staining protocol during the seven days aligned with the FDA staining as an end-point protocol, to study the differential cellular responses of the professional mineralizers and the calcifying cells. So, in figure 14A – 14D, under DMEM culture conditions, all four cell lines revealed a similar number of FDA green positive cells as well as in Figures 14E – 14H, where the cells proliferated under calcification conditions. The bright field representative pictures illustrated the absence of Alizarin Red stain in vascular smooth muscle cells (Figure 14K, 14L, 14O and 14P) – IM1 and IM3 – and the professional mineralizers – SaOs-2 and OCCM (Figure 14I, 14J, 14M and 14N) under calcification and proliferation conditions.

Figure 13 and 14 displayed notable differences around the SaOs-2 cells as well as the Alizarin Red staining results in both vascular smooth muscle cells used (IM1 and IM3).

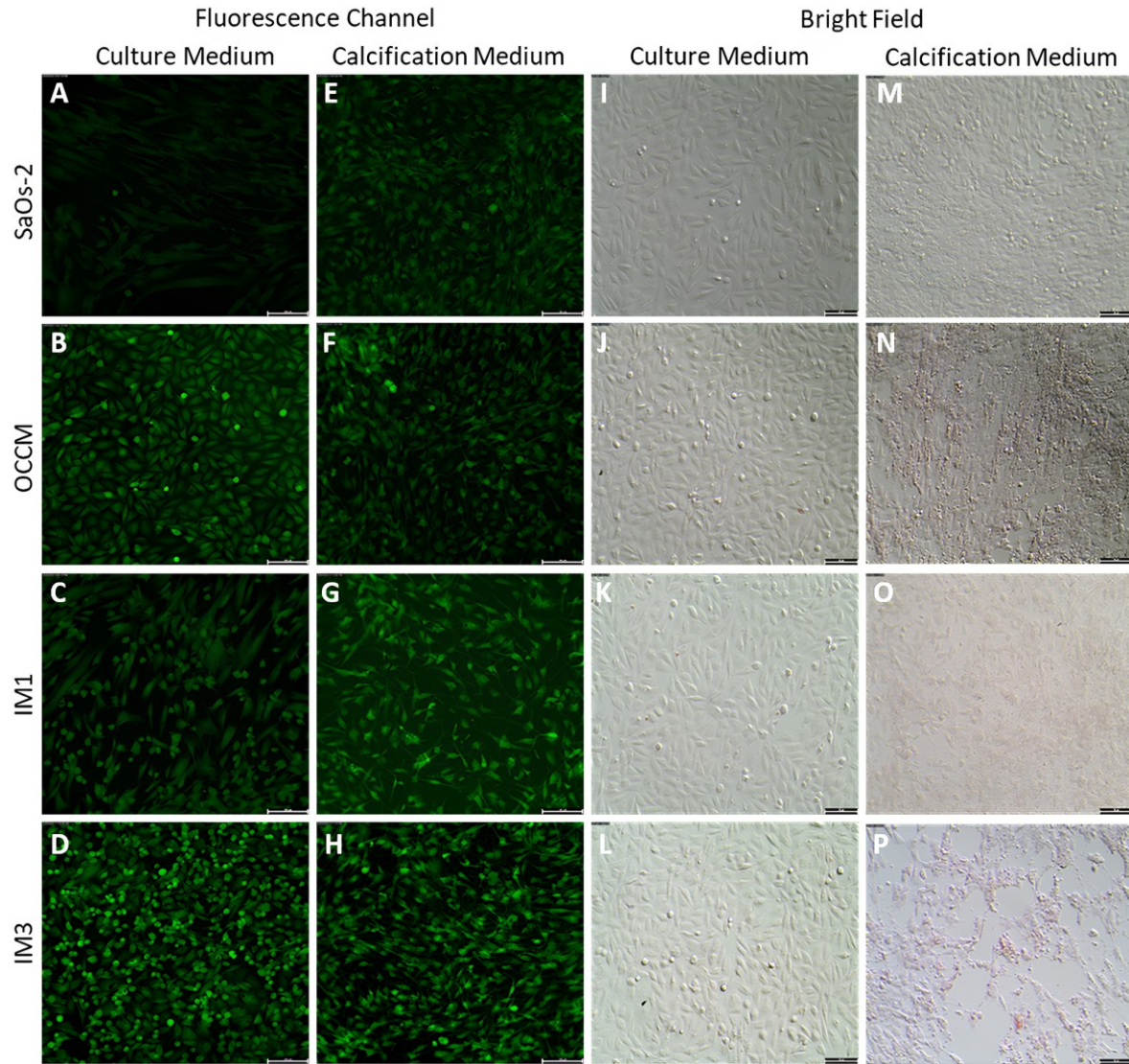


Figure 14: Live staining of IM1, IM3, Saos-2, and OCCM cells over seven days incubated with mFA-mRuby in the culture and calcification medium.

FDA and Fetuin-A staining of the 4 cell lines incubated in culture medium (A-D, I-L) and calcification medium (E-H, M-P). Live cells were stained with green (FDA), and red for fetuin-A signal, and both mediums were studied using the fluorescence channels (overlap of the green and red channels) available on the microscope. Significant calcification lesions were not observed. The existence of calcification lesions was verified in the alizarin red staining protocol with the bright field. The fetuin-A staining was used during cell culture in the medium and as an end-point staining protocol.

5. DISCUSSION

5.1 Production and Expression of Recombinant Fetuin-A Probes

Fetuin-A/ α_2 -Heremans Schmid (HS) is a negatively charged glycoprotein produced in the liver and released into the bloodstream. Human Fetuin-A is widely found in literature due to its involvement and contribution to various biological processes such as a strong anti-inflammatory effect as it is reported to be a potent crystal-bound inhibitor preventing the development of hydroxyapatite crystals (Jahnen-Dechent et al., 2011). The tertiary protein structure of Fetuin-A incorporates a calcium phosphate binding site that can bind calcium phosphate pre-nucleation clusters, amorphous calcium phosphate, and crystalline apatite (Brylka & Jahnen-Dechent, 2013). Therefore, Fetuin-A binds with high-affinity calcium phosphate and calcium carbonate, which inhibits the formation of new calcium-phosphate complexes without influencing the existent mineral deposits. These findings corroborated a deeper and thorough investigation into the correlation between this plasma protein, Fetuin-A, and the calcification of smooth muscle cells in the human body, culminating in health pathologies like atherosclerosis or CKD.

The present dissertation aimed to study the expression and production of two Fetuin-A variants, namely i) the expression and amplification of the plasmid of Fetuin-A variants in *Escherichia Coli* cells; ii) transfection in Chinese Hamster Ovary (CHO) cells and iii) purification and quantification of Fetuin-A variants produced. Thus, chicken-Fetuin-A and m-Fetuin-A-mRuby were strategically chosen for the present work. The chicken-Fetuin-A was synthesized as requested by Berlin BioGenes and, therefore employed to gain insights into the methodologies for optimizing high-yield protein production, in the initial phases of the study. The m-Fetuin-A-mRuby was employed in the experimental protocol as it facilitates i) daily monitoring during protein production in CHO cells (chapter 3.6.3), due to the transition from a colourless to an orange-coloured medium, indicating the expression of the desired protein; ii) an easy tracking of the target protein localization, due to the fluorescence intensity, during image acquisition via microscopy, that provides important insights into the concentration and distribution of the protein along the cell culture matrix.

Protein production required a deep understanding of each method employed and their fundamental role in achieving both high-yield expression of the desired proteins and a highly purified sample. Protocols like transfection in CHO cells, Western Dot Blot, IMAC chromatography, Spectrophotometer, SDS-Page, and Coomassie staining.

Both Fetuin-A plasmids were introduced into CHO cells, in the protocol known as transfection, to express the proteins desired and produce the recombinant proteins. CHO cells were the transfection system chosen due to their high-density suspension growth and their satisfactory yield and quality of recombinant protein expression (Z. M. Li et al., 2022). The Western Dot Blot (chapter 3.6.4) was undertaken after, revealing successful protein production during the five days. Given the laboratory's prior experience with protein expression in CHO cells of m-Fetuin-A-mRuby, this method was only executed with chicken Fetuin-A. As this was the first instance of the use of chicken Fetuin-A plasmid in the laboratory it was essential to analyze and track the progression of protein expression and production every day. Therefore, the results demonstrated a consistent increase in the output of the target protein, as desired.

In terms of the protein yield of each round of expression and protein production, the spectrophotometer results revealed that the two chicken Fetuin-A gels disclosed concentrations of 1,55 mg and 2,56mg per mL of elution, which indicated a total production of 3.1mg (Elutions 3 and 4, 1mL each) and 7,68 mg (Elutions 1,2 and 3, 1mL each) of chicken Fetuin-A, respectively. For the m-Fetuin-A-mRuby a concentration of 9,1mg per mL of elution was obtained, which results in a total production of 27.3mg of protein as three elutions (E3, E4, E5) of 1mL each were aliquoted. These findings implied a significantly higher yield of the m-fetuin-A-mRuby compared to the two rounds of chicken Fetuin-A expression. As the protein expression occurred in a mammalian cell line (Chinese hamster ovary cells) and the murine plasmid originated from a mammal, it likely exhibited a higher affinity with the CHO cell system than the avian plasmid (chicken plasmid). Therefore, that might have resulted in a better optimization and efficient recombinant production system, due to the closer phylogenetic relationship between the mammalian expression cell line and the mammalian gene in the murine plasmid, resulting in more efficient post-translational modifications. As described in the literature, CHO cells are commonly used in mammalian protein expression, further explaining the observed yield differences (Z. M. Li et al., 2022).

On the SDS-gel of recombinant m-fetuin-A-mRuby, theoretical molecular weight in the range of 70 to 90 kDa, given that ruby is also a protein, was set as a guiding reference, following the literature (Chekol Abebe et al., 2022). For the chicken Fetuin-A a smaller molecular weight in the range of 51 to 67 kDa was considered. Therefore, the three gels produced intended to separate the proteins in each sample based on their molecular weight, as evidenced by the satisfactory results illustrated in Figure 10. The Coomassie staining protocol subsequently employed permitted the visualization of the protein separation on the acrylamide gels from the previously performed SDS-PAGE.

The intensity of the staining in each band correlated directly to the protein concentration allowing the assessment of the protein expression levels and the purity of each sample. In the three gels, thicker prominent bands were noted around the MW range corresponding to the reference size for mFA-mRuby and chicken Fetuin-A. These bands were notably clearer than other proteins present, detected at significantly lower concentrations, that appeared as faint bands in higher and lower molecular weight in all three gels. The m-Fetuin-A-mRuby exhibited a lower purification level due to the presence of additional proteins from elution 2 to 5, alongside the target protein. However, the two chicken Fetuin-A gels revealed similar top-notch purification levels. These gels contrary to that of the m-Fetuin-A-mRuby sample, showed higher purified samples, characterized by a prominent concentration of the target protein and a faint presence of other proteins with distinct molecular weights. The lower level of purification might result from some setbacks during the immobilized metal affinity (IMAC) chromatography. Inefficient elutions, where the non-specific proteins remained in the samples, could have been one of the complications. Furthermore, a high concentration of non-target proteins may have interfered with the effective binding of the desired protein, but also with the elution of the unwanted proteins during their respective extract phase.

The outcomes of this study demonstrated a highly satisfactory alignment with the initial predictions, affirming the validity of the methodological framework employed. The level of congruency reflected the efficacy of each protocol implemented and paved the way for subsequent experimental procedures

5.2 Calcium-Phosphate Precipitation Inhibition Assay – Fetuin-A

The Calcium-Phosphate Precipitation Inhibition Assay is a method widely used to study how different substances affect delicate balances in biological mineralization. The assay uses conditions in which calcium and phosphate ions typically would precipitate to enable researchers to evaluate the efficacy of inhibitors such as proteins, polymers, or drugs in slowing or stopping crystal growth. The study of a substance's inhibition activity is vital for understanding the mechanism underlying the development of mineral precipitates. This insight is fundamental to generate techniques to prevent soft tissue calcification in blood vessels, for example, and for establishing the balance for normal maturation and mineralization. This assay has been valuable in studies of bone and mineral metabolism as well as in research on pathological calcification such as ectopic calcification.

The data obtained from spectrophotometric measuring was analysed using the GraphPad Software features (Figure 11) and provided information on the inhibitory potential of the Fetuin-A probes tested – Chicken Fetuin-A and the m-Fetuin-A-mRuby. The graph provided an important parameter to assess the protein effectiveness/affinity: the Turning Point also known as the IC_{50} value. This parameter gives a perception of the concentrations required for a prominent shift in the inhibition behaviour of the protein. The nomenclature IC_{50} translate to value of the half maximal inhibitory concentration and allows the determination of the inhibition potency of the probes tested, by indicating the concentration of protein needed to inhibit the growth of mineral crystals by 50%, based on the concentrations of calcium (5mM) and phosphate (3mM) added. The IC_{50} value endorsed the assessment of each protein efficacy, a lower IC_{50} value indicating that a smaller quantity of protein was required to achieve a half-maximal effect, thus suggesting an elevated potency. On the other hand, a higher IC_{50} value indicated a need for a higher amount of protein to achieve the same level of inhibition activity. The IC_{50} value was calculated with the GraphPad Software. According to the values acquired, the mFetuin-A-mRuby exhibited a IC_{50} value of 0.49 μ M, significantly lower than that of the chicken Fetuin-A, 3.88 μ M. Thus, the mFetuin-A-mRuby had a substantially higher inhibitory activity compared to the chicken Fetuin-A probe, further supporting the previously described hypothesis. Therefore, to achieve the same desired level of inhibition, a lower concentration of m-Fetuin-A-mRuby is necessary.

5.3 Cell Culture-based Calcification Assay

Following the production of m-Fetuin-A-mRuby, the next phase aimed to mimic the biological conditions present within the human body through a calcification assay. Four cell lines were chosen concretely for this assay. SaOs-2 (human osteosarcoma cell line) and OCCM (immortalized murine cementoblast cell line) were selected, due to their professional mineralizer activity. These cell lines are widely used as models for studying the bone formation, metabolism, and mineralization essential for bone tissue engineering (Declercq et al., 2004). Thus, they were ideal for mimicking the behaviour of professional mineralizing cells under the experimental conditions studied.

Vascular smooth muscle cells were chosen due to their capacity to alter their phenotype from contractile cells to osteogenic, under pathological conditions, characterized by the development of calcifying vesicles and consequent mineral deposits (Durham et al., 2018). These calcifying cells are associated with pathological conditions and are widely associated with diseases such as hypertension, chronic kidney disease, *diabetes mellitus*, or atherosclerosis, therefore VSMCs are ideal to investigate *in vitro* pathological calcification and to comprehend the mechanism and factors behind these complex biological processes. The ability to mimic calcifying conditions and utilize the cells directly associated with these biological events within the human body offers a more accurate approximation of *in vivo* human conditions. Also, the employment of two VSMC lines (IM1 and IM3) from different patients was a crucial strategy to enhance the reliability and accuracy of the results, better modelling of the calcification conditions, compare if the calcification response alters or not between patients and have more consistency, precise and well-founded outcomes.

Therefore, a cell culture-based assay was implemented, as this type of study is fundamental in biomedical research for in-depth study of biological processes, such as calcification, and bridging the gap between laboratory research and clinical applications. The opportunity to perform this assay with these four cell lines enables a more accurate and precise testing environment to assess therapeutic strategies to prevent, reverse, or eliminate calcifying deposits correlated to ectopic calcification.

Figures 13 and 14 illustrate the representative microscope pictures, with the green and red fluorescence channels and the bright field, of the four cell lines – SaOs-2, OCCM, IM1, and IM3 – under culture and calcification medium. The fluorescence channels enabled the visualization of the green signal emitted by the FDA staining, the red signal corresponding to the mFetuin-A-mRuby in combination with the fluorescence ruby. The bright field imaging provided a more refined view of the Alizarin Red Staining, as this method provides an overview of the staining outcome to be observed with the naked eye. These mosaic images were distinguished by the protocol adopted.

Figure 14 employed on the incorporation of mFa-mRuby as a component of the mediums. Both figures 13 and 14, the Fetuin-A end-point staining protocol (Chapter 3.8.2) was implemented.

In Figure 13, the fluorescence channels revealed a bright green signal in the culture medium, confirming FDA-positive cells in the four cell lines. This insight indicated the presence of viable cells within the wells of all four cell lines, thereby implying a healthy cell proliferation during the seven-day assay. However, the calcification medium appears to have a detrimental effect on SaOs-2, as evidenced by a lower number of FDA-positive cells and a faint green signal, highlighting a minimal cell density, in comparison to the outcome of the culture medium condition. A decrease in FDA-positive cells in the OCCM calcification medium culture was also detected, indicating possible cell apoptosis induced by the media composition or the hypothesis that the cells create shields connected to the extracellular matrix and are flushed away during the washing steps within the staining protocols. In the red fluorescence channel, SaOs-2 exhibited a very faint red signal in the form of specks only in the calcification medium, which may suggest the potential presence of calcifying lesions or calcified dead cells. On the other hand, the OCCM cells did not reveal any red mFa-mRuby signal, suggesting the nonexistence of microcalcifications in both conditions. Even though, SaOs-2, osteogenic cells and OCCM, highly differentiated mesenchymal cells of the periodontal ligament (PDL), are different cell lines, both contributing to biological processes related to mineralization, with the balanced deposition of calcium phosphate minerals in bone and cementum, respectively (Bao et al., 2013; Dvorakova et al., 2023). As cementum and bone have similar compositions of minerals, organic matrix, and water, that finding may justify their similar behaviour under calcification conditions and why diseases that affect the properties of bone often also affect the cementum. Therefore, for both cell lines physiologically higher concentrations of calcium and phosphate promote tissue mineralization, however, if these mineral levels are pathologically high the surrounding environment can become detrimental. As under pathophysiological conditions, phosphate and calcium plasma concentrations range from 2.85 – 2.95 mmol/L, those mineral levels can potentially initiate unbalanced mineralization, also known as calcification. Hence, the calcification assay (chapter 3.8) established a concentration of 3 mM phosphate and 4.3 mM calcium in the medium, significantly higher than pathophysiological conditions and approaching levels observed in disease states, which contemplate a phosphate concentration around 4.4mM (Bhargava et al., 2023). This excessive concentration of phosphate could have led to cell death and dystrophic calcification of dead tissues, as evidenced in ectopic calcification cases. Thus, as calcification and cell death are widely reported to have a strong correlation, the combination of 3 mM phosphate and 4.3 mM calcium may instead of triggering calcification, as expected, stimulate cell apoptosis.

This finding may explain the lower number of FDA-positive cells observed in the fluorescence results in the OCCM cells under calcification medium, the absence of viable SaOs-2 cells, and the presence of small red specks, that may indicate calcified dead cells. For this reason, an in-depth investigation of the effective and optimal combination of the phosphate and calcium concentrations would represent a significant advancement, enabling a proper study of active calcification with significant cell survival, without inducing calcification through cell apoptosis.

On the other hand, as expected, some prominent signs of the presence of possible calcifying lesions were observed in the vascular smooth muscle cells IM3. This hypothesis is based on the red signal observed in the representative image (Figure 13F), pointed with white arrows, that highlighted the m-Fetuin-A-mRuby potentially bonded to the calcification lesions, suggestive of its role in cell signalling and cellular interactions. Additionally, some red specks in the fluorescence image of the IM1 cells (Figure 13E) were detected, which may suggest the potential existence of microcalcifications. As the calcification medium has high levels of calcium and phosphate, these mineral concentrations exceed their solubility, mimicking pathological conditions. This high concentration of extracellular phosphate could lead to the inhibition of the production of calcification inhibitors and promote the production of calcifying extracellular vesicles, secreted by SMCs (smooth muscle cells), that aggregate and form microcalcifications. Bigger calcification lesions tend to arise from the continuous accumulation of microcalcifications and maturation of the minerals (Blaser & Aikawa, 2018; Goettsch et al., 2016; Hutcheson et al., 2016; Lee et al., 2020).

Thus, the actual existence of calcifying lesions could only be confirmed by the Alizarin red staining, as evidenced by the bright field results. As expected, none of the four cell lines revealed any staining, under culture conditions, therefore confirming the absence of calcified lesions has been predicted previously based on the fluorescence images. Given that the culture medium supplied all the minerals and nutrients beneficial for cellular proliferation, the cells were maintained in an optimal growth environment, which effectively didn't contribute to the development of microcalcification. Under calcification conditions, a clear distinction between the smooth muscle cells – IM1 and IM3 – and the professional mineralizers – SaOs-2 and OCCM - was witnessed. The Alizarin Red staining highlighted, with a red pigment, the calcified lesions present in the cell's wells. Smooth muscle cells revealed a presence of red colouring on the cells on the bright field, confirming the development of calcified lesions over the seven days of the assay, supporting the initial hypothesis proposed based on the fluorescence channel. Conversely, the absence of red colouring on the representative images in the SaOs-2 and OCCM cells indicated the nonexistence of calcification in these cells' lines.

This finding was previously corroborated by the absence of a red signal in the fluorescence channel pictures of the OCCM cells. The negative staining of the SaOs-2 cells supported the previous hypothesis, confirming that the red specks observed in the red fluorescence channel represent calcified dead cells, as the Alizarin red protocol exclusively stains mineral deposits.

In Figure 14, it's possible to verify high cell viability, according to the FDA green signal mostly homogenous in all four cell lines, again translating a healthy cell proliferation during the seven days of the assay, in both culture and calcification mediums. No significant differences were observed in the microscopy pictures, either between the four cell lines or across the two mediums. The absence of fluorescence signals in all the conditions and cell lines in the red channel might indicate the non-existence of calcified lesions, as the m-Fetuin-A-mRuby incorporated in the medium would have bonded to the microcalcifications and emitted a red signal. As the four cell lines were continuously in contact with the Fetuin-A probe throughout the seven-day experiment, this variable appears to have been a decisive condition, influencing the outcomes illustrated in Figures 13 and 14, particularly as the smooth muscle cells exhibited contrasting findings. VSMCs, described previously, secrete mineralizing vesicles, apoptotic bodies and calcifying extracellular vesicles, under calcification conditions.

Thus, the concentration of Fetuin-A (20 μ /mL) in the medium, during the seven days, appeared to protect these cell lines from the development of microcalcifications by preventing uncontrolled mineralization. Based on the literature, Fetuin-A seems to play a crucial role in regulating mineralization, by buffering excess extracellular minerals, as this protein is reported to be taken up from the medium into vesicular structures inside the VSMCs and to be present in matrix vesicles and apoptotic bodies in the extracellular environment. Fetuin-A prevents the uncontrolled mineralization of the VSMCs and the apoptosis of these cells by inhibiting mineral precipitation of calcium and phosphate (in high concentration in calcification medium) inside the intracellular vesicles. The main role of fetuin-A in the calcification assay is perceived as primarily stabilizing mineral phases intra- and extracellular vesicles till the mineral precursors, like calcium and phosphate, have reached the region of mineralization and therefore inhibited these vesicles mineralization (Brylka & Jahnen-Dechent, 2013). Therefore, this hypothesis may provide a plausible explanation for the absence of mFa-mRuby red signal in IM1 and IM3 fluorescence images (Figure 14I and Figure 14J). As the end-point staining uncovered that the lack of signalling, may indicate that the Fetuin-A probe did not bind to any type of calcifications. However, this aim can only be confirmed by the Alizarin Red Staining results. Therefore, the bright field images confirmed the identical results between calcifying cells – IM1 and IM3 – and the professional mineralizers – SaOs-2 and OCCM - under both conditions.

The absence of red colouring on the Alizarin Red staining confirmed the non-development of microcalcifications in neither condition over the seven days of the assay as corroborated by the hypothesis described in the fluorescence channel pictures.

Accordingly, the evident difference between Figures 13 and 14 might imply that the duration of contact between the cells and the higher concentration of Fetuin-A in the mediums appeared to influence cell viability, proliferation, survival, and especially the unfolding of microcalcification in the cells. These findings unveiled the impactful role of Fetuin-A as a potent calcification inhibitor.

6. CONCLUSION

Ectopic calcification is a pathologic condition described as the inadequate deposition of calcium-phosphate precipitate in soft tissues, which can have debilitating consequences, ranging from common vascular calcification to rare hereditary calcification. Although the pathophysiology of this condition resembles the physiological process of bone tissue mineralization, it is important to note that calcification and mineralization are fundamentally different processes, but both are characterized by highly complex regulatory mechanisms. Thus, the main objective of this thesis as the study of the behaviour of professional mineralizing cells – osteosarcoma cells (SaOs-2) and cementoblast (OCCM) and calcifying cells - vascular smooth muscle cells IM1 and IM3 - under proliferation conditions and, more importantly, under pathological calcification conditions. Understanding the molecular mechanisms and factors that trigger tissue transformation into calcified lesions, is essential for finding and developing effective treatment therapies. Over the past decade, the plasma glycoprotein α_2 -Heremans Schmid, also known as Fetuin-A, has come into the spotlight for investigation in the field of ectopic calcification, as being reported to be a calcification inhibitor.

To study the properties of this emerging protein, it was crucial to produce, purify, and assess the inhibitory activity of the protein probes: Chicken Fetuin-A (produced to deliver to Berlin BioGenes, for antibody production) and m-Fetuin-A-mRuby (to use as a protein staining). These methodologies enable the synthesis of a functional mFa-mRuby, for the upcoming tests. So, the calcification assays performed were critical for the interpretation of the impact of medium supplementation with higher concentrations of calcium, phosphate, and Fetuin-A. Those findings enabled an understanding of the correlation between calcification and cell survival, enhancing the importance of an in-depth investigation of the optimal combination of phosphate and calcium to properly investigate active calcification of professional mineralization cells without inducing cell death. Also, allowed a wider perception of the role of Fetuin-A and its impactful inhibition activity in ectopic calcification, by preventing mineral precipitation.

In the final analysis, the future of Fetuin-A as either a therapeutic agent or a diagnostic tool against ectopic calcification seems promising, as it aligns the natural role and function of this plasma carrier protein to its ability to control calcium and phosphate metabolism.

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7. ATTACHMENTS

7.1 Uniprot Protein Comparison



Figure 15: Blast Alignment of the amino acids of the protein chains of fetuin-A variants from mammals: Mouse, Cotja and two different protein chains lengths from chicken using the *Uniprot* Software.

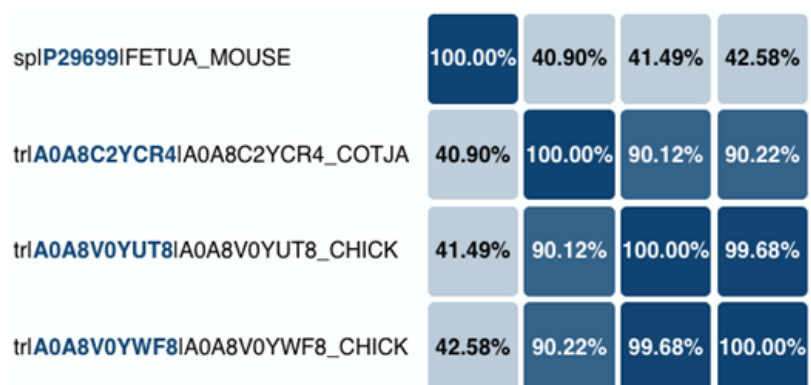


Figure 16: Percentage Identity Matrix from *Uniprot* Software of the four different fetuin-A protein variants from mammals: Mouse, Cotja and Chicken.

7.2 Chicken Fetuin-A Sequence 3D Image



Figure 17: Potential representation of a 3D-Model of the protein structure of Fetuin-A derived from chicken using the ChimeraX software.

7.3 Neubauer Chamber

A cell culture sample was taken from the Erlenmeyer flask and diluted by a factor of 1:10 with PBS. Trypan blue was used to determine the cell culture viability as it only colours dead cells blue, so the live cells could be counted. So, a dilution of 1:20 with trypan blue (trypan blue in PBS, 0.5% w/v) was performed. A sample of 7 μ L of cell culture + trypan blue was pipetted into the Neubauer chamber. The cells counted were inside the 4 squares represented (Figure 19). Subsequently, the number of cells in the Erlenmeyer flask was calculated with Equation 1.

Equation 1: $\frac{\text{cell count}}{\text{squares}} \times \text{Neubauer chamber factor} \times \text{dilution factor}$

Neubauer chamber factor = 10000

Dilution factor = 20, if trypan blue was used (1: 1)

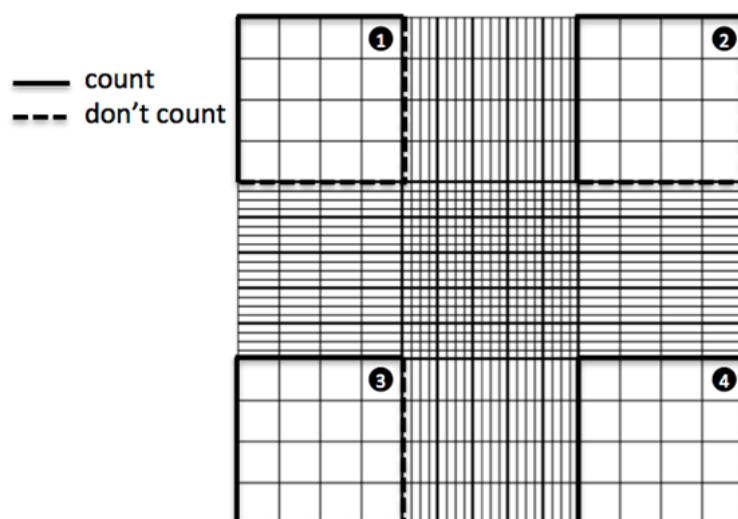


Figure 18:Neubauer Chamber Layout for counting live/dead cells.

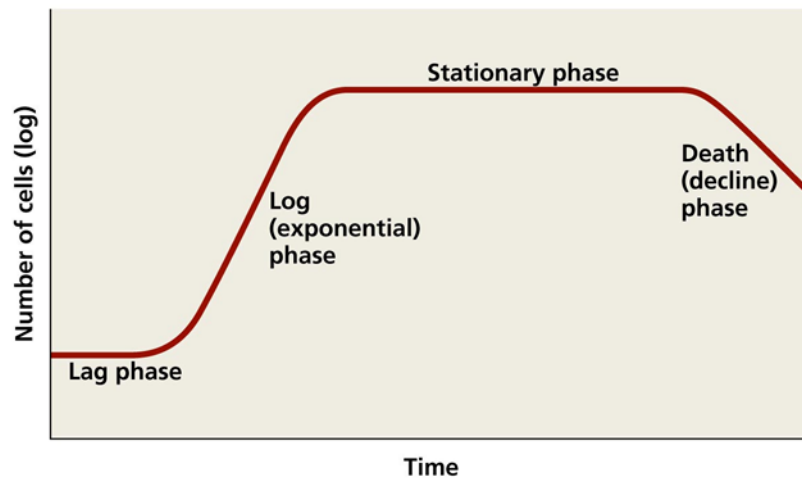
7.4 PureYield™ Plasmid Midiprep System

The PureYield™ Plasmid Midiprep System protocol was used to isolate and analyze plasmid DNA from *Escherichia coli* cells. In this study was important to quantify the production of chicken Fetuin-A plasmid by the *E. coli* cells. The following protocol was used.

- A 100 mL transformed *E. coli* bacterial cell culture grow overnight at 37°C and 250 rpm;
- Two 50 mL falcon were filled with cell culture and centrifuge at 5000 g for 10 minutes;
- The supernatant was discarded, and each pellet was resuspended in 3mL Cell Resuspension Solution;
- 3mL of cell Lysis Solution was added to each falcon. The mixture was gently inverted 3-5 times. Incubation for 3 minutes at room temperature;
- Centrifugation of the lysate at 15000 g for 15 minutes;
- The PureYield™ clearing and binding columns were stacked and the lysate was added to the column;
- Vacuum was added to allow the sample to go through the filter;
- 5 mL of Endotoxin Removal wash and 20mL of column wash solution were added consequently and a vacuum was applied;
- 1.5mL Eppendorf was placed in the base of the elution device with the cap open;
- 600μL of nuclease-free water was added to the DNA binding column and applied vacuum.

- The DNA was collected and saved.

7.5 Cell Culture Growth Curve Assay



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Figure 19: General representation of a Cell Growth Curve

7.6 Calcification Assay

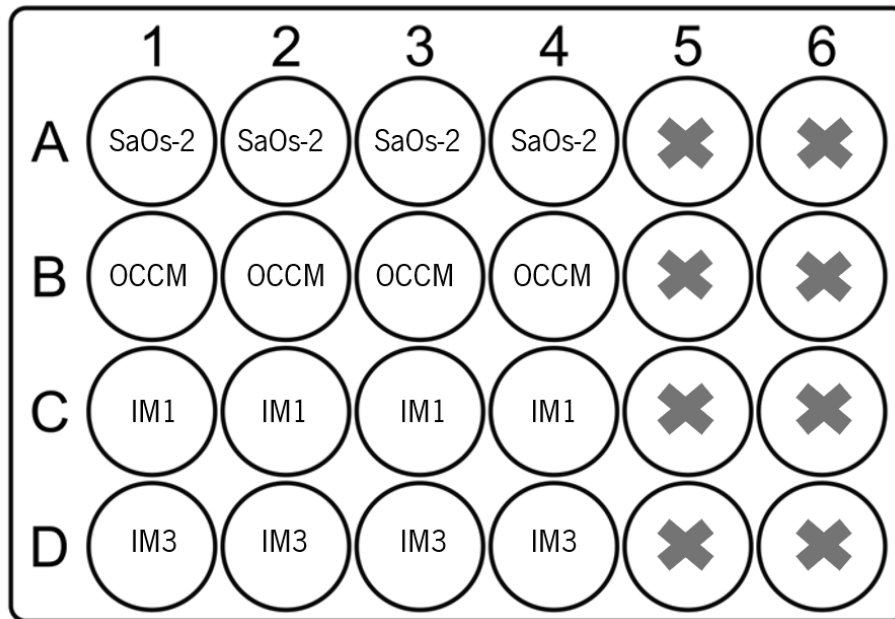


Figure 20: 24-well-plate display used for the calcification assay.

The cell lines used: osteosarcoma cells (SaOs-2), cementoblast (OCCM) and two different cell lines from vascular smooth muscle cells from two different patients (IM1 and IM3). Before the addition of the calcification medium the culture medium was used in all the wells. The calcification medium was added to all the wells on column 2 and 4. At the end the culture medium on column 1 and 3 was compared to the calcification conditions. The impact of the presence of Fetuin-A was studied as well, so in one of the 24-well plate the target protein was in contact with the cells during the seven-day experiment; in the second 24-well plate the Fetuin-A was used as an end-point staining on the last day of the study. Each Fetuin-A study was conducted in duplicate (chapter 3.8.1 and 3.8.2).