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Development and Characterization of a Nanoparticle-Loaded Thermoresponsive Hydrogel for Local TIMP-3 mRNA Delivery in Metastatic Melanoma

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Abstract

Metastatic melanoma represents one of the most aggressive forms of skin cancer, characterized by high invasive potential and profound alterations within the tumor microenvironment. In this process, a key role is played by silencing of tissue inhibitor of metalloproteinases-3 (TIMP-3), a tumor suppressor protein that regulates extracellular matrix remodeling (e.g. angiogenesis, apoptosis). This thesis was aimed at the development and characterization of an innovative local delivery system based on the introduction of polymeric nanoparticles into a thermoresponsive hydrogel to deliver TIMP-3 mRNA. The methodology involved the synthesis of pBAE nanoparticles (NPs) modified with oligopeptides (lysine and histidine) optimized for mRNA encapsulation. These NPs are integrated into a Poloxamer 407/188 hydrogel to transition from liquid to gel at body temperature, which is injectable and localized. Physicochemical characterization of NPs revealed good dimensions (~ 200 - 250 nm) and stability. Flow cytometry studies were carried out on murine melanoma cell lines (B16-F1 and B16-F10) and found that the "Hydrogel+NPs" system can release biologically active nanoparticles in a gradual and continuous way for up to 72 hours. Through confocal microscopy, we observed effective cellular internalization and unexpected nuclear localization of TIMP-3, suggesting a role in intra-nuclear modulatory activity. Finally, Western blot analysis confirmed the expression of TIMP-3 protein in glycosylated form (~ 35 kDa), demonstrating that the delivered mRNA is fully functional. We have demonstrated that the combination of hydrogel and pBAE nanoparticles is an attractive platform for local gene therapy, which can sustain protein expression over time and overcome the limitations of conventional systemic treatments for melanoma.

Table of Contents

List of Tables	IV
List of Figures	V
1 Introduction	1
1.1 Melanoma	1
1.1.1 Epidemiology and risk factors	2
1.1.2 Tumor progression and metastasis	3
1.1.3 Current treatments	8
1.2 Tumor microenvironment and extracellular matrix remodeling	9
1.2.1 ECM and progression of melanoma	10
1.2.2 Matrix metalloproteinases (MMPs) and tissue inhibitors (TIMPs)	13
1.3 Role of TIMP-3 in melanoma	13
1.3.1 Structure and operation of TIMP-3	14
1.3.2 TIMP-3 as a regulator of tumor progression	16
1.3.3 RNA-based Therapeutic Strategies Targeting TIMP-3 in Melanoma	18
1.4 Polymeric NPs as a drug delivery systems	18
1.4.1 Polymeric nanoparticles for nucleic acid delivery	19
1.4.2 Poly(Beta-amino ester)(pBAE) NPs	20
1.4.3 Oligopeptide end-modified pBAE nanoparticles	21
1.5 Thermoresponsive hydrogels for controlled release	22
1.5.1 Hydrogels as local delivery systems	22
1.5.2 Poloxamer 188/407	24
2 Aim of Thesis	26
2.1 Main Objective	26
2.2 Specific Objectives	26

3	Materials and Methods	28
3.1	Materials	28
3.2	Cell lines	28
3.3	Methods	29
3.3.1	Production of NPs	29
3.3.2	Preparation of Poloxamer 188/407 hydrogel	30
3.3.3	Qualitative of protein expression by Confocal microscopy . .	30
3.3.4	Protein quantification by BCA assay	32
3.3.5	Titration of primary antibody(Dot Blot)	33
3.3.6	Western blot	33
3.3.7	Flow cytometry	36
4	Results and discussion	37
4.1	DLS characterization of nanoparticles	37
4.2	Validation of the Hydrogel+NPs Delivery System by Flow Cytometry Using mCherry	38
4.3	Evaluation of intracellular TIMP-3 expression by confocal microscopy	43
4.4	Western Blot	48
5	Conclusions	57
	Bibliography	65

List of Tables

1.1	Clinical features of common nevi, atypical nevi, and melanoma . . .	3
3.1	Experimental setup for confocal microscopy treatments and staining.	31
4.1	DLS characterization of KH-pBAE nanoparticles.	38
4.2	Protein concentrations and sample preparation for Western Blot analysis of B16F10 lysates collected after 24 h.	49
4.3	Protein concentrations and sample preparation for Western Blot analysis of B16F10 lysates collected after 48 h.	49
4.4	Protein concentrations and sample preparation for Western Blot analysis of B16F1 lysates collected after 48 h.	49

List of Figures

1.1	Schematic representation of skin structure showing epidermis, dermis, and the location of melanocytes in the basal layer [1].	2
1.2	MAPK, PI3K-AKT signalling, and cell-cycle regulation under normal conditions permits balanced control of basic cell functions [2]. . . .	5
1.3	In melanomas, genetic alterations lead to constitutive pathway activation with loss of cellular homeostasis [2].	5
1.4	(C) The PD-1–PD-L1 immune checkpoint is primarily regulated by interferon- γ signalling [2].	6
1.5	The progression of cutaneous melanoma from benign nevi to metastatic disease. The change from radial to vertical growth is associated with more invasiveness and tumor angiogenesis leading to dissemination by blood vessels. Adapted from [18].	7
1.6	Stages of cutaneous melanoma progression from localized disease to metastatic spread. Early stages are confined to the epidermis, while advanced stages involve lymph nodes and distant organs. Created with BioRender.com.	8
1.7	Structure and organization of the extracellular matrix in connective tissue with key components such as collagen fibers, elastin, proteoglycans and glycoproteins. Reproduced from [27].	11
1.8	Comparison between normal extracellular matrix (ECM) and increased matrix stiffness in cancer. Tumor-associated ECM is characterized by increased collagen deposition, cross-linking, and altered cell–matrix interactions, promoting tumor progression and invasion [30].	12
1.9	Predicted three-dimensional structure of TIMP-3 highlighting the spatial organization of the protein and the location of selected amino acid residues and variants. Adapted from [42].	15

1.10	Schematic representation of the main tumor-suppressive functions of TIMP-3 in cancer progression, including inhibition of angiogenesis, invasion, metastasis, EMT, and promotion of apoptosis. Adapted from [54].	17
1.11	Schematic representation of polymeric nanoparticle-mediated nucleic acid delivery, including cellular uptake, endosomal escape, intracellular trafficking, and release of genetic material. Adapted from Bishop et al., 2015 [63].	19
1.12	Chemical structure of a poly(<i>beta</i> -amino ester) (pBAE) polymer. The backbone contains ester linkages and amine groups (R), allowing nucleic acid complexation [70].	21
1.13	Schematic representation of oligopeptide end-modified pBAEs (OM-pBAEs) forming polyplexes with nucleic acid cargo. Terminal modifications (R, K, H, D) confer specific functional properties including charge, endosomal escape, and colloidal stability. Image created with BioRender.	21
1.14	Schematic representation of a nanoparticle-loaded hydrogel, showing the polymeric network, encapsulated nanoparticles, drug molecules, and mechanisms of release via diffusion, polymer degradation, and swelling Created by Biorender.	23
1.15	Generic chemical structure of Poloxamer 188/407, showing the ABA triblock with hydrophilic PEO blocks and hydrophobic PPO block (adapted from Chen et al., 2024 [79]).	24
1.16	Chemical structures of Poloxamer 407 and Poloxamer 188 showing the ratio of PEO and PPO units [81].	24
3.1	Schematic representation of the hydrogel preparation steps. I: weighing poloxamers; II: addition of 10X PBS; III: addition of MilliQ water; IV: stirring at 4°C for 72 hours. Created by Biorender	30
3.2	Schematic representation of the Transwell setup used for hydrogel and nanoparticle treatments. The inset shows the hydrogel with embedded nanoparticles. Created by Biorender	31
3.3	Schematic representation of the “blot sandwich” assembly for protein transfer. The figure illustrates the order of layers from the clear cassette face to the black cassette face, including fiber pads, filter papers, SDS-PAGE gel, and the PVDF transfer membrane [85]. . .	35
4.1	Flow cytometry analysis of mCherry expression in B16F10 cells treated with free mCherry-loaded nanoparticles and Hydrogel+mCherry NPs at 24 h and 48 h. Data are presented as mean $\pm$ SD.	39

4.2	Flow cytometry analysis of mCherry expression in B16F10 (A) and B16F1 (B) melanoma cells treated with free mCherry-loaded nanoparticles or Hydrogel+mCherry NPs at 24 h, 48 h, and 72 h. Data are presented as mean $\pm$ SD.	41
4.3	Flow cytometry analysis of mCherry expression in B16F10 (A) and B16F1 (B) melanoma cells treated with free mCherry-loaded nanoparticles or Hydrogel+mCherry NPs at 24 h, 48 h, and 72 h using six experimental replicates for the Hydrogel+NPs condition. Data are presented as mean $\pm$ SD together with individual replicate values.	42
4.4	Negative control conditions used for confocal microscopy analysis of intracellular TIMP-3 localization in B16-F10 melanoma cells. . . .	44
4.5	Models of nuclear activities associated with metalloproteinase family proteins and their inhibitors. TIMP-3 may act as a transcriptional or proteolytic modulator, similarly to what has been documented for other members of the MMP/TIMP family (adapted from Jobin et al., 2017 [95]).	46
4.6	Confocal microscopy images of B16-F10 melanoma cells treated with TIMP-3-loaded nanoparticles. From left to right: DAPI nuclear staining (blue), CellMask staining (red), AF568 fluorescence associated with TIMP-3 detection (green), and merged channels. Scale bar: 20 μ m.	47
4.7	Confocal microscopy images of B16-F10 melanoma cells treated with Hyd+TIMP-3 NPs. From left to right: DAPI nuclear staining (blue), CellMask staining (red), AF568 fluorescence associated with TIMP-3 detection (green), and merged channels.	48
4.8	Dot Blot analysis was performed to optimize the dilution of the secondary antibody used for TIMP-3 detection. The primary anti-TIMP-3 antibody was maintained at a dilution level of 1:1000 and the secondary antibody was tested at dilutions of 1:1000 and 1:2000. Higher dilutions (1:5000 and 1:10000) did not result in detectable signals and were not included.	50
4.9	Western Blot analysis of TIMP-3 expression in B16-F10 cells after 24 h of treatment. (A) TIMP-3 immunodetection showing bands between 30 and 37 kDa, corresponding to the glycosylated forms of TIMP-3. (B) β -actin immunodetection used as loading control. (C) Densitometric quantification of the TIMP-3 signal normalized to the negative control. Quantification was performed on the band between 35 and 37 kDa	52

4.10	Western blot analysis of TIMP-3 expression in B16F10 cells after 48h. (A) TIMP-3 expression (B) Vinculin as a loading control (C) Densitometric quantification of TIMP-3 expression normalized to Vinculin and expressed relative to the negative control.	54
4.11	Western blot analysis and densitometric quantification of TIMP-3 expression in B16F1 cells after 48 h of treatment. Vinculin was used as the loading control. TIMP-3 expression levels are reported relative to the negative control.	56

Chapter 1

Introduction

1.1 Melanoma

Melanoma, also known as malignant melanoma, is a potentially serious form of skin cancer characterized by the uncontrolled growth of melanocytes, the cells responsible for pigment production [1]. Normal melanocytes are located in the basal layer of the epidermis, the outermost layer of the skin, and produce melanin, that protects skin cells from damage caused by ultraviolet (UV) radiation(**figure1.1**). The non-cancerous proliferation of melanocytes leads to benign lesions such as moles (melanocytic nevi) and freckles, whereas their malignant transformation results in the development of melanoma. From a clinical point of view, melanoma can be classified into different stages: it is defined as in situ when it is confined to the epidermis, invasive when it penetrates the dermis, and metastatic when it spreads to other tissues of the body. Skin cancer is generally divided into two main categories: melanoma and non-melanoma skin cancers. Although melanoma is the most common type of skin cancer of skin cancer, it is highly aggressive, with a greater ability to invade surrounding tissues and spread to other organs. When melanoma originates in the skin, it is referred to as cutaneous melanoma, which is the most common form; however, it can also develop in other locations, such as mucosal membranes that line internal surfaces like the lips. Once melanoma progresses and spreads to other parts of the body, it rapidly becomes a highly lethal disease [2].

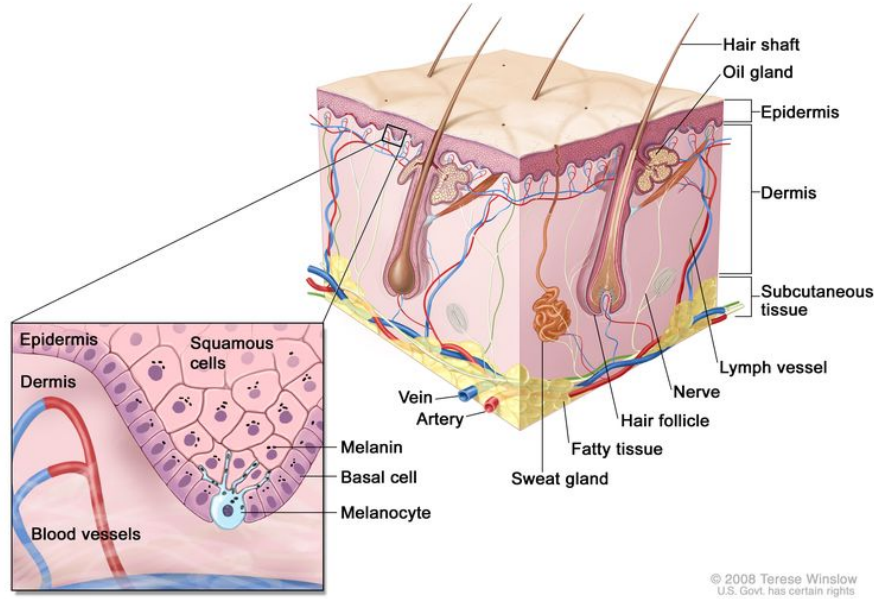


Figure 1.1: Schematic representation of skin structure showing epidermis, dermis, and the location of melanocytes in the basal layer [1].

1.1.1 Epidemiology and risk factors

Compared to other cancers, the incidence of cutaneous melanoma (CM) is increasing at a significantly faster rate [3]. According to data from the American Cancer Society, approximately 100,640 new cases and 8,290 deaths related to melanoma were estimated in the United States in 2024 [4]. The epidemiological data indicates that melanoma incidence is influenced by age and sex. The incidence increases with age with the highest number of new cases between 65 and 74 years and a median age at diagnosis of 64 years [5]. Sex is also different from age: Melanoma is more common in females in younger age groups and males have a higher incidence after 55 years [6].

Melanoma is a complex disease, and its development is associated with an interplay of environmental, genetic, phenotypic and behavioral factors. Exposure to ultraviolet radiation (UVR) is the major environmental risk factor with about 60-70% of melanoma cases [7]. UV radiation causes DNA damage in different ways such as direct DNA modification and accumulation of ROS, oxidative stress, and inflammatory reactions such as macrophage and neutrophil infiltration. Malignant melanocytes are formed from these processes [8].

Phenotypic characteristics also play an important role in melanoma susceptibility. Individuals with fair skin, light-colored eyes, blonde or red hair, and a tendency to freckle or burn have a significantly higher risk of developing melanoma [3]. This

increased susceptibility is mainly due to lower levels of protective eumelanin and a higher amount of pheomelanin, which is more sensitive to UV damage [6]. The presence and number of melanocytic nevi are amongst the most important clinical risk factors. Moles can represent either a precursor lesion or a strong marker of melanoma risk (**Table 1.1**). About 25% of melanomas develop from pre-existing nevi, and nearly 80% of patients report changes in existing lesions [6]. In addition, the total number of nevi, especially atypical or dysplastic nevi, is directly related to melanoma risk: the higher the number, the greater the risk of developing the disease.

Table 1.1: Clinical features of common nevi, atypical nevi, and melanoma

Clinical Feature	Common Nevi	Atypical Nevi	Melanoma
Size	Usually <5 mm	Varies, often >5 mm	Varies, may be >10–15 mm
Shape	Round; well-defined borders	Irregular; ill-defined borders	Irregular; notched borders
Color	Even	Variable; shades of brown; may include very dark regions; erythema often present	Variable; shades of brown to black; may also include red (inflammation), white (regression), and blue (pigment deep in dermis)

Genetic factors also play a role in melanoma risk. Mutations in key genes like BRAF and NRAS happen to be found in about 60% and 15-20% of melanomas, respectively [9], and although these mutations do not cause melanoma themselves, they can affect susceptibility when combined with environmental factors such as UV exposure. Melanoma has a high mutation rate as an outcome with up to 100 mutations per megabase, which makes it one of the cancers with the highest mutation rates [10].

Family history is another important risk factor. People from families with a genetic predisposition have a 5-10% increased risk of melanoma. Immunosuppression also has been found to be a factor in melanoma development.

Finally, the anatomical presentation of melanoma is influenced by environmental exposure patterns. Melanoma is more common in areas exposed to intense and intermittent sunlight, such as the back in males and limbs in females [11].

Early exposure to high-intensity ultraviolet radiation is also an important risk factor for melanoma development.

1.1.2 Tumor progression and metastasis

The malignant transformation of melanocytes into metastatic melanoma is the result of a complex process that involves a close interaction between exogenous

and endogenous factors, as well as between tumor-intrinsic factors and components of the immune system. Although melanocytes divide rarely (less than twice per year) [12], the proliferative index progressively increases during the evolution of melanocytic neoplasms, accompanied by a continuous accumulation of point mutations and copy number alterations [13].

Comparative studies have shown that cutaneous melanomas have a high mutational burden (more than 10 mutations per megabase) and a strong signature of UV exposure. In particular, the C→T transitions associated with UVB and G→T transitions associated with UVA radiation are typical molecular signatures of UV-induced damage. Not only with direct DNA damage, UVA radiation can also cause mutations indirectly through free radicals produced by the interaction with melanin.

Apart from genetic changes, melanoma progression is also affected significantly by the tumor environment. Melanoma cells, stromal cells, endothelial cells, and immune cells all interact to accelerate tumor formation, invasion, and metastasis. Melanoma is able to evade immune surveillance through immune checkpoint molecules and immune-suppressive factors, as well as immune checkpoints and immunosuppressive factors, which can be produced by immune cells of the tumor microenvironment. In addition to the genetic changes, inflammatory conditions in the tumor microenvironment and chronic inflammation can promote angiogenesis and promote the survival and growth of malignant cells. These interactions are an important factor in the progression of the disease and the development of resistance to therapy.

Like other tumors, melanoma progression is a sequential genetic process that results in activation of key oncogenic pathways. The activating *BRAF*^{V600} mutation is already present in benign nevi, while other mutations, such as those in the telomerase reverse transcriptase (TERT) promoter, are required to progress to intermediate lesions and melanoma in situ. Acquisition of invasive capacity requires additional mutations in genes involved in cell cycle regulation (CDKN2A) or chromatin remodeling (ARID1A, ARID1B, ARID2), and metastatic disease is associated with mutations in PTEN and TP53. At the protein level, these alterations lead to the hyperactivation of key signaling pathways, particularly the MAPK pathway and the PI3K/AKT/mTOR pathway [2] (**Figure 1.2**). Melanoma cells are also able to evade the immune system through several mechanisms. A central role is played by the activation of immune checkpoints. The interferon pathway, together with the JAK and STAT pathways, regulates the expression of the PD-1 immune checkpoint. Following the recognition of tumor antigens by T lymphocytes, interferons induce the expression of PD-L1 and PD-L2 on tumor cells (**figure1.3**); the binding of these ligands to PD-1 leads to suppression of T-cell effector activity and inhibition of the antitumor immune response (adaptive immune resistance) [14]. Additional immunosuppressive mechanisms include the downregulation of

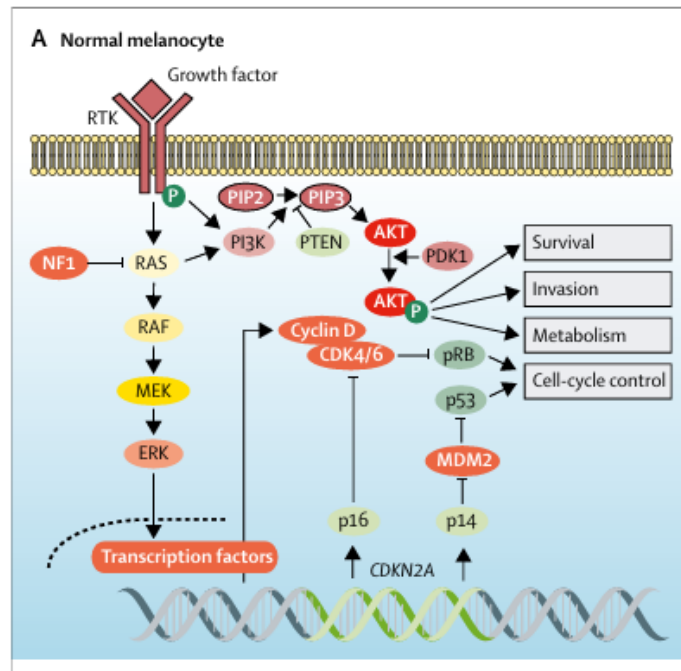


Figure 1.2: MAPK, PI3K-AKT signalling, and cell-cycle regulation under normal conditions permits balanced control of basic cell functions [2].

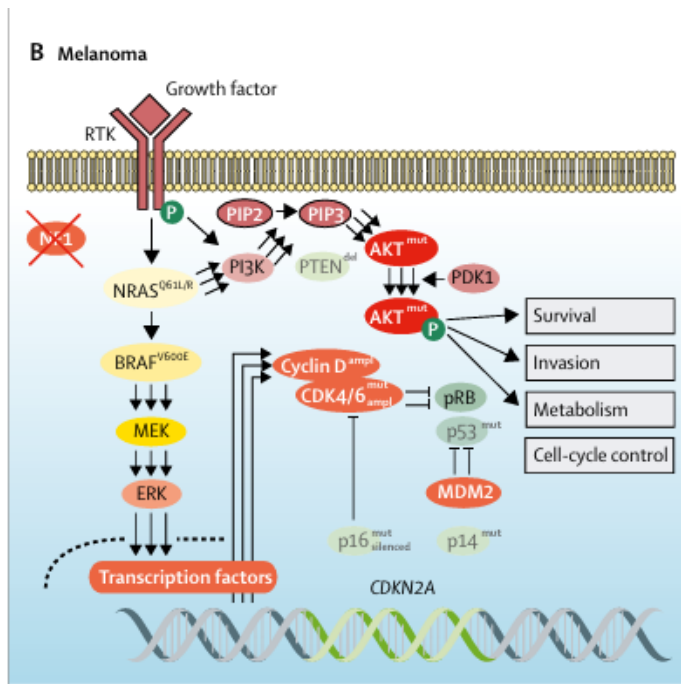


Figure 1.3: In melanomas, genetic alterations lead to constitutive pathway activation with loss of cellular homeostasis [2].

tumor-associated antigens and major histocompatibility complex class I (MHC-I), as well as the secretion of inhibitory factors such as transforming growth factor beta (TGF- β) [15].

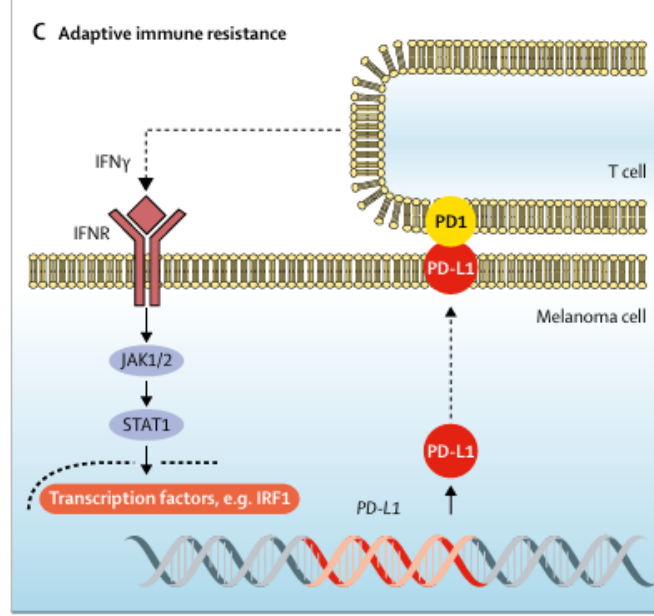


Figure 1.4: (C) The PD-1–PD-L1 immune checkpoint is primarily regulated by interferon- γ signalling [2].

Metastasis represents the final outcome of this evolutionary process and consists of the dissemination of tumor cells to distant anatomical sites and their adaptation to new tissue microenvironments [16]. This process is regulated by a complex interaction between tumor cells, host factors, and homeostatic mechanisms. The metastatic process is multistep and includes several sequential phases: tumor proliferation, neovascularization, immune evasion, invasion of surrounding tissues, intravasation into blood or lymphatic vessels, survival in circulation, extravasation, and colonization of distant tissues [17]. Interactions between tumor cells and non-neoplastic stromal cells play a crucial role in the progression of the invasion–metastasis cascade [17]. A key mechanism underlying invasion and metastasis is a process similar to epithelial–mesenchymal transition (EMT). Although melanoma does not originate from epithelial cells, tumor cells acquire mesenchymal-like features, including increased migratory capacity, invasiveness, resistance to apoptosis, and enhanced production of extracellular matrix components [17]. During this transition, melanoma cells can undergo a reversible phenotypic switch between a proliferative state and an invasive state, contributing to tumor heterogeneity and the ability to adapt to different microenvironments [17]. Tumor invasion also requires interaction with the extracellular matrix and the

expression of adhesion molecules such as integrins, cadherins, and immunoglobulin superfamily molecules, which facilitate cell migration and adhesion [17]. At the same time, degradation of the extracellular matrix is a crucial step for tumor cell dissemination. Another fundamental element in melanoma progression is angiogenesis, defined as the formation of new blood vessels. Tumor vascularization consists of the development of microvessels in the dermis at the base of invasive melanoma and is associated with the vertical growth phase in thin melanomas [18] (**figure1.5**). Different degrees of vascularization have been described (absent, sparse, moderate, and prominent), with higher vascularization observed in thicker and ulcerated tumors [19]. Melanomas with prominent vascularization show greater tumor thickness and are associated with a higher risk of recurrence and mortality [19].

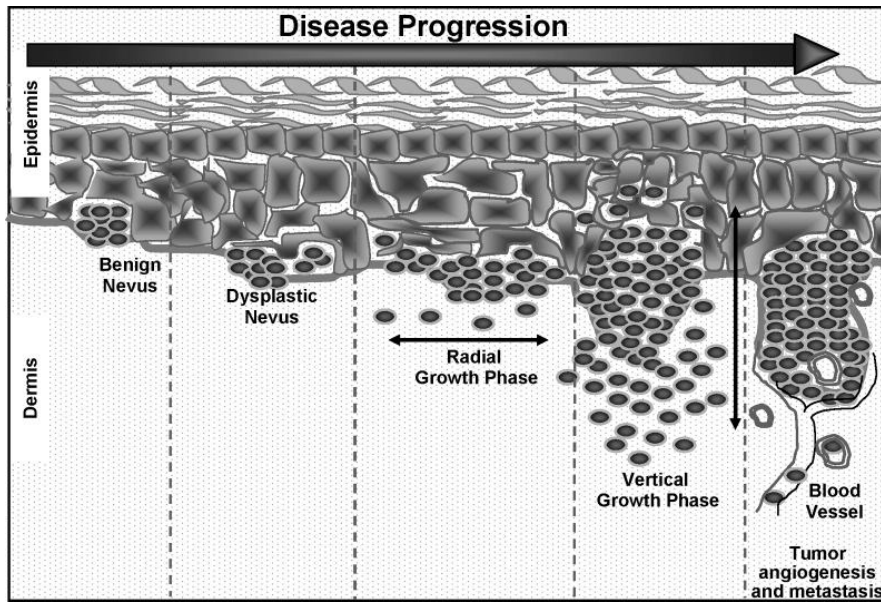


Figure 1.5: The progression of cutaneous melanoma from benign nevi to metastatic disease. The change from radial to vertical growth is associated with more invasiveness and tumor angiogenesis leading to dissemination by blood vessels. Adapted from [18].

Angiogenesis is mainly regulated by vascular endothelial growth factor (VEGF), which promotes the formation of new blood vessels and supports tumor growth and dissemination [17]. In addition, tumor cells may directly contribute to vascular structure formation through processes such as vascular mimicry. In addition to the classical model of dissemination through the vascular system, alternative metastatic mechanisms have been proposed, such as angiotropism, in which tumor

cells migrate along the external surface of blood vessels without entering the circulation, representing an additional mode of melanoma spread [17]. From a clinical perspective, melanoma progression is described by the American Joint Committee on Cancer (AJCC) staging system, which represents the main tool for disease classification and prognostic evaluation. Based on the TNM classification, this system considers the characteristics of the primary tumor, lymph node involvement, and the presence of distant metastases. Melanoma is classified into stages ranging from 0 to IV (**figure1.6**). Stage 0 corresponds to melanoma in situ, confined to the epidermis. Stages I and II represent localized disease, mainly defined by tumor thickness and the presence of ulceration. Stage III is characterized by regional spread, including lymph node involvement or local metastases, while stage IV indicates distant metastasis and is associated with the worst prognosis.

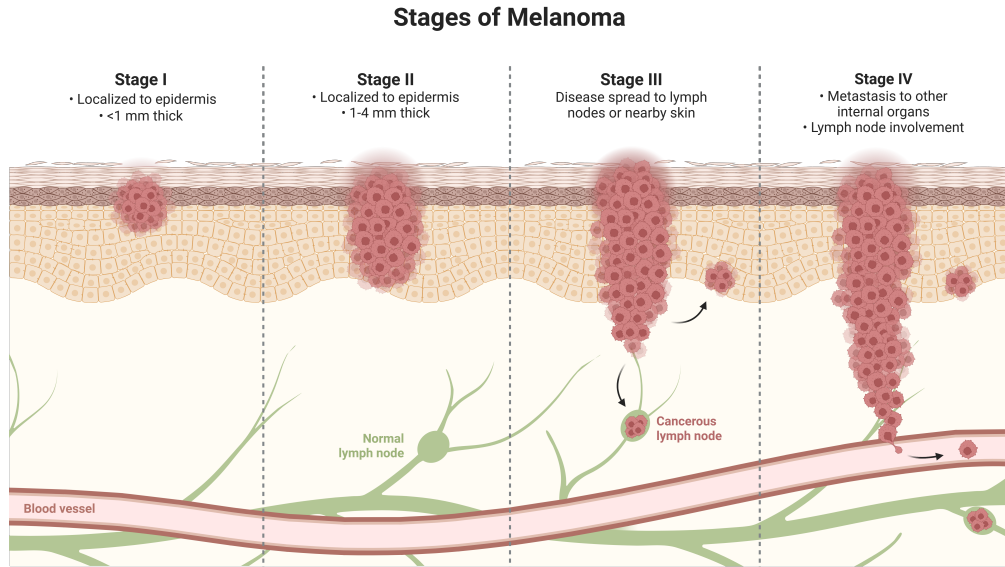


Figure 1.6: Stages of cutaneous melanoma progression from localized disease to metastatic spread. Early stages are confined to the epidermis, while advanced stages involve lymph nodes and distant organs. Created with BioRender.com.

As knowledge of melanoma biology has increased, staging systems have continuously improved. Since the 1990s, the introduction of lymphatic mapping and sentinel lymph node biopsy has represented a major advancement in the clinical management of melanoma [20].

1.1.3 Current treatments

The treatment of cutaneous melanoma strongly depends on the stage of the disease and requires a multidisciplinary approach. In recent years, significant therapeutic

progress has been achieved thanks to a better understanding of tumor genetic alterations and anti-tumor immune responses [21]. Targeted therapies are characterized by rapid responses, whereas immunotherapy is associated with more durable responses [22]. Surgery is the standard treatment for localized melanoma and consists of wide local excision of the primary tumor with appropriate margins [21]. In patients with advanced disease, mastectomy can be considered in selected cases, especially in oligometastatic disease [23]. Chemotherapy, especially dacarbazine, was previously used in advanced melanoma, but it has low response rates and limited survival benefit. Currently, it is limited to selected cases. Targeted therapies, with a focus on BRAF mutations, are a successful approach in about 40-50% of melanoma patients; however, the effectiveness of these treatments is limited by resistance, which typically occurs in a few months and is most likely to be less successful in the long term. Resistance mechanisms include reactivation of the MAPK pathway and activation of alternative pathways such as PI3K/AKT, which promote tumor survival and invasiveness [25]. Treatment resistance represents one of the major challenges in advanced melanoma. In targeted therapy, resistance is associated with: reactivation of oncogenic signaling pathways increased tumor plasticity EMT-like phenotype transition [25]. In immunotherapy, resistance is linked to immune evasion mechanisms and the presence of “cold tumors” with low T-cell infiltration. Strategies are being developed to overcome resistance, including combination therapies and approaches aimed at converting “cold” tumors into “hot” tumors. Radiotherapy is mainly used for palliative purposes, especially in patients with brain metastases [26] New therapeutic approaches are currently under investigation, including: tumor-infiltrating lymphocyte (TIL) therapy cancer vaccines new immune checkpoint inhibitors photodynamic therapy and nanotechnology-based approaches [26]. These strategies aim to overcome resistance mechanisms and improve clinical outcomes.

1.2 Tumor microenvironment and extracellular matrix remodeling

Tumor progression and metastasis formation do not depend only on the intrinsic properties of cancer cells, but are strongly influenced by their dynamic interaction with the surrounding microenvironment. The tumor microenvironment (TME) is a complex and highly organized system composed not only of tumor cells, but also of stromal components such as fibroblasts, immune cells, endothelial cells, and a network of soluble factors that regulate many biological processes. The TME plays a critical role in melanoma, in terms of tumour growth, invasion, metastatic potential and response to therapies. The extracellular matrix (ECM) is a key component. Traditionally thought of as a simple structural support, the ECM

is now considered a dynamic entity that influences cell activity with the help of biochemical and mechanical signals. Over the course of cancer, the ECM is continuously remodeled and changes its composition and organization; hence its metastasis process. A key role in these processes is played by specific proteolytic enzymes, particularly matrix metalloproteinases (MMPs), which are responsible for ECM component degradation. The activity of MMPs is regulated by endogenous inhibitors such as TIMPs (tissue inhibitors of metalloproteinases) that are the mediators of matrix synthesis and matrix degradation.

1.2.1 ECM and progression of melanoma

The extracellular matrix (ECM) is a dynamic 3D network of macromolecules and it serves as a structural support for tissues and thus plays an important role in regulating cell behavior. In addition to mechanical function, the ECM is a means to communicate between cells and the external environment and in certain cases cell adhesion, proliferation, differentiation and survival. Its composition is highly dynamic and can continuously change in response to biochemical and mechanical signals. From a structural point of view, the ECM is mainly composed of collagen, proteoglycans and glycosaminoglycans, elastin, laminins, fibronectin, and other glycoproteins. Collagen is the main structural component, while proteoglycans and glycosaminoglycans contribute to tissue hydration and the ability of the matrix to bind growth factors. Elastin provides elasticity to tissues, whereas fibronectin and laminins are involved in matrix organization and cell adhesion. The ECM is also organized into different compartments, including the pericellular matrix, which is directly involved in cell signaling and interactions, and the interstitial matrix, which mainly provides structural support. A specialized example of pericellular matrix is the basement membrane, which creates an adhesive and functional microenvironment for cells and contributes to tissue homeostasis [27].

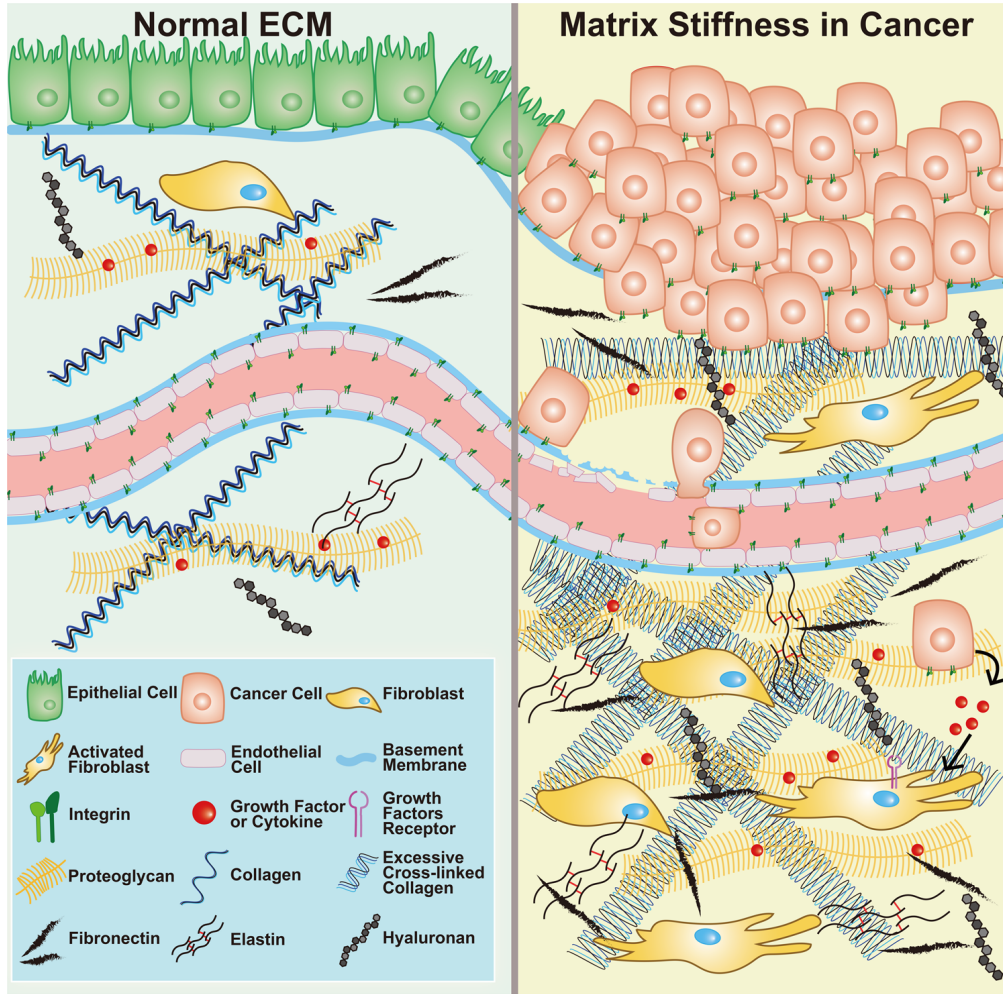


Figure 1.8: Comparison between normal extracellular matrix (ECM) and increased matrix stiffness in cancer. Tumor-associated ECM is characterized by increased collagen deposition, cross-linking, and altered cell–matrix interactions, promoting tumor progression and invasion [30].

A central role is played by the bidirectional interaction between tumor cells and the microenvironment. Melanoma cells not only respond to ECM signals, but also actively remodel it through interactions with cancer-associated fibroblasts and other stromal cells [31]. In this context, integrins mediate the adhesion to ECM components such as collagen, laminin, and fibronectin, activating signaling pathways that promote tumor cell migration and invasion [29]. In particular, the interaction with the basement membrane and its components is essential for the transition from the radial growth phase (RGP) to the vertical growth phase (VGP), which represents a key step in melanoma progression toward invasive and

metastatic disease [29]. These ECM changes have clinical implications. A stiffer and disorganized matrix not only promotes invasion and metastasis, but also leads to treatment resistance in that it limits drug penetration and modulates the immune response [32].

Finally, ECM remodeling is a central process in tumor progression and is closely related to the activity of specific proteolytic enzymes, particularly matrix metalloproteinases (MMPs) responsible for ECM degradation and tumor invasion [33].

1.2.2 Matrix metalloproteinases (MMPs) and tissue inhibitors (TIMPs)

In the tumor context, MMPs promote invasion by degrading the extracellular matrix and the basement membrane, creating a path that allows tumor cells to migrate and invade surrounding tissues, eventually leading to metastasis [34]. This process is not only structural, but also leads to the release of growth factors trapped within the matrix, contributing to cell proliferation and angiogenesis [34]. The activity of MMPs is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs), which control their proteolytic activity and contribute to maintaining matrix homeostasis [35]. However, in tumors, an imbalance between MMPs and TIMPs is observed, with increased proteolytic activity that promotes tumor invasiveness and progression [36]. Among the different MMPs, MMP-2 and MMP-9 are particularly important, as they are strongly associated with invasion and metastasis and are able to degrade type IV collagen of the basement membrane [37]. Among the TIMPs, TIMP-3 plays a particularly important role, as it is the only member of the family that is stably localized in the extracellular matrix. TIMP-3 has a broad inhibitory profile, not only against MMPs but also against other metalloproteinases such as ADAMs, which are involved in the regulation of key signaling pathways such as the MAPK pathway, frequently altered in melanoma [38]. In addition, TIMP-3 is known to promote apoptosis, further contributing to the control of tumor growth [39]. Loss of TIMP-3 expression has been associated with poor prognosis in several cancers, including malignant melanoma, where it acts as a tumor suppressor and a negative regulator of the metastatic cascade [40]. For this reason, therapeutic strategies aimed at increasing TIMP-3 expression may represent a promising approach for melanoma treatment.

1.3 Role of TIMP-3 in melanoma

The balance between matrix metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs) plays a crucial role in the regulation of tumor invasion. Among

the TIMP family members, tissue inhibitor of metalloproteinases-3 (TIMP-3) is of particular interest because of its unique characteristics and its role in controlling tumor progression [35]. Unlike the other TIMPs, TIMP-3 is strongly associated with the extracellular matrix, where it exerts a local and persistent regulation of proteolytic activity. In addition to inhibiting MMPs, TIMP-3 is involved in the regulation of several biological processes related to tumor progression, including angiogenesis, cell migration, and invasiveness. For this reason, TIMP-3 has been widely known as a tumor suppressor and its reduced expression is frequently associated with increased tumor aggressiveness [35]. Recently in melanoma, TIMP-3 expression has been found to decrease with disease progression showing a crucial role in the metastatic cascade. TIMP-3 levels are believed to be linked with more migratory and invasive abilities of melanoma cells, highlighting its negative role as a tumor control regulator. Overall, TIMP-3 represents a key mediator in the interaction between tumor cells and the tumor microenvironment, as well as a potential therapeutic target.

1.3.1 Structure and operation of TIMP-3

Among the tissue inhibitors of metalloproteinases (TIMPs), TIMP-3 belongs to a family of four endogenous inhibitors composed of TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Although these proteins were initially identified for their ability to inhibit matrix metalloproteinases (MMPs), they are now known to regulate a broader range of metalloproteinases, including members of the ADAM and ADAMTS families [41]. The different TIMP family members, TIMP-3 displays unique structural and functional properties. Unlike the other TIMPs, which are mainly soluble proteins, TIMP-3 is tightly associated with the extracellular matrix (ECM) through electrostatic interactions with sulfated glycosaminoglycans, particularly heparan sulfate (HS) and chondroitin sulfate (CS) [42] [43]. This characteristic allows TIMP-3 to remain localized at sites of proteolysis, exerting a local and prolonged inhibitory activity.

Structural human TIMP-3 is synthesized as a precursor protein containing a 23-amino-acid signal peptide required for cellular secretion [44]. The mature protein is composed of approximately 211–218 amino acids and is organized into two main domains: an N-terminal domain of approximately 120 amino acids and a C-terminal region of approximately 80 amino acids [41]. Its tertiary structure is stabilized by six disulfide bonds formed by twelve cysteine residues, generating a wedge-shaped conformation complementary to the catalytic site of target metalloproteinases [45] (figure1.9).

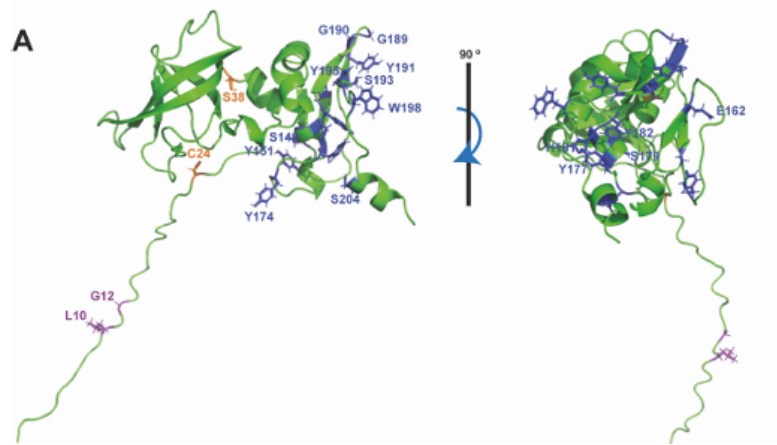


Figure 1.9: Predicted three-dimensional structure of TIMP-3 highlighting the spatial organization of the protein and the location of selected amino acid residues and variants. Adapted from [42].

The N-terminal domain is mainly responsible for the direct inhibition of metalloproteinases, whereas the C-terminal domain contributes to interactions with ECM components [42]. TIMP-3 exists in both unglycosylated (24 kDa) and glycosylated (30 kDa) forms; the glycosylated isoform is the predominant form in biological systems and is associated with greater stability and stronger ECM binding [46]. TIMP-3 is considered a multi-functional regulator of extracellular proteolysis, and in addition to several MMPs, such as MMP-2 and MMP-9, TIMP-3 is also able to inhibit ADAM10, ADAM17, and ADAMTS47. This broad inhibitory activity enables TIMP-3 to control ECM remodeling, the release of soluble bioactive factors, and several cellular signaling pathways.

ADAM proteases are responsible for ectodomain shedding, which results in the release of growth factors, receptors, and cytokines from the cell membrane. Through this mechanism, ADAMs contribute to cell proliferation, angiogenesis, and modulation of the immune response [47]. Therefore, inhibition of ADAM proteases by TIMP-3 contributes to the regulation of important signaling pathways involved in tumor progression. TIMP-3 also exerts an important anti-angiogenic activity through inhibition of VEGF-mediated signaling. In particular, TIMP-3 is able to interfere with VEGFR2 and other pathways involved in blood vessel formation [48].

Recent studies have shown that extracellular TIMP-3 availability is dynamically regulated by the balance between ECM binding and endocytosis mediated by low-density lipoprotein receptor-related protein 1 (LRP1) [42]. TIMP-3 can be internalized either alone or in complex with target metalloproteinases such as MMP-1 through specific interactions with LRP1 [49]. Moreover, binding of TIMP-3 to heparan sulfate can reduce LRP1-mediated endocytosis, increasing the extracellular

persistence of the protein [50].

1.3.2 TIMP-3 as a regulator of tumor progression

TIMP-3 plays a fundamental role as a regulator of extracellular matrix homeostasis and of the proteolytic processes involved in tumor invasion, angiogenesis, and metastasis. One of the main mechanisms through which TIMP-3 limits tumor progression is the inhibition of extracellular matrix degradation. MMPs are involved in the digestion of collagen, proteoglycans, and basement membrane components, allowing tumor cells to migrate and invade surrounding tissues. In particular, MMP-2 and MMP-9 are strongly associated with melanoma metastatic progression because of their ability to degrade type IV collagen of the basement membrane and promote tumor dissemination [51]. By inhibiting these proteases, TIMP-3 contributes to maintaining ECM structural integrity and limits the invasive ability of tumor cells.

The strong association of TIMP-3 with the extracellular matrix allows a local and persistent regulation of proteolytic activity at sites of tumor remodeling. This characteristic significantly contributes to its tumor-suppressive function within the tumor microenvironment.

TIMP-3 regulates the structure and remodeling of the matrix as well as tumor angiogenesis. Tumor growth and metastasis are determined by the development of new blood vessels that are controlled by the VEGF/VEGFR2 pathway. TIMP-3 acts as an inhibitor of angiogenic activity by blocking the interaction of VEGF with VEGFR2, thus limiting endothelial cell proliferation and migration and decreasing the supply of nutrients and oxygen to the tumor [52]. Also, reduced degradation of ECM limits the release of growth factors trapped in the extracellular matrix, which is related to tumor growth.

Another important aspect is the role of TIMP-3 in the regulation of apoptotic pathways. Many studies have shown that TIMP-3 overexpression mediates apoptosis in both in vitro and in vivo in different tumor cell lines, such as melanoma [53]. It seems to be mainly mediated by the stabilization of pro-apoptotic receptors involved in the extrinsic cell death pathway (such as TNF-R1, and TRAIL-R1), which activate caspase-8 and caspase-3 in this pathway. TIMP-3 is also the only member of the TIMP family able to consistently induce apoptosis in tumor cells, highlighting a distinctive biological function compared to the other metalloproteinase inhibitors (**figure1.10**).

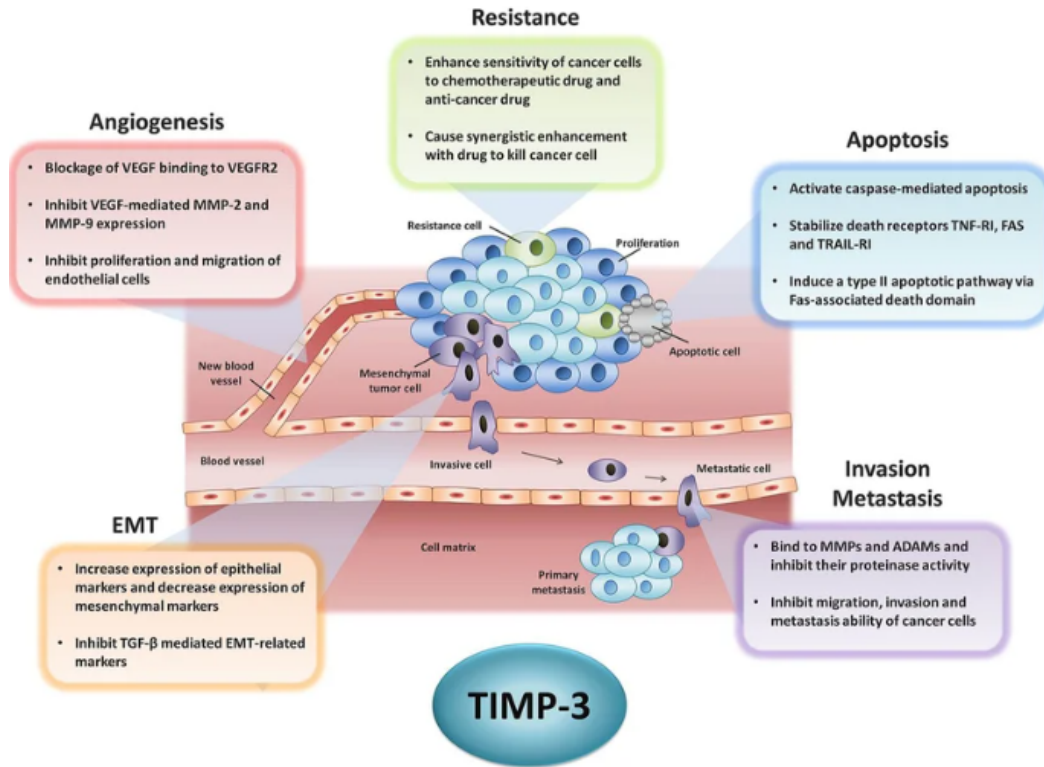


Figure 1.10: Schematic representation of the main tumor-suppressive functions of TIMP-3 in cancer progression, including inhibition of angiogenesis, invasion, metastasis, EMT, and promotion of apoptosis. Adapted from [54].

TIMP-3 also participates in the regulation of important inflammatory pathways through the inhibition of ADAM17, also known as TNF- α converting enzyme (TACE)[55]. ADAM17 is involved in the release of several pro-inflammatory molecules and growth factors from the cell membrane; therefore, its inhibition by TIMP-3 contributes to the modulation of the tumor microenvironment and to the reduction of pro-survival and pro-invasive signaling.

Loss or reduction of TIMP-3 expression has been associated with increased tumor aggressiveness and poor prognosis in several cancers, including melanoma. Lower TIMP-3 levels alter the proteolytic balance of the tumor microenvironment, resulting in ECM degradation, angiogenesis, cell migration and metastasis. Therefore, restoring TIMP-3 expression has been proposed as a promising antitumor therapeutic strategy. From the late 1990s to the early 2000s, several studies have examined adenovirus-mediated delivery to increase TIMP-3 expression in tumor cells, which has been shown to reduce tumor invasiveness and growth [56].

1.3.3 RNA-based Therapeutic Strategies Targeting TIMP-3 in Melanoma

The tumor microenvironment in metastatic melanoma is characterized by an upregulation of metzincin proteases (MMPs, ADAMs, ADAMTSs) and concurrent downregulation of TIMP-3, creating a proteolytic landscape that favors invasion, angiogenesis, and metastatic dissemination [56]. Due to the inherent instability of RNA molecules, strategies to modulate TIMP-3 expression, such as mRNA or siRNA therapeutics, require advanced delivery systems to protect the nucleic acid, facilitate cellular uptake, and ensure controlled intracellular release [55].

RNA-based approaches aim to restore TIMP-3 activity and reestablish proteolytic balance, suppress angiogenesis and induce apoptosis in melanoma cells through the extrinsic death-receptor pathway (TNF-R1, FAS, TRAIL-R1, caspase-8 and later caspase-3 activation [56]). By targeting TIMP-3 expression at the RNA level, these therapies provide a rationale for the subsequent use of polymeric nanoparticles for better stability, bioavailability and localization of therapeutic RNA in tumor tissue, resulting in reduced off-target effects and enhanced efficacy.

1.4 Polymeric NPs as a drug delivery systems

In recent years, nanotechnology-based drug delivery systems have emerged as promising strategies to overcome the limitations associated with conventional therapies. In particular, the delivery of drugs and genetic material faces several challenges related to their instability in biological environments, rapid degradation, and limited ability to efficiently reach the tumor site. For this reason, nanomedicines have attracted increasing interest due to their ability to improve the stability, targeting, and release efficiency of therapeutic compounds [57].

Nanocarriers like liposomes, dendrimers, metallic nanoparticles and polymeric nanoparticles have been developed for biomedical and therapeutic applications [58] [59]. The physicochemical properties of nanoparticles such as size, surface charge, and composition play an important part in their uptake, biodistribution, and therapeutic cargo release capacity in clinical applications. Of the different types of nanoparticle systems that are developed for drug and gene delivery, biodegradable polymeric nanoparticles have attracted particular attention because of their biocompatibility, versatile nature and their ability to change chemistry. Poly(β -amino ester)s (pBAEs) are considered as a promising platform for nucleic acid delivery since they can compress genetic material and encourage cellular internalization.

1.4.1 Polymeric nanoparticles for nucleic acid delivery

Polymeric nanoparticles represent advanced systems for the intracellular delivery of nucleic acids, such as DNA, mRNA, and siRNA, into tumor cells, including melanoma. These platforms protect genetic material from enzymatic degradation, enhance stability in biological environments, and allow controlled release of the therapeutic cargo [60].

The physicochemical properties of polymeric nanoparticles—such as size, surface charge, hydrophobicity, and polymer composition are crucial for efficient cellular uptake, penetration through the extracellular matrix, and proper cargo release [61]. Studies have shown that nanoparticles sized between 10 and 200 nm with positive surface charge enhance membrane adsorption and internalization via clathrin- or caveolae-mediated endocytosis, improving delivery efficiency [62]. A schematic representation of the main steps of polymeric nanoparticle-mediated nucleic acid delivery, including cellular uptake, endosomal escape, intracellular trafficking, and release of the genetic material, is shown in **figure1.11**.

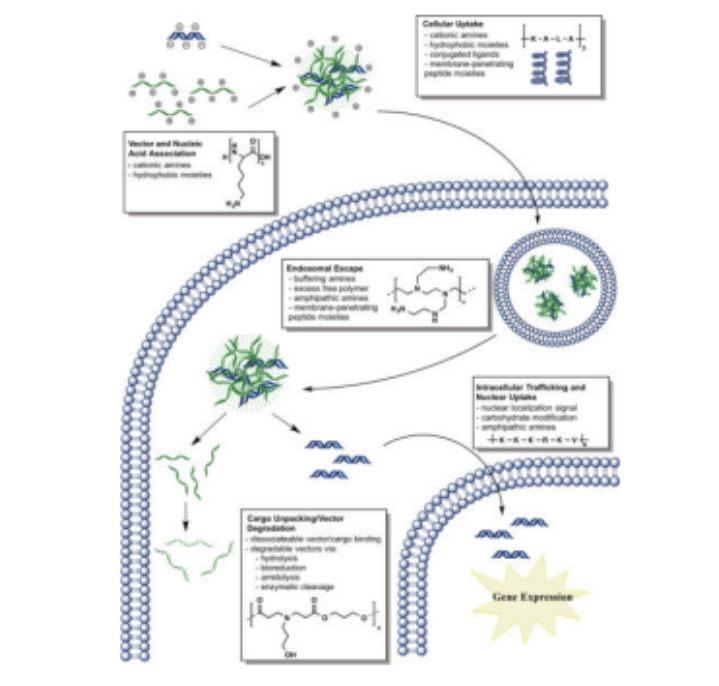


Figure 1.11: Schematic representation of polymeric nanoparticle-mediated nucleic acid delivery, including cellular uptake, endosomal escape, intracellular trafficking, and release of genetic material. Adapted from Bishop et al., 2015 [63].

Polymeric nanoparticles can exploit both passive targeting, via the enhanced permeability and retention (EPR) effect of solid tumors, and active targeting

through surface ligands or peptides, increasing specificity for melanoma cells while reducing uptake by healthy tissues [57]. Some systems are designed to release cargo in response to physiological stimuli, such as acidic pH, enzymatic activity, or redox stress in the tumor microenvironment [64].

The most studied polymers for nucleic acid delivery are poly(β -amino ester)s (pBAEs). These cationic polymers effectively complex DNA and RNA into stable nanoparticles, promoting cellular internalization and protecting the nucleic acids until intracellular release [65]. Terminal modifications with oligopeptides further improve targeting, cargo protection, and transfection efficiency in tumor cells [62].

Therapeutic advantages of polymeric nanoparticles include increased stability and bioavailability of genetic material, reduced systemic toxicity, the ability to co-deliver multiple types of cargo (drugs + nucleic acids), and controlled and sustained release of therapeutic agents [65].

1.4.2 Poly(Beta-amino ester)(pBAE) NPs

Poly(*beta*-amino ester) (pBAE) polymers are a class of biodegradable cationic polymers widely studied as vectors for nucleic acid delivery since their first use in 2000 by Lynn and colleagues [65]. Over the past twenty years, interest in pBAEs has grown due to their ability to modulate gene expression in immune and tumor contexts, showing preclinical efficacy in several gene therapy [66].

pBAEs are mainly synthesized via Michael Addition reactions between diacrylates and primary or secondary diamines, or through ring-opening polymerization. These methods allow fast, cost-effective, and scalable synthesis while enabling structural tuning of the polymer according to desired properties. The wide variety of available monomers provides high chemical versatility, which is crucial to optimize nucleic acid binding and release [67].

The structure of pBAEs (**figure1.12**) offers several advantages over other carriers. In particular, their pH-dependent charge promotes electrostatic interaction with nucleic acids under slightly acidic conditions, enhancing polyplex formation. The hexyl (C6) monomers in pBAEs can be biodegradable by hydrolysis or enzymatic cleavage by esterases which form low-toxicity degradation products for mammalian cells [68]. They also allow for freeze drying and redispersion without loss of functionality and better distribution of nanoparticles [69].

pBAEs are composed of a high number of protonable amino groups, which have a positive charge and enable rapid complexation with nucleic acids. This leads to the formation of nanoparticles with high encapsulation efficiency (>70%), optimizing both loading capacity and stability [70]. These properties make pBAEs ideal candidates for the delivery of a wide range of nucleic acids, including plasmids, siRNA, and microRNA, with direct applications in gene therapy and immunotherapy.

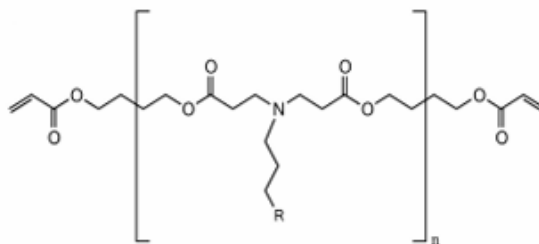


Figure 1.12: Chemical structure of a poly(*beta*-amino ester) (pBAE) polymer. The backbone contains ester linkages and amine groups (R), allowing nucleic acid complexation [70].

1.4.3 Oligopeptide end-modified pBAE nanoparticles

Oligopeptide end-modified poly(*beta*-amino ester)(OM-pBAE) polymers are a hybrid synthetic-biological platform designed to enhance hydrophilicity and cationic charge of pBAEs, improving nucleic acid encapsulation and transfection efficiency [71]. The choice of terminal oligopeptide (**figure1.13**) gives specific properties to the polymer: arginine (R) and lysine (K) modifications maintain a stable positive charge at physiological pH, promoting tight condensation of genetic cargo and efficient cellular uptake; histidine (H) modifications facilitate endosomal escape via the “proton sponge” effect; and aspartic acid (D) modifications reduce nonspecific interactions, improving colloidal stability [72].

Cysteine termination allows redox-sensitive crosslinking or site-selective conjugation, further enhancing nanoparticle stability and functionality. Studies have also shown that combining different OM-pBAE variants improves gene delivery efficiency, and adjusting the hydrophilic/hydrophobic balance of these polymers positively affects nanoparticle stability, loading capacity, and transfection performance [73].

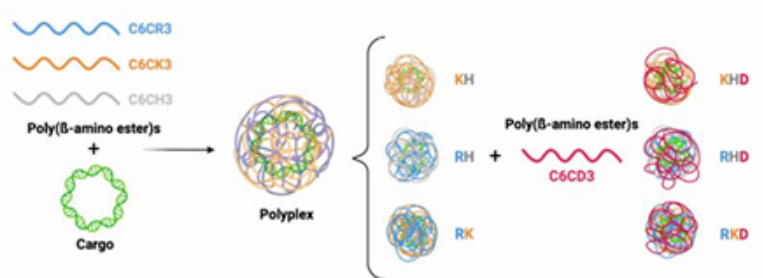


Figure 1.13: Schematic representation of oligopeptide end-modified pBAEs (OM-pBAEs) forming polyplexes with nucleic acid cargo. Terminal modifications (R, K, H, D) confer specific functional properties including charge, endosomal escape, and colloidal stability. Image created with BioRender.

pBAE nanoparticles offer a safe and flexible platform for nucleic acid delivery in melanoma, overcoming limitations of viral vectors [74]. Branched pBAEs efficiently delivered a Bax-encoding plasmid, inducing apoptosis with minimal cytotoxicity [75]. They also enabled STING agonist delivery, enhancing interferon responses and NK cell recruitment [76], and were used in mRNA vaccines to trigger antigen-specific T cell responses and tumor regression [76]. These studies highlight the potential of pBAEs and OM-pBAEs for melanoma immunotherapy.

Although the promising properties of pBAE nanoparticles for nucleic acid delivery, systemic administration presents some limitations. The particles can be rapidly degraded by plasma enzymes, cleared via renal or hepatic pathways, and may cause systemic toxicity due to their cationic charge [76]. In addition, untargeted release reduces the effective concentration of therapeutic RNA at the tumor site, limiting treatment efficacy [75].

For this reason, pBAE nanoparticles can be incorporated into thermoresponsive hydrogels, which allow local and controlled release of the therapeutic cargo, protect the RNA, and improve cellular uptake in tumor cells [74]. This strategy reduces off-target effects and maintains effective RNA concentrations at the site of interest, providing a natural link to the following section on hydrogel-based delivery systems.

1.5 Thermoresponsive hydrogels for controlled release

Therapeutic nanoparticles have also been incorporated into hydrogels, creating platforms for controlled drug release. Embedding the particles in the gel matrix forms a dual-barrier system, where the nanoparticles must first be gradually released from the network and then internalized by cells via endocytosis. This approach allows prolonged retention of the drug at the target site, modulation of release kinetics based on the gel's physicochemical properties, and reduced systemic side effects.

1.5.1 Hydrogels as local delivery systems

Hydrogels are soft materials composed of three-dimensional networks of hydrophilic polymers, capable of absorbing large amounts of water while maintaining structural integrity. Gelation can occur through reversible physical bonds sensitive to stimuli or through covalent chemical bonds, which provide greater mechanical stability [77]. Combining a tissue-like environment, biocompatibility, and tunable physicochemical properties, hydrogels have been widely used in 3D cell cultures, local drug delivery, wound dressings, and tissue engineering [78].

Stimuli-responsive hydrogels are particularly interesting for drug delivery applications [79]. Thermosensitive hydrogels can transition from a liquid state at room temperature to a solid gel at body temperature, allowing injection in situ and gel formation at the target site [63]. This makes them suitable for injectable applications, such as tissue regeneration and cancer treatment [80]. Local therapy allows drug delivery directly at the affected site, avoiding systemic side effects [81]. Hydrogels provide biocompatibility, biodegradability, and soft mechanical properties, but traditional hydrogels, such as alginate-based ones, require surgical procedures for insertion and do not respond to environmental stimuli [78]. For this reason, stimuli-sensitive hydrogels have been developed, which can respond to temperature, light, pH, ionic strength, or pressure [80]. In recent decades, thermosensitive hydrogels have been used for intratumoral delivery of chemotherapeutic drugs, offering sustained release and reducing side effects [81]. Therapeutic molecules are often encapsulated in nanoparticles before being loaded into hydrogels, forming nanoparticle-loaded hydrogels (NLH), which improve stability, mechanical strength, and controlled degradation [79] (**figure1.14**).

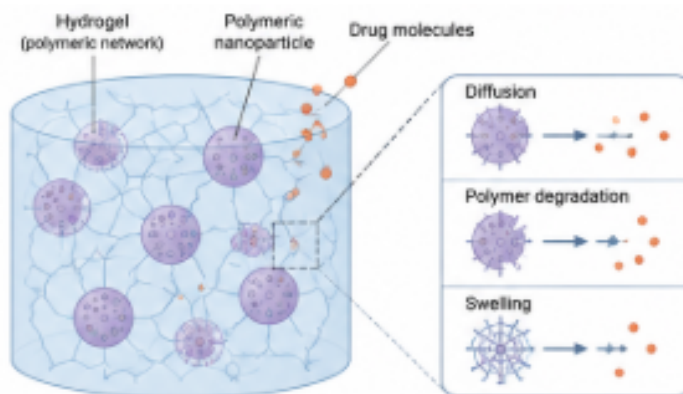


Figure 1.14: Schematic representation of a nanoparticle-loaded hydrogel, showing the polymeric network, encapsulated nanoparticles, drug molecules, and mechanisms of release via diffusion, polymer degradation, and swelling Created by Biorender.

Hydrogels can be derived from natural or synthetic sources. Natural polymers include alginate, chitosan, hyaluronic acid, collagen, hydroxypropyl methylcellulose, and gelatin [63], while the most studied synthetic polymers for thermosensitive hydrogels include polyethylene glycol (PEG), polyacrylamide (PAM), poly(vinyl alcohol) (PVA), polymethylacrylate (PMMA), and PEO-PPO copolymers such as poloxamers, which offer high mechanical strength and low immunogenicity [74].

1.5.2 Poloxamer 188/407

Poloxamers are ABA type triblock copolymers composed of two hydrophilic poly(ethylene oxide)(PEO) blocks and a central hydrophobic poly(propylene oxide)(PPO) block [75] (**figure1.15**). Due to their amphiphilic structure, these polymers show thermoresponsive behavior, transitioning from sol to gel near body temperature, and are generally recognized as safe (GRAS), with low toxicity and minimal immunogenicity [76]. Gelation occurs through micelle aggregation at the critical micelle concentration (CMC) and critical micellization temperature (CMT), forming a stable three-dimensional network [77].

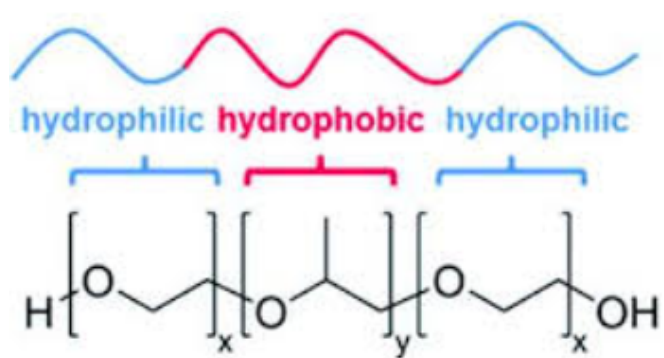


Figure 1.15: Generic chemical structure of Poloxamer 188/407, showing the ABA triblock with hydrophilic PEO blocks and hydrophobic PPO block (adapted from Chen et al., 2024 [79]).

Poloxamer 407 (P407) has been widely studied for biomedical applications because of its biocompatibility and regulatory approval. However, when used alone, it has limitations such as rapid gel erosion, low mechanical strength, and burst release of drugs, which reduce long-term efficacy. To overcome these issues, it is often combined with poloxamer 188 (P188). Thanks to its lower molecular weight and higher PEO/PPO ratio, P188 increases the gelation temperature and improves gel stability, reducing erosion and allowing for more fluid, injectable formulations [81] (**figure1.16**).



Figure 1.16: Chemical structures of Poloxamer 407 and Poloxamer 188 showing the ratio of PEO and PPO units [81].

Poloxamer-nanoparticle composite hydrogels are generally prepared using the “cold method”, where the poloxamer is dissolved in cold water and preformed

polymeric nanoparticles are dispersed under vigorous mixing before heating induces gelation. This combination couples thermoresponsive gelation with controlled, targeted nanoparticle release, optimizing drug retention at the target site and improving mechanical properties, stability, and controlled degradation of the system [82].

These P407/P188 binary systems provide a versatile platform for local delivery of drugs and therapeutic agents, including chemotherapeutics and nanoparticles, with applications in ocular, nasal, transdermal, and oncological therapies [83].

Chapter 2

Aim of Thesis

2.1 Main Objective

The main objective of this master's thesis was to evaluate and characterize a hydrogel system loaded with TIMP-3 mRNA-containing nanoparticles, previously studied in terms of formulation and rheological properties, to determine its local therapeutic efficacy in metastatic melanoma. The study focused on the controlled release behavior of the nanoparticles and hydrogel in vitro, their internalization by target cells, and the functionality of the nanoparticles as mRNA carriers.

2.2 Specific Objectives

Validation of the Hydrogel+NPs Delivery System

- Evaluate the ability of the Hydrogel+NPs system to provide sustained release of biologically active nanoparticles over time.
- Assess the efficiency of nanoparticle delivery and cellular internalization mediated by the hydrogel-based platform.

Evaluation of Intracellular TIMP-3 Localization

- Determine the intracellular localization and distribution of TIMP-3 following delivery of TIMP-3-loaded nanoparticles.
- Investigate the influence of the hydrogel carrier on the intracellular delivery and distribution of the therapeutic cargo compared with free nanoparticles.

Evaluation of TIMP-3 Protein Expression

- Confirm the successful expression of TIMP-3 protein following mRNA delivery mediated by the nanoparticle system.
- Evaluate the contribution of the hydrogel to sustaining nanoparticle-mediated TIMP-3 expression over time.

Chapter 3

Materials and Methods

3.1 Materials

Lysine-end modified poly(β -amino ester) (K), histidine-end modified poly(β -amino ester) (H), TIMP-3 mRNA (PureBoost™ Modified TIMP3 mRNA), eGFP mRNA (PureBoost™ EGFP mRNA), mCherry mRNA, sodium acetate buffer (12.5 mM, pH 5.2), HEPES buffer (20 mM, pH 7.4), sucrose (4 wt%), and RNase-free water. Poloxamer 407 (Kolliphor® P407) obtained from BASF, Poloxamer 188 (Lutrol® F68) purchased from Sigma-Aldrich, and phosphate buffered saline (PBS) 1X, MilliQ water. Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 50% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin. Trypsin, Jet Messenger® Transfection Reagent (Sartorius), 4% formaldehyde, Tween 20/TritonX-100, blocking solutions consisting of 5% (w/v) powdered milk in PBS-T, where PBS-T contained 0.05% Tween 20. primary anti-TIMP-3 antibody, secondary AF568 antibody, DAPI, CellMask, Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), including BCA Reagent A and BCA Reagent B, bovine serum albumin (BSA). Pure TIMP-3, Armesharm system, Ripa buffer, sonicator. Acrylamide/bis-acrylamide solutions (Solutions A, B, C, G), SDS, TEMED, ammonium persulfate (Solution G), isopropanol, loading buffer, β -mercaptoethanol, protein marker, XCell SureLock™ Mini-Cell, PVDF membrane, methanol, filter papers, fiber pads, blotting cassette components (clear and black faces), secondary antibody (HRP- or fluorophore-conjugated), orbital shaker.

3.2 Cell lines

For this study, two murine melanoma cell lines, B16-F1 and B16-F10, were used. Both originate from the parental B16 melanoma, with the B16-F1 subline characterized by low metastatic potential, while B16-F10 was obtained through successive

in vivo selections, exhibiting high metastatic capacity, particularly to the lungs. B16-F1 and B16-F10 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin.

Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air. Subcultures were performed when cells reached 70–90% confluence using 75 cm² flasks. The B16-F10 line was purchased from ATCC (CRL-6475TM), while B16-F1 was kindly provided by the Microenvironment and Metastasis Laboratory at the Centro Nacional de Investigaciones Oncológicas in Madrid, Spain.

3.3 Methods

3.3.1 Production of NPs

The nanoparticles were prepared using lysine- and histidine-end modified poly(β -amino ester) (pBAE) polymers in a 60:40 weight ratio. For TIMP-3 mRNA-loaded nanoparticles (T3-NPs), the polymer-to-nucleic acid molar ratio (N/P ratio) was 75:1, while for fluorescent protein-loaded nanoparticles, eGFP and mCherry, the N/P ratio was 25:1.

The preparation was done as follows. First, the K/H polymer mixture was dissolved in 12.5 mM sodium acetate (AcONa, pH 5.2) in an Eppendorf tube. Next, the mRNA (TIMP-3, eGFP, mCherry at 0.5 mg/mL) was dissolved in the same buffer in a second Eppendorf tube. The mRNA solution was then added to the Eppendorf containing the polymer solution. The mixture was pipetted gently to ensure proper mixing and then incubated at room temperature for 20 minutes to allow the nanoparticles to self-assemble.

After incubation, the nanoparticle solution was transferred to the same amount of RNase-free water and mixed by gentle pipetting to complete the nanoprecipitation process. Then, HEPES buffer (20 mM, pH 7.4) containing 4 wt% sucrose was used as a cryoprotectant and lyoprotectant. The final solution consisted of approximately one third nanoparticle suspension, one third RNase-free water, and one third HEPES buffer to produce a stable nanoparticle solution for all downstream experiments.

The polymer solution used for nanoparticle formation was 12.5 mg/mL in sodium acetate buffer and the mRNA solution was 0.5 mg/mL. This protocol resulted in nanoparticles with a hydrodynamic diameter below 250 nm, a low polydispersity index (PDI < 0.2), and a high encapsulation efficiency (>80% for TIMP-3 and mCherry nanoparticles) suitable for incorporation into hydrogel in the experimental treatments. The nanoparticles formed and size distribution were confirmed using dynamic light scattering (DLS).

3.3.2 Preparation of Poloxamer 188/407 hydrogel

The injectable system investigated in this thesis consists of a thermosensitive hydrogel containing Poloxamer 407 and Poloxamer 188, which was used as a carrier in combination with KH nanoparticles previously loaded with TIMP-3 mRNA/mCherry mRNA. The hydrogel system was employed in all experimental setups to evaluate its capacity to release nanoparticles and to study release kinetics.

The hydrogel was prepared by dissolving Poloxamer 407 and Poloxamer 188 in PBS at concentrations of 18% and 4.5% (w/w), respectively, under vigorous stirring at 4 °C for at least 72 hours. Gentle mixing was applied to minimize air entrainment and interfacial stress.

A 90% weight hydrogel formulation was prepared and maintained until the day of the transfection experiment. On the day of the treatment, 24-well Transwell inserts containing the cultured B16-F1 or B16-F10 cells were filled with the hydrogel at 90% of the final volume and the remaining 10% was composed of preformed KH nanoparticles. Such a process ensured that the hydrogel remained stable but allowed nanoparticles to be incorporated directly into each experiment enabling consistent evaluation of nanoparticle release and delivery to the cells.

During hydrogel preparation, poloxamers were first weighed and combined with concentrated PBS (10X). MilliQ water was added to reach 90% of the final weight and stirred at 4 °C for 72 hours.



Figure 3.1: Schematic representation of the hydrogel preparation steps. I: weighing poloxamers; II: addition of 10X PBS; III: addition of MilliQ water; IV: stirring at 4°C for 72 hours. Created by Biorender

3.3.3 Qualitative of protein expression by Confocal microscopy

This experiment was performed to evaluate the internalization and intracellular localization of TIMP-3-loaded nanoparticles, in order to determine the efficiency of their release and the spatial distribution of the therapeutic mRNA within B16-F10 cells. Confocal microscopy also allows the observation of co-localization with cellular structures and the comparison of different treatments (including Hydrogel

+ NPs and controls).

B16-F10 cells were seeded on 12 mm glass coverslips placed in 24-well plates, which were sterilized by exposure to UV light for 15 minutes. The coverslips were initially coated with 400 μ L of 0.1% gelatin in PBS and incubated for 1 hour at 37 °C in a humidified atmosphere containing 5% CO₂. After removing the gelatin, cells were seeded at a density of 100,000 cells/mL.

The following day, the cells were subjected to various treatments:

Condition	Primary Ab anti-TIMP-3 (1:50)	Secondary Ab AF568 (1:1000)	DAPI (1:1000)	CellMask (1:1000)
Negative control (DAPI)	—	—	✓	—
Negative control (DAPI + CellMask)	—	—	✓	✓
Negative control (DAPI + CellMask + AF568)	—	✓	✓	✓
Positive control (DAPI + AF568)	✓	✓	✓	—
Positive control (DAPI + CellMask + AF568)	✓	✓	✓	✓
TIMP-3 NPs	✓	✓	✓	✓
Hyd + NPs	✓	✓	✓	✓

Table 3.1: Experimental setup for confocal microscopy treatments and staining.

for the positive control, Jet Messenger reagent was used following the protocol [84]; for the Hyd+NPs condition, the DMEM was removed, a Transwell insert was applied, and the hydrogel containing 90% Hyd and 10% TIMP-3-loaded nanoparticles was added inside the Transwell(**figure3.2**).

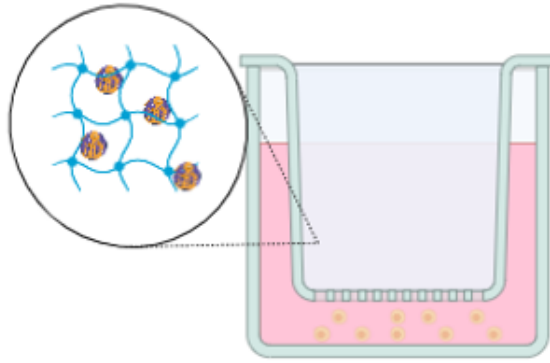


Figure 3.2: Schematic representation of the Transwell setup used for hydrogel and nanoparticle treatments. The inset shows the hydrogel with embedded nanoparticles. Created by Biorender

After 24 hours of treatment, the culture medium was removed, and the cells were washed twice with PBS 1X. Fixation was performed by adding 250 μ L of

4% formaldehyde in PBS 1X for 20 minutes at room temperature. The cells were then permeabilized with 0.1% Tween 20/Triton X-100 in PBS 1X for 30 minutes at room temperature and washed twice with PBS 1X.

Cells were incubated with blocking solution (5% powdered milk in PBS-T, where PBS-T contained 0.05% Tween 20) for both primary and secondary antibody steps, always under gentle agitation, for 1 hour at room temperature.

The coverslips were subsequently incubated with the primary anti-TIMP-3 antibody (rabbit, 1:50 dilution) overnight at 4 °C under agitation, followed by incubation with the secondary AF568 antibody (anti-rabbit, 2 µg/mL) for 1 hour at room temperature, protected from light using aluminum foil.

Finally, the cells were treated with DAPI at 20 µg/mL, diluted 1:1000 in PBS 1X, and with CellMask at 5 µg/mL, diluted 1:1000 in PBS 1X, in the appropriate wells for 10 minutes at room temperature. After two washes with PBS 1X, the glass coverslips were mounted on glass slides, and the samples were ready for analysis using confocal microscopy.

3.3.4 Protein quantification by BCA assay

Total protein quantification in the samples was performed using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). This method uses the reduction of copper ions ($\text{Cu}^{2+} \rightarrow \text{Cu}^+$) by proteins in an alkaline environment (Biuret reaction) and then a soluble complex with two molecules of BCA (bicinchoninic acid) is formed to produce a purple colour at 562 nm. The absorbance is proportional to the protein concentration in the samples.

Bovine serum albumin (BSA) was used to prepare a set of reference standards covering the kit's working range, including a blank consisting of only PBS as diluent. The working reagent (WR) was prepared by mixing BCA Reagent A and BCA Reagent B at a volumetric ratio of 50:1 (Reagent A : Reagent B). The total volume of WR required was calculated using the formula:

$$V_{\text{WR,total}} = (N_{\text{standards}} + N_{\text{samples}}) \times R \times V_{\text{WR,sample}}$$

The prepared working reagent is stable for several days when stored at room temperature in a closed container.

For each well, 25 µL of sample or standard was added to the microplate, followed by 200 µL of working reagent. The plate was shaken for 30 seconds and incubated at 37 °C for 30 minutes. After incubation the plate was equilibrated to room temperature, the absorbance was measured at 562 nm, subtracting the mean blank absorbance from each reading to obtain the corrected absorbance for each sample.

The standard curve was constructed by plotting the corrected absorbance of each BSA standard against the concentration. The protein concentration of unknown

samples was calculated by interpolating their absorbance values on the standard curve which provides a good measurement of the total protein in each sample.

3.3.5 Titration of primary antibody(Dot Blot)

This experiment was performed to determine the optimal dilution of the secondary antibody for subsequent Western Blot analysis. The PVDF membrane was first cut into multiple pieces according to the number of dilutions to be tested. Small equidistant spots were applied on each piece. On the first row, spots of the sample treated with TIMP-3-loaded nanoparticles, previously quantified by BCA assay, were placed. For each spot, 2 μ L of solution was applied across five serial dilutions of the sample (1:10, 1:20, 1:40, 1:80, and 1:160), prepared by diluting 10 μ L of the sample in Milli-Q water. On the second row, pure TIMP-3 was applied in the same manner to serve as a reference standard, allowing assessment of the antibody's sensitivity and optimal dilution. After all spots were applied, the membrane was left at room temperature for 15 minutes to allow protein adsorption.

The blocking buffer was then applied, and the membrane was incubated for 30 minutes at room temperature under gentle agitation. The primary antibody, anti-TIMP-3 (rabbit), was applied at a dilution of 1:1000 on each membrane piece and incubated for 1 hour at room temperature under agitation. After incubation, the membrane was washed three times with PBST, each wash lasting 10 minutes.

The secondary antibody was then applied in different dilutions (1:1000, 1:2000, 1:5000, and 1:10000) on each membrane piece and incubated for 30 minutes at room temperature under agitation, followed by the same washing procedure. Finally, the membrane was analyzed using the Armesharm system to determine the optimal dilution of the secondary antibody for future Western Blot experiments.

3.3.6 Western blot

Western Blot analysis was performed to evaluate the expression and amount of TIMP-3 in cells treated with nanoparticles and hydrogel, in order to compare the efficacy of different treatments and to study the release and internalization of the mRNA over time. This experiment allows the analysis of both intracellular proteins and those released into the culture medium, comparing the signal between samples treated with TIMP-3, negative controls, positive controls, and hydrogel-nanoparticle systems. Measuring protein levels at three selected time points (24 h, 48 h, and 72 h) enables the study of expression kinetics and the progressive release of the therapeutic mRNA.

The preparation of protein samples for Western Blot analysis began with the seeding of B16-F10 and B16-F1 cells in 24-well plates, using a density of 80,000 cells/mL for B16-F10 to ensure cell viability until the final 72-hour time point,

and 100,000 cells/mL for B16-F1. The following day, the cells were transfected with different treatments, including a negative control, a positive control using Jet Messenger according to the manufacturer’s protocol [84], eGFP-loaded nanoparticles, TIMP-3-loaded nanoparticles, hydrogel alone, and hydrogel combined with TIMP-3-loaded nanoparticles. For hydrogel treatments, 24-well Transwell inserts were used. Each treatment was performed in triplicate, and the samples from the three replicates were combined into a single Eppendorf tube per treatment.

At the end of the first 24-hour interval, the condition of the cells was checked under a microscope. The supernatant from each well was collected separately, and for each treatment, the supernatants of the three replicates were combined into a single Eppendorf tube. For these type of samples, only the final high-speed centrifugation at 14,000 rpm for 10 minutes at 4 °C was performed to remove any debris and obtain the clear fraction.

For the preparation of cell lysates, the wells were washed with PBS 1X, the PBS was removed, and 150 μ L of trypsin was added per well, allowing it to act for 5 minutes at room temperature. Trypsin facilitates cell detachment by digesting extracellular matrix proteins and adhesion molecules. The trypsin activity was then neutralized with 300 μ L of DMEM per well, and the detached cells were transferred to dedicated Eppendorf tubes for each treatment.

The lysed samples were then subjected to a first centrifugation at 4,000 rpm for 10 minutes at 4 °C to form the cell pellet. The pellet was resuspended in 180 μ L of lysis buffer, mixed thoroughly, sonicated for 2 minutes, and frozen at -80 °C for 5 minutes, repeating this sequence twice. Finally, the lysates were centrifuged at 14,000 rpm for 10 minutes at 4 °C, and the supernatant was collected into new Eppendorf tubes. The prepared lysates, together with the previously collected supernatants, were ready for total protein quantification using the BCA assay and for subsequent loading on the Western Blot.

After protein samples were prepared and quantified using the BCA assay, the samples were prepared for SDS-PAGE analysis. Gels were assembled according to the specified acrylamide concentrations, preparing the running gel and stacking gel separately [85]. Acrylamide/bis-acrylamide solutions (Solution A and B), SDS, TEMED, and ammonium persulfate (Solution G) were mixed according to the required volumes and Solution G was added last to start polymerization. The running gels were covered with isopropanol to prevent dehydration and after about 10 minutes the isopropanol was removed using filter paper. The stacking gel, water, Solutions C and A, SDS, and TEMED were mixed and a gel comb was inserted to form the wells.

Protein samples, normalized for total protein content, were mixed with loading buffer containing β -mercaptoethanol and heated at 95 °C for 10 minutes to denature the proteins. Twentyfive microliters of each sample were loaded into the gel wells along with a protein marker. Electrophoresis was performed using the XCell

SureLock™ Mini-Cell at a constant voltage of 180 V for approximately 1 hours, monitoring the bromophenol blue dye front until it reached the bottom of the gel.

Following electrophoresis, proteins were transferred to a PVDF membrane using the XCell IITM Blot Module. The PVDF membrane was wetted in 100% methanol for 30 seconds, then equilibrated with transfer buffer together with filter papers for 2 minutes. The blot “sandwich” was assembled according to the proper order (figure3.3), and transfer was performed for 1.5 hours at a constant ampere of 330 mA, taking into account the negative charge of the proteins under these conditions.

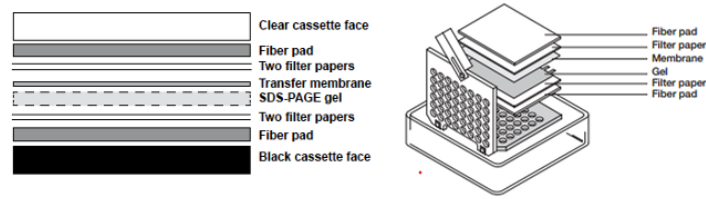


Figure 3.3: Schematic representation of the “blot sandwich” assembly for protein transfer. The figure illustrates the order of layers from the clear cassette face to the black cassette face, including fiber pads, filter papers, SDS-PAGE gel, and the PVDF transfer membrane [85].

After transfer, the membrane was incubated at room temperature for 1 hour on an orbital shaker with blocking buffer containing 5% non-fat milk in PBST to reduce non-specific binding. Following the blocking step, the membrane was cut horizontally at about 100 kDa. The upper part of the membrane was used for Vinculin detection as a loading control and the lower part was used for TIMP-3 detection. The upper membrane section was incubated overnight at 4°C with the primary anti-Vinculin antibody diluted 1:1000 in blocking buffer and the lower membrane section was incubated overnight at 4°C with the primary anti-TIMP-3 antibody diluted in blocking buffer at the working concentration.

After overnight incubation, both membrane sections were washed three times with PBST for 10 minutes each and briefly rinsed with two changes of PBST. The upper membrane section was then incubated for 1 hour at room temperature under agitation with an HRP-conjugated anti-mouse secondary antibody diluted 1:1000, whereas the lower membrane section was incubated under the same conditions with an HRP-conjugated anti-rabbit secondary antibody diluted 1:1000. After incubation, both membrane sections were washed three times with PBST for 10 minutes each.

Vinculin was expected to appear at approximately 116–130 kDa and was used as a loading control. TIMP-3 was expected to be detected as bands corresponding to its non-glycosylated form (~24–27 kDa) and glycosylated form (~30–35 kDa). Protein bands were visualized using the Amersham imaging system.

3.3.7 Flow cytometry

Flow cytometry analysis was performed to study the release and cellular uptake of mCherry-labeled nanoparticles and hydrogel formulations over time. Using mCherry as a standard fluorescent marker allows to measure the internalization of nanoparticles and evaluate the delivery efficiency of the hydrogel and nanoparticle system in target cells. This method allows for the analysis of release dynamics, uptake rate and comparison to a range of treatments including free nanoparticles, hydrogel-encapsulated nanoparticles and controls.

For flow cytometry, cells were seeded in plates of the desired density and incubated overnight at 37~°C. The following day, cells were transfected with different treatments, including a negative control, a positive control using Jet Messenger according to the manufacturer's protocol, mCherry-loaded nanoparticles, hydrogel alone, and hydrogel containing mCherry-loaded nanoparticles. For the hydrogel and nanoparticle treatment, the number of wells was increased to assess variability in the results.

Samples were collected at three designated time points: 24 h, 48 h, and 72 h. At each time point, the supernatant from each well was aspirated and the cells were washed once with PBS 1X. Then, 150 μ L of trypsin was added per well, and cells were incubated for 5 minutes at 37 °C to allow detachment from the plate surface. After detachment, 350 μ L of fixation/neutralization solution was added per well, consisting of one-third 4% formalin and two-thirds complete DMEM containing 10% FBS, 1% glycerol, and 1% penicillin/streptomycin. The total volume per well was 500 μ L, and plates were covered with aluminum foil to protect the samples from light.

The entire volume of each well was collected into Eppendorf tubes and centrifuged for 5 minutes at 1500 $\times$ g at 4 °C. The supernatant was carefully removed without disturbing the pellet, which was then resuspended in 100 μ L of PBS 1X. The cell suspension was transferred to 96-well plates, making the samples ready for flow cytometry analysis.

Chapter 4

Results and discussion

4.1 DLS characterization of nanoparticles

Before each transfection experiment, we conducted dynamic light scattering (DLS) analysis to evaluate the physicochemical properties of the nanoparticle formulation and determine whether it was suitable for future cellular experiments. We also examined the hydrodynamic diameter and polydispersity index (PDI) since these are important parameters to determine nanoparticle stability, homogeneity, and transfection efficiency.

Nanoparticle size is an important parameter because it influences cellular uptake, intracellular trafficking, and nanoparticle stability in biological environments. Similarly, the PDI provides information regarding the homogeneity of particle size distribution within the formulation. Low PDI values indicate a homogeneous nanoparticle population, whereas high PDI values may suggest aggregation or formulation instability. According to the literature, polymeric nanoparticles intended for nucleic acid delivery generally show optimal cellular internalization when presenting sizes below approximately 200–250 nm and PDI values lower than 0.2 [86, 87].

For each nanoparticle preparation, DLS measurements were performed in triplicate, and the reported values represent the average ranges obtained from different independent formulations prepared during the experimental period.

Table 4.1: DLS characterization of KH-pBAE nanoparticles.

Formulation	Hydrodynamic diameter range (nm)	PDI range
mCherry-loaded KH-pBAE NPs	160–220	0.06–0.20
TIMP-3-loaded KH-pBAE NPs	200–260	0.14–0.22

Both mCherry-loaded and TIMP-3-loaded nanoparticles showed reproducible physicochemical properties, with hydrodynamic diameters and PDI values compatible with homogeneous and stable nanoparticle formulations, making them suitable for subsequent transfection experiments.

4.2 Validation of the Hydrogel+NPs Delivery System by Flow Cytometry Using mCherry

To evaluate the functionality of the Hydrogel+NPs system, mCherry loaded nanoparticles were used as a fluorescent reporter model. The use of fluorescent proteins is a widely employed strategy for monitoring delivery efficiency, cellular internalization, and transfection capability of nanoparticle based systems [88]. In particular, mCherry allows the direct and quantitative detection of fluorescent signal expression in treated cells, making it possible to assess whether nanoparticles released from the hydrogel maintain their biological functionality over time.

The aim of this analysis was not limited to the evaluation of nanoparticle transfection efficiency alone, but also included a broader characterization of the entire Hydrogel+NPs system. The purpose of the study was to explore the ability of the hydrogel to release nanoparticles simultaneously and thus maintain their stable nature and the ability to be absorbed by melanoma cells. To evaluate the efficiency of the system, flow cytometry was used, as this approach allows fast and quantitative analysis of the number of mCherry-positive cells and is very popular in terms of nanoparticle uptake and delivery at the single-cell level [89, 90]. This approach allowed the comparison between free nanoparticles and hydrogel released nanoparticles at different experimental time points, providing information regarding both delivery efficiency and release dynamics of the system.

The experiments were initially performed on B16F10 melanoma cells at 24h and 48h to evaluate whether nanoparticles released from the hydrogel retained their transfection capability over time. We observed negligible mCherry expression

in the negative control group as shown in Figure 4.1, which means that there is no background fluorescence and that the gating is correct. On the other hand, in cells loaded with free mCherry nanoparticles, we saw a significant percentage of mCherry-positive cells at both time points for nanoparticle-mediated transfection.

No significant fluorescent signal was detected in cells with hydrogel alone, suggesting that the hydrogel matrix did not contribute to mCherry fluorescence. Cells treated with the Hydrogel+mCherry NPs system displayed detectable mCherry expression at 24 h and 48 h, but at lower levels than free nanoparticles. These results indicate that nanoparticles released from the hydrogel were still able to be internalized by B16F10 cells and remained biologically active after release from the matrix.

The lower percentage of mCherry-positive cells reported in the Hydrogel+mCherry NPs condition might be related to the controlled release of the hydrogel. In this case, the hydrogel likely acted as a temporary reservoir, gradually regulating nanoparticle diffusion into the extracellular environment [91, 80, 81]. As a consequence, cells were exposed to nanoparticles more progressively compared to direct treatment with free nanoparticles, while maintaining detectable transfection efficiency over time.

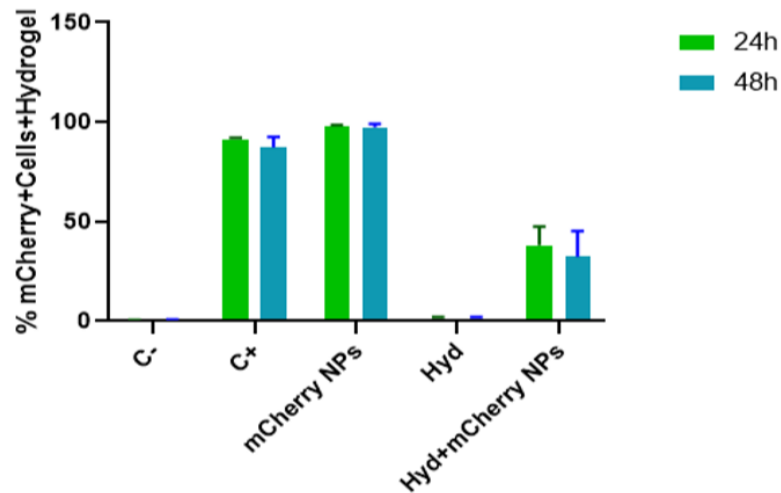


Figure 4.1: Flow cytometry analysis of mCherry expression in B16F10 cells treated with free mCherry-loaded nanoparticles and Hydrogel+mCherry NPs at 24 h and 48 h. Data are presented as mean $\pm$ SD.

These preliminary findings supported the capability of the Hydrogel+NPs system to release biologically active nanoparticles over time. Based on these encouraging results, the study was further extended by including an additional experimental time point (72 h) and a second melanoma cell line, B16F1, in order to better characterize the temporal behavior of the system and evaluate its biological reproducibility in

different melanoma cell models.

As shown in Figure 4.2, both cell lines showed very low mCherry expression in the negative control and in samples treated with hydrogel alone, further confirming the absence of nonspecific fluorescence associated with the hydrogel matrix and the correctness of the gating strategy used during flow cytometry analysis. In contrast, samples treated with free mCherry-loaded nanoparticles showed high percentages of mCherry-positive cells at all analyzed time points, confirming the high internalization and transfection efficiency mediated by the NPs.

As a result, the Hydrogel+mCherry NPs treatment showed that both cell lines showed detectable levels of mCherry expression at 24 h, 48 h and 72 h, which showed that nanoparticles released from the hydrogel are still internalized and can also induce fluorescent reporter expression even after long incubation periods. However, in comparison to free nanoparticles, the signal in the Hydrogel+NPs system was lower and more heterogeneous. This trend suggests that nanoparticle release from the hydrogel matrix did not occur immediately or uniformly, but was influenced by the temporal behavior of the system and by the interaction between the hydrogel and nanoparticles [92]. From the comparison between the two cell lines, differences in the biological behavior of the system could also be observed. In particular, B16F10 cells showed higher variability in the Hydrogel+mCherry NPs treatment, especially at the 48 h time point, as highlighted by the high standard deviation values. In contrast, B16F1 cells showed a slightly more stable and reproducible trend over time, while still maintaining lower expression levels compared to free nanoparticles. These differences may not be exclusively associated with intrinsic biological characteristics of the two cell lines, but also with technical aspects related to the preparation and distribution of the Hydrogel+NPs system. In particular, hydrogel handling represents a critical step of the experimental protocol, since the system rapidly changes its physical properties depending on temperature, making homogeneous distribution within the transwells more difficult [79]. During the mixing process between hydrogel and nanoparticles, excessively rapid handling or release of the material could promote the formation of bubbles or microheterogeneities within the matrix, potentially affecting local nanoparticle distribution and contributing to the variability observed in mCherry expression levels [92, 81].

The high variability observed in the Hydrogel+mCherry NPs treatment suggested the need for further investigation of the reproducibility of the system. For this reason, additional experiments were performed by increasing the number of experimental replicates, from three to six wells, for the Hydrogel+NPs treatment, in order to obtain a more accurate evaluation of the biological and technical variability associated with the release kinetics and temporal behavior of the system.

As shown in Figure 4.3, the Hydrogel+mCherry NPs treatment continued to show detectable levels of mCherry expression in both cell models and at all analyzed

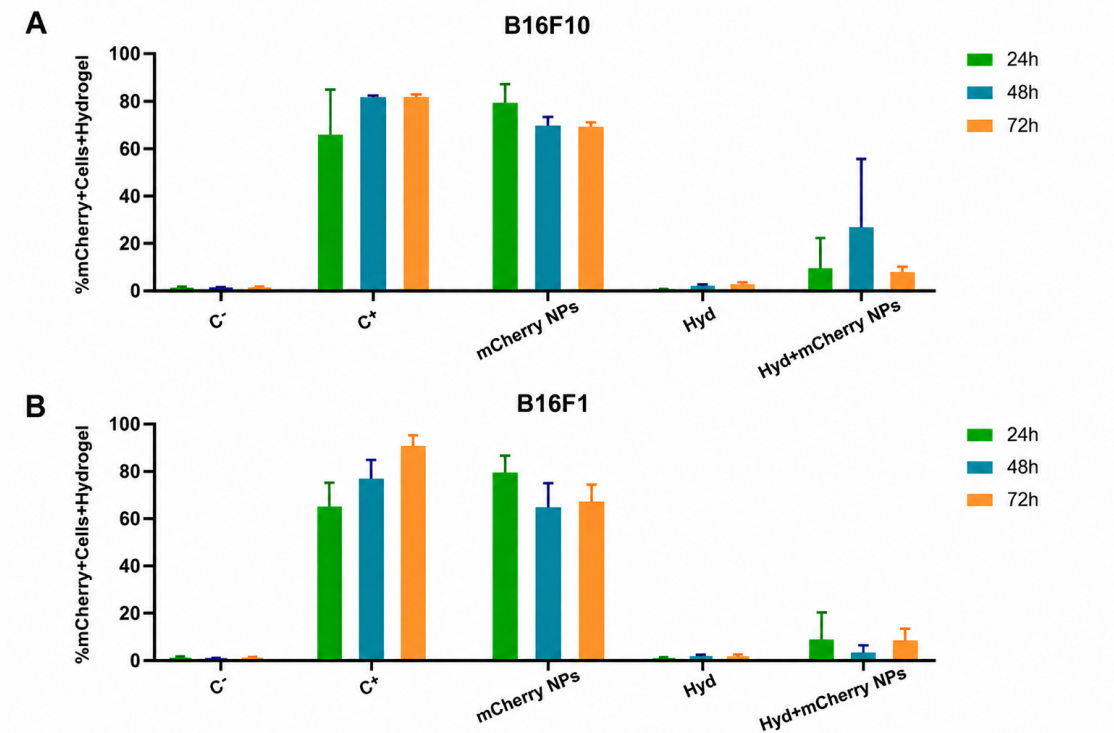


Figure 4.2: Flow cytometry analysis of mCherry expression in B16F10 (A) and B16F1 (B) melanoma cells treated with free mCherry-loaded nanoparticles or Hydrogel+mCherry NPs at 24 h, 48 h, and 72 h. Data are presented as mean $\pm$ SD.

time points, confirming that nanoparticles released from the hydrogel maintained their biological activity and their ability to induce fluorescent reporter expression over time.

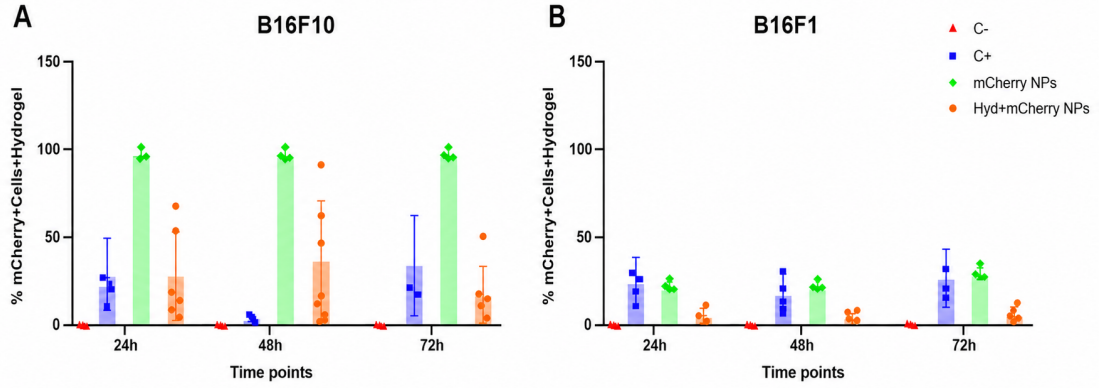


Figure 4.3: Flow cytometry analysis of mCherry expression in B16F10 (A) and B16F1 (B) melanoma cells treated with free mCherry-loaded nanoparticles or Hydrogel+mCherry NPs at 24 h, 48 h, and 72 h using six experimental replicates for the Hydrogel+NPs condition. Data are presented as mean $\pm$ SD together with individual replicate values.

Overall, the results obtained in B16F10 cells were more evident and consistent compared to those observed in B16F1 cells. In the first cell line, the Hydrogel+mCherry NPs treatment showed marked variability among the different wells, highlighting a heterogeneous distribution of mCherry expression levels. The visualization of individual experimental values almost suggested the presence of two distinct subgroups within the replicates, with some wells showing significantly higher transfection levels compared to others. Despite this heterogeneity, the broad trend across the different experiments was positive and indicated that the system was able to release nanoparticles that were still functioning and biologically active.

In contrast, the results obtained in B16F1 cells appeared to be lower and more homogeneous over time. This might also be explained by the lower cell density observed in the experiment, which may have affected the overall internalization efficiency and the detection of the fluorescent signal. The increased number of replicates also confirmed that the variability observed in the Hydrogel+NPs system represented a recurrent characteristic of the experimental model rather than an occasional event.

Despite these limitations, the overall results validated the functionality of the Hydrogel+NPs system, confirming both the role of the hydrogel as a controlled-release platform and the ability of the nanoparticles to maintain biological activity and transfection capability during prolonged incubations [80, 81]. These findings support the potential use of the hydrogel-based delivery system as a platform for sustained nanoparticle release and prolonged cellular exposure over time.

4.3 Evaluation of intracellular TIMP-3 expression by confocal microscopy

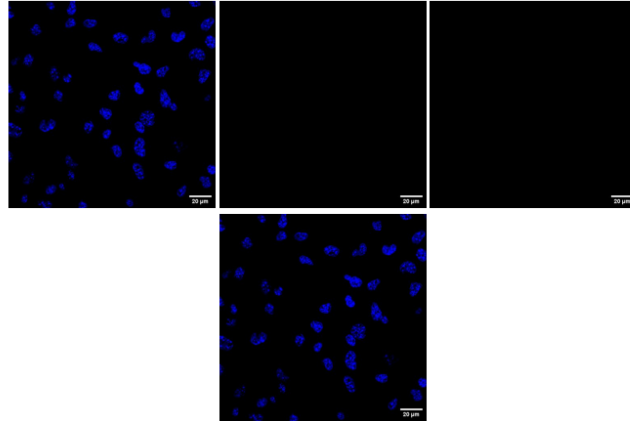
Confocal microscopy analysis was performed to evaluate the intracellular localization of TIMP-3 in B16-F10 melanoma cells treated with TIMP-3-loaded nanoparticles and with the Hydrogel+NPs system. This approach allowed a qualitative observation of nanoparticle internalization and of the distribution of the expressed protein inside the cells.

Different control conditions were included in order to distinguish the specific fluorescent signal from possible background fluorescence. DAPI staining was used to identify cell nuclei, while CellMask allowed the visualization of cell morphology and plasma membrane organization. TIMP-3 staining was detected through the green fluorescence emitted by the AF568 conjugated secondary antibody.

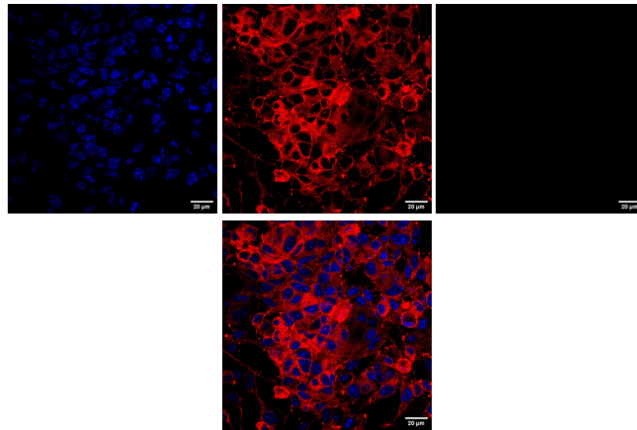
Negative controls were used to verify the absence of nonspecific fluorescence generated by the different staining reagents, whereas positive controls transfected with JetMessenger confirmed the correct intracellular expression of TIMP-3. Subsequently, treatments with TIMP-3 NPs and Hyd+TIMP-3 NPs were compared in order to evaluate the intracellular presence and distribution of TIMP-3 following nanoparticle-mediated mRNA delivery. Particular attention was dedicated to comparing free nanoparticles with nanoparticles incorporated within the hydrogel matrix in order to verify whether the Hydrogel+NPs system was still able to release functional nanoparticles capable of reaching the cells and inducing intracellular TIMP-3 expression [93].

As shown in Figure 4.4, in the DAPI-only control, only the blue nuclear signal was observed, confirming the absence of fluorescence in the CellMask and AF568 channels. In the DAPI + CellMask sample, cell morphology and plasma membrane organization were clearly visible, while no detectable green signal was observed in the AF568 channel, confirming the absence of nonspecific fluorescence associated with the TIMP-3 detection system. In the DAPI + CellMask + AF568 control, a weak diffuse green intracellular signal was observed. Since no TIMP-3 mRNA-loaded nanoparticles were present in this condition, this fluorescence was associated with a possible basal endogenous expression of TIMP-3 in B16-F10 cells, as TIMP-3 is known to be physiologically expressed in several cell types and tissues [94].

A — DAPI only



B — DAPI + CellMask



C — DAPI + CellMask + AF568

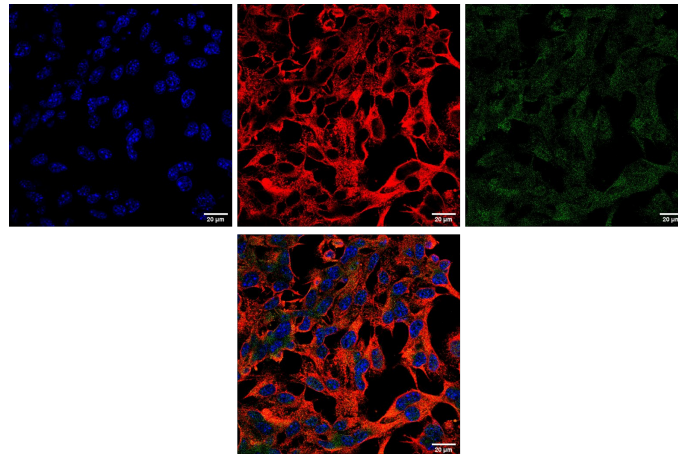


Figure 4.4: Negative control conditions used for confocal microscopy analysis of intracellular TIMP-3 localization in B16-F10 melanoma cells.

After confirming the specificity of the staining conditions through negative control, we further investigated the same cells with TIMP-3-loaded nanoparticles. As shown in Figure 4.6, the TIMP-3-loaded nanoparticles show a significantly higher green fluorescent signal than with negative control. The AF568 signal associated with TIMP-3 staining through the fluorescent secondary antibody showed that TIMP-3 exogenously expressed TIMP-3 after nanoparticle mRNA delivery.

One of the interesting things that we found in the images is the specific intracellular localization of the fluorescent signal of TIMP-3. Contrary to the classical view of TIMPs, TIMP-3 seems to be mostly localized in the nuclear region and has a distribution pattern. This may seem quite unanticipated, but it is clear that in recent literature TIMPs are in the "moonlighting" or pleiotropic proteins [95] group. These proteins, despite being composed of a single polypeptide chain, are capable of performing independent and biochemically distinct functions depending on their subcellular location [96]. Specifically, it has already been reported that at least two other family members, TIMP-1 and TIMP-4, have been identified within the nuclear compartment [95]. The mechanism by which exogenous TIMP-3, delivered via nanoparticles (NPs), reaches the nucleus may be linked to processes of cellular re-entry or non-canonical intracellular trafficking. Literature indicates that TIMP-3 can be internalized through LRP1-mediated endocytosis (Low-density lipoprotein-related protein 1) Once inside, the protein could translocate to the nucleus by utilizing nuclear localization signals (NLS), similar to those identified in many members of the matrix metalloproteinase (MMP) family [95].

Functionally, the nuclear presence of an inhibitor such as TIMP-3 suggests potential roles in transcriptional regulation or the modulation of cellular stress signals. As illustrated in Figure 4.5, the presence of enzymes and inhibitors from the metalloproteinase family in the nucleus is associated with critical functions ranging from the degradation of nuclear substrates (such as PARP1 or XRCC1) to direct transcriptional regulation [96]. This provides a solid biological rationale for the TIMP-3 localization observed in B16F10 cells, suggesting that the inhibitor may act as an intra-nuclear modulator. To confirm that this signal does not result from overlap artifacts [95]. Another important observation concerned cell morphology. In the negative control conditions, cells showed a well-defined morphology, with clear cellular boundaries visible through CellMask staining, suggesting good cellular integrity and viability. In contrast, cells treated with TIMP-3 NPs appeared less organized and showed less defined contours, with morphological alterations compatible with cellular stress or possible apoptotic-like conditions.

Although this observation may suggest a potential antitumoral effect associated with TIMP-3 expression, consistent with the tumor-suppressive and anti-angiogenic functions previously described for TIMP-3 [97], it is currently not possible to determine with certainty whether these alterations were actually caused by the biological action of exogenously expressed TIMP-3 or by a possible cytotoxic effect

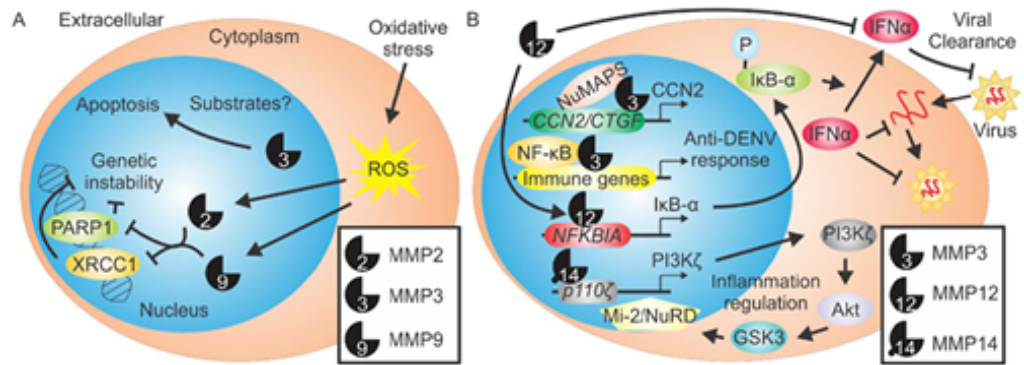


Figure 4.5: Models of nuclear activities associated with metalloproteinase family proteins and their inhibitors. TIMP-3 may act as a transcriptional or proteolytic modulator, similarly to what has been documented for other members of the MMP/TIMP family (adapted from Jobin et al., 2017 [95]).

associated with the nanoparticles themselves. For this reason, further experiments and additional controls, for example using nanoparticles loaded with non-therapeutic mRNAs such as eGFP, will be necessary in order to better distinguish the specific contribution of TIMP-3 from possible effects related to the nanoparticle system itself.

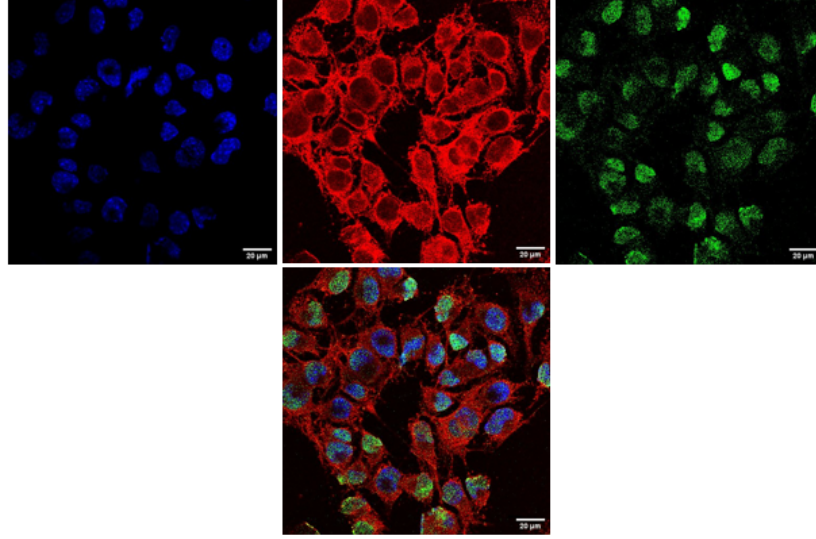


Figure 4.6: Confocal microscopy images of B16-F10 melanoma cells treated with TIMP-3-loaded nanoparticles. From left to right: DAPI nuclear staining (blue), CellMask staining (red), AF568 fluorescence associated with TIMP-3 detection (green), and merged channels. Scale bar: 20 μm .

After TIMP-3 NPs analysis, the final condition was treated with Hyd+TIMP-3 NPs. As shown in Figure 4.7, treatment with Hyd+TIMP-3 NPs also produced a fluorescent signal related to TIMP-3 and the hydrogel nanoparticles were able to deliver the mRNA and to induce TIMP-3 expression in the intracellular setting.

Compared to free nanoparticles, the signal observed in the Hyd+TIMP-3 NPs treatment appeared less intense, suggesting a more gradual release of nanoparticles from the hydrogel matrix [98]. This behavior is consistent with the role of the hydrogel as a controlled release system, capable of modulating cellular exposure to nanoparticles over time.

Another interesting observation concerned cell morphology. Cells showed a generally preserved morphology and well-defined cellular boundaries. Unlike what was observed in the TIMP-3 NPs treatment, no marked morphological alterations were evident, suggesting that the more progressive exposure to nanoparticles may have contributed to better preservation of cellular integrity.

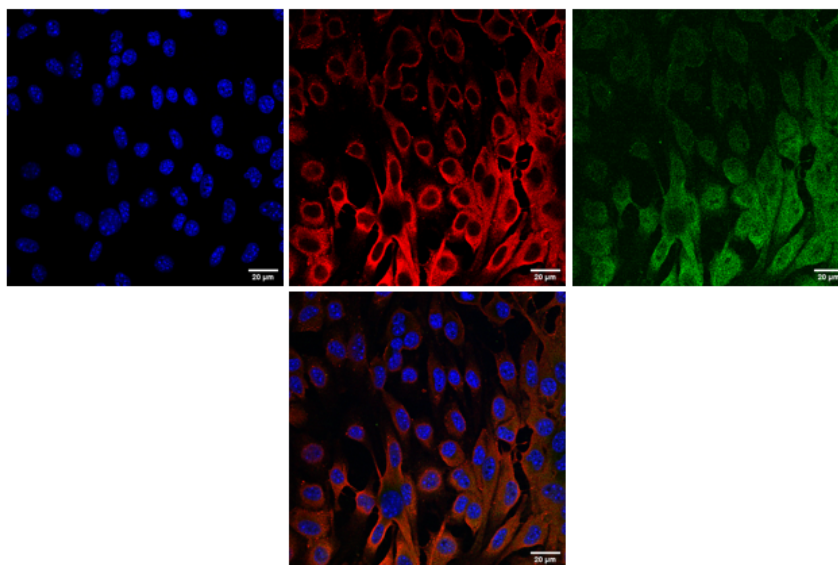


Figure 4.7: Confocal microscopy images of B16-F10 melanoma cells treated with Hyd+TIMP-3 NPs. From left to right: DAPI nuclear staining (blue), CellMask staining (red), AF568 fluorescence associated with TIMP-3 detection (green), and merged channels.

Finally, the confocal microscopy results showed that TIMP-3 NPs and Hyd+TIMP-3 NPs were able to promote intracellular TIMP-3 expression. As expected, with free nanoparticles, the fluorescent signal was stronger; with nanoparticles added in the hydrogel, the signal was weaker but still clearly detectable. Furthermore, differences in cell morphology between the two treatments suggest that the hydrogel may provide a more gradual exposure to the nanoparticles, allowing for a more controlled release of the treatment over time.

4.4 Western Blot

Before proceeding with Dot Blot and Western Blot experiments, total protein quantification was performed using the BCA assay. This step was essential to determine the protein concentration of the different samples and to ensure the loading of comparable amounts of protein in subsequent analyses. Furthermore, protein quantification allowed the evaluation of the quality of the obtained protein extracts and the selection of the most appropriate conditions for the optimization of TIMP-3 detection.

Table 4.2: Protein concentrations and sample preparation for Western Blot analysis of B16F10 lysates collected after 24 h.

Sample	Protein concentration ($\mu\text{g mL}^{-1}$)	V_{protein} (μL)	V_{buffer} (μL)	V_{total} (μL)	$V_{\text{H}_2\text{O}}$ (μL)
C-	3343.18	5.98	5.0	25.0	14.0
C+	2964.83	6.75	5.0	25.0	13.3
GFP	3353.50	5.96	5.0	25.0	14.0
TIMP3	2787.30	7.18	5.0	25.0	12.8
Hyd	3005.90	6.65	5.0	25.0	13.3
Hyd TIMP3	2871.40	6.97	5.0	25.0	13.0

Table 4.3: Protein concentrations and sample preparation for Western Blot analysis of B16F10 lysates collected after 48 h.

Sample	Protein concentration ($\mu\text{g mL}^{-1}$)	V_{protein} (μL)	V_{buffer} (μL)	V_{total} (μL)	$V_{\text{H}_2\text{O}}$ (μL)
C-	4318.0	4.63	5.0	25.0	15.4
C+	3084.9	6.48	5.0	25.0	13.5
GFP	3850.3	5.19	5.0	25.0	14.8
TIMP3	3334.5	6.00	5.0	25.0	14.0
Hyd	3120.0	6.41	5.0	25.0	13.6
Hyd+TIMP3	2013.8	9.93	5.0	25.0	10.1

Table 4.4: Protein concentrations and sample preparation for Western Blot analysis of B16F1 lysates collected after 48 h.

Sample	Protein concentration ($\mu\text{g mL}^{-1}$)	V_{protein} (μL)	V_{buffer} (μL)	V_{total} (μL)	$V_{\text{H}_2\text{O}}$ (μL)
C-	2813.54	7.11	5.0	25.0	12.9
C+	2131.28	9.38	5.0	25.0	10.6
GFP	2418.70	8.27	5.0	25.0	11.7
T3	1359.90	14.71	5.0	25.0	5.3
Hyd	1381.50	14.48	5.0	25.0	5.5
Hyd+T3	1334.90	14.98	5.0	25.0	5.0

This analysis allowed for the quantification of protein concentrations of the

selected samples that were later used in protein detection experiments.

To determine the correct antibody conditions for TIMP-3 detection, a Dot Blot assay was first performed. Moreover, this analysis allows for a quick evaluation of the signal quality obtained in different experimental conditions and determine the desired parameters for the next test.

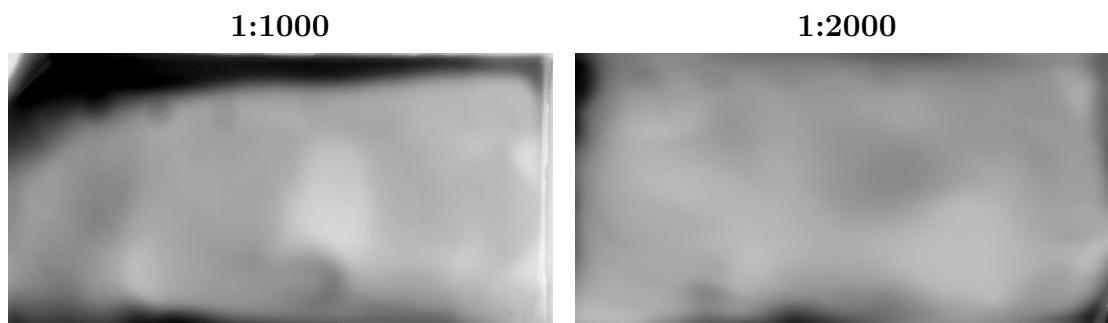


Figure 4.8: Dot Blot analysis was performed to optimize the dilution of the secondary antibody used for TIMP-3 detection. The primary anti-TIMP-3 antibody was maintained at a dilution level of 1:1000 and the secondary antibody was tested at dilutions of 1:1000 and 1:2000. Higher dilutions (1:5000 and 1:10000) did not result in detectable signals and were not included.

As shown in Figure 4.8, we performed the Dot Blot assay for the different dilutions of the secondary antibody while the primary anti-TIMP-3 antibody was kept at the same dilution of 1:1000. Among all the conditions tested, only the secondary antibody dilutions of 1:1000 and 1:2000 caused a detectable signal and no appreciable signal at the higher dilutions of 1:5000 and 1:10000. Although the overall signal intensity was relatively low, the membrane incubated with the secondary antibody dilution of 1:1000 was the most clearly visible signal and therefore higher sensitivity to detection was obtained in this case. The 1:2000 dilution produced a weaker and less clear signal.

The interpretation of the results was limited by the presence of a high background signal and a non-uniform distribution of chemiluminescence across the membrane surface.

Effect that may have been caused by a local accumulation of the chemiluminescent detection solution, possibly favored by slight unevenness of the support surface on which the membrane was placed during image acquisition. As a consequence, the stronger background signal may have reduced the visibility of the spots of interest, making their identification more difficult. In addition, the spots containing pure TIMP-3 were difficult to distinguish, possibly due to partial degradation of the protein standard during storage. Despite these limitations, the 1:1000 dilution of the secondary antibody provided the most clearly detectable signal and was therefore selected for all subsequent Western Blot experiments. Following the optimization of

the experimental conditions, Western Blot experiments were performed to evaluate TIMP-3 protein expression in the different experimental groups. Several cell lines and time points were initially analyzed; however, only the conditions that provided the most reliable and informative results are presented in this section. The clearest and most reproducible signals were obtained from B16-F10 cell lysates collected after 24 and 48 hours and from B16-F1 cell lysates collected after 48 hours.

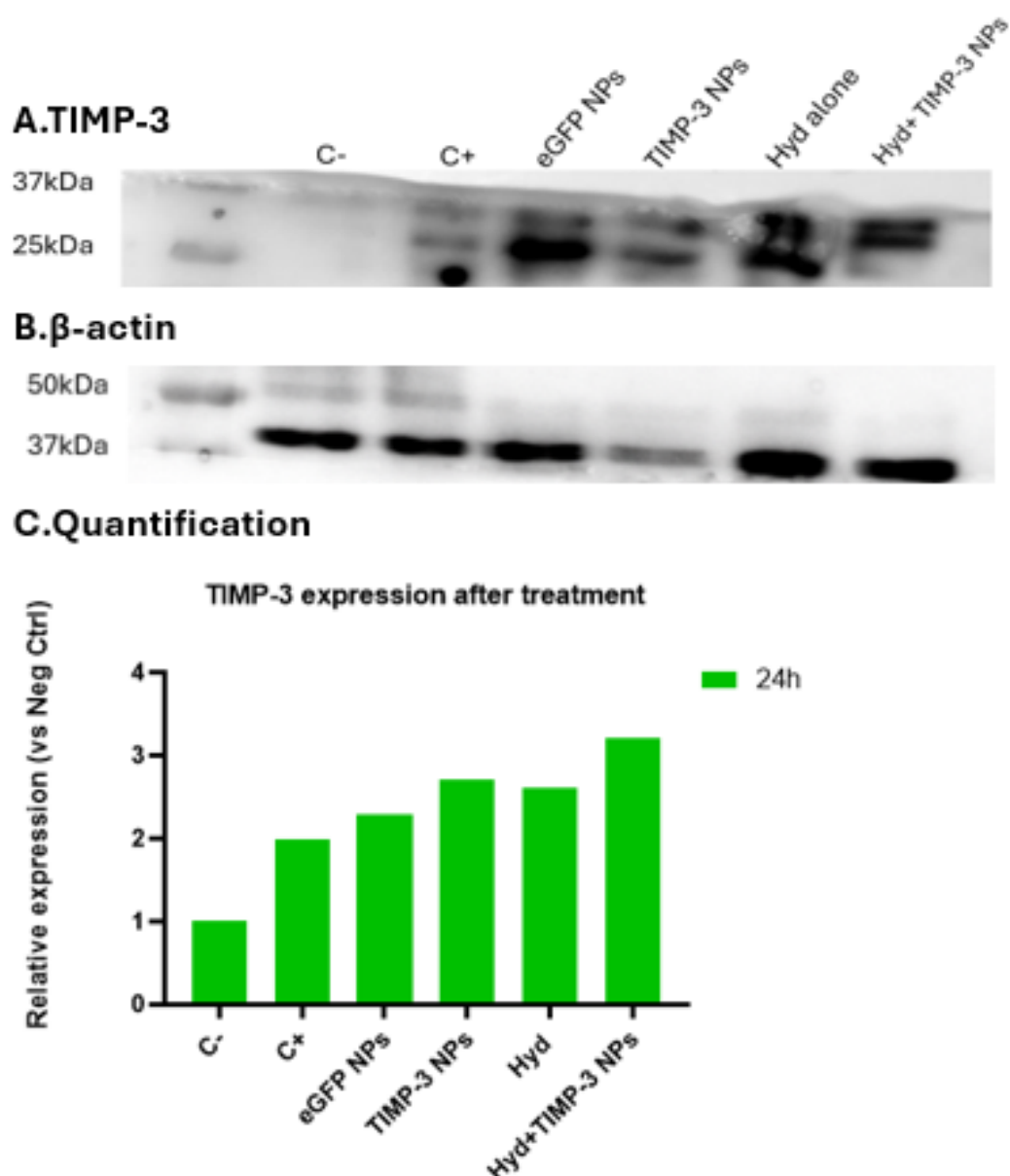


Figure 4.9: Western Blot analysis of TIMP-3 expression in B16-F10 cells after 24 h of treatment. (A) TIMP-3 immunodetection showing bands between 30 and 37 kDa, corresponding to the glycosylated forms of TIMP-3. (B) β -actin immunodetection used as loading control. (C) Densitometric quantification of the TIMP-3 signal normalized to the negative control. Quantification was performed on the band between 35 and 37 kDa

As shown in Figure 4.9, the membrane incubated with the anti- β -actin antibody was used as a loading control. In general, bands were detected in all experimental

conditions and showed comparable intensities, indicating a relatively homogeneous protein loading among the different samples analyzed.

Regarding the membrane incubated with the anti-TIMP-3 antibody, bands were observed between approximately 30 and 37 kDa, a molecular weight range compatible with the glycosylated forms of TIMP-3. No evident bands were detected in the negative control, whereas clearly distinguishable signals were observed in the other experimental conditions. In the Hyd+TIMP-3 NPs treatment, the signal appeared as two distinct bands, a pattern consistent with the different glycosylation variants of TIMP-3 described in the literature, which may influence protein stability and turnover [99].

An interesting observation was the presence of a detectable signal in the eGFP NPs and Hydrogel alone samples, although these conditions were not designed to induce exogenous TIMP-3 expression.

This finding may be explained by considering the endogenous inhibitory role of TIMP-3 and its complex regulatory mechanisms [100]. The absence of a detectable signal in the negative control is consistent with the phenotype of melanoma cells, such as B16-F10, in which TIMP-3 expression is often strongly downregulated or silenced in order to promote tumor progression and invasiveness [99]. This silencing commonly occurs through epigenetic mechanisms, such as promoter hypermethylation, or through post-transcriptional regulation mediated by oncogenic microRNAs, particularly miR-21, which promotes the degradation of TIMP-3 transcripts [100].

The appearance of bands in the Hyd alone and eGFP NPs samples may therefore suggest an induction of endogenous TIMP-3 expression or an accumulation of basal protein levels in response to the experimental treatment. Indeed, TIMP-3 expression is known to be upregulated by various growth factors and cytokines through the activation of signaling pathways such as ERK/MAPK and PI3K/Akt [101]. The presence of the hydrogel or the cellular uptake of nanoparticles may act as physical or biochemical stimuli capable of partially reactivating these signaling pathways in B16-F10 cells.

Furthermore, TIMP-3 is the only member of the TIMP family that is able to bind tightly to the extracellular matrix (ECM) through specific lysine- and arginine-rich binding domains. The presence of the hydrogel in the “Hyd alone” condition may have acted as a biochemical scaffold capable of retaining and concentrating the small amounts of endogenously produced TIMP-3, making them more readily detectable by immunochemical analysis.

Densitometric quantification was performed considering the band located between approximately 35 and 37 kDa, which appeared to be the most representative and best-defined among those observed. The obtained values were normalized to the negative control, which was set to a value of 1. The quantitative analysis confirmed the qualitative observations obtained from the membrane. The negative

control showed values close to zero, consistent with the basal silencing of TIMP-3 expression in untreated tumor cells [99]. An increase in signal intensity was observed in all treated conditions; however, the highest expression levels were detected in the TIMP-3 NPs and Hyd+TIMP-3 NPs groups, with the latter showing the highest overall value, suggesting an effective synergy between nanoparticle-mediated TIMP-3 delivery and the possible stabilizing effect provided by the hydrogel matrix [102].

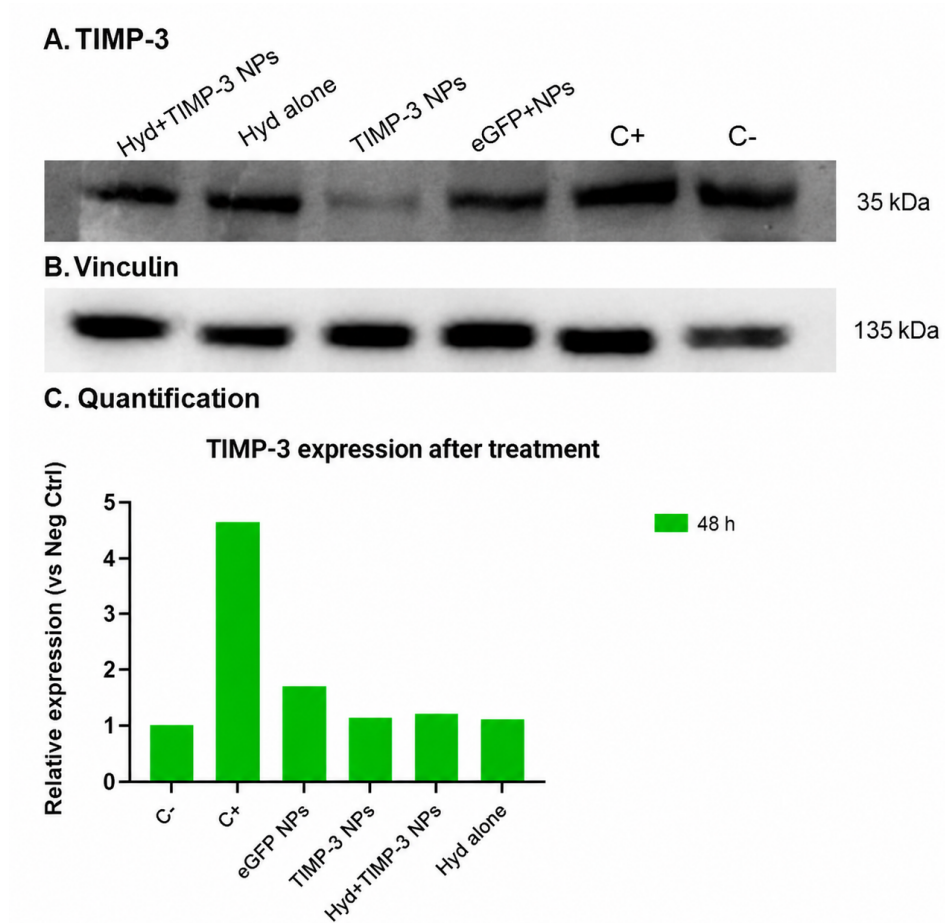


Figure 4.10: Western blot analysis of TIMP-3 expression in B16F10 cells after 48h. (A) TIMP-3 expression (B) Vinculin as a loading control (C) Densitometric quantification of TIMP-3 expression normalized to Vinculin and expressed relative to the negative control.

To evaluate the persistence of TIMP-3 expression over time, Western blot analysis was performed on B16F10 cells after 48 h of treatment (**Figure 4.10**). In this experiment, Vinculin was used as the loading control instead of β -Actin, as its molecular weight allowed a more convenient membrane-cutting strategy while

avoiding potential interference with the protein of interest. Vinculin bands were clearly detectable and showed comparable intensity across all samples, indicating uniform protein loading.

Regarding TIMP-3, a band was detected at approximately 35 kDa, consistent with the glycosylated form of the protein [102]. Compared with the 24 h time point, a detectable signal was also observed in the negative control. This finding suggests the possible presence of endogenous TIMP-3 expression in B16F10 cells and is consistent with the results obtained from confocal microscopy, where intracellular localization attributable to TIMP-3 was also observed. The positive control showed the highest level of TIMP-3 expression, as confirmed by densitometric analysis. Among the experimental groups, cells treated with eGFP NPs displayed the strongest signal, while the Hyd alone group showed expression levels comparable to those observed in the other treated samples. TIMP-3 NPs resulted in a moderate level of TIMP-3 expression, whereas the Hyd+TIMP-3 NPs group showed a clearly detectable signal that was higher than the negative control, suggests that the mRNA delivered by the nanoparticles remained biologically active even after prolonged incubation.

Figure 4.11 shows TIMP-3 protein expression in B16F1 cells after 48 h of treatment. The well-defined band was found to be about 35 kDa, which is consistent with the glycosylated form of TIMP-3, while the loading control Vinculin was generally similar in all samples. The positive control showed the highest expression level in the densitometry, which is proof of a good transfection protocol as well as a good detection technique. Just like B16F10 cells at the same time, it was also detected in the negative control with TIMP-3 expression. Of the treated groups, eGFP NPs and TIMP-3 NPs showed expression levels that are very similar to the negative control and Hyd alone showed the lowest signal intensity. The most significant result was observed in the Hyd+TIMP-3 NPs group, which showed the biggest increase in TIMP-3 expression of all experiments, which approached two-fold that obtained in the negative control condition. The fact that TIMP-3 mRNA-loaded nanoparticles were present in the hydrogel matrix indicates that even after 48 h of incubation, TIMP-3 protein expression was still present.

If comparing the two cell lines during the same time point, the effect for the Hyd+TIMP-3 NPs treatment appeared to be more pronounced in B16F1 cells than in B16F10 cells, implying a cell line-dependent response to the hydrogel-based nanoparticle delivery system.

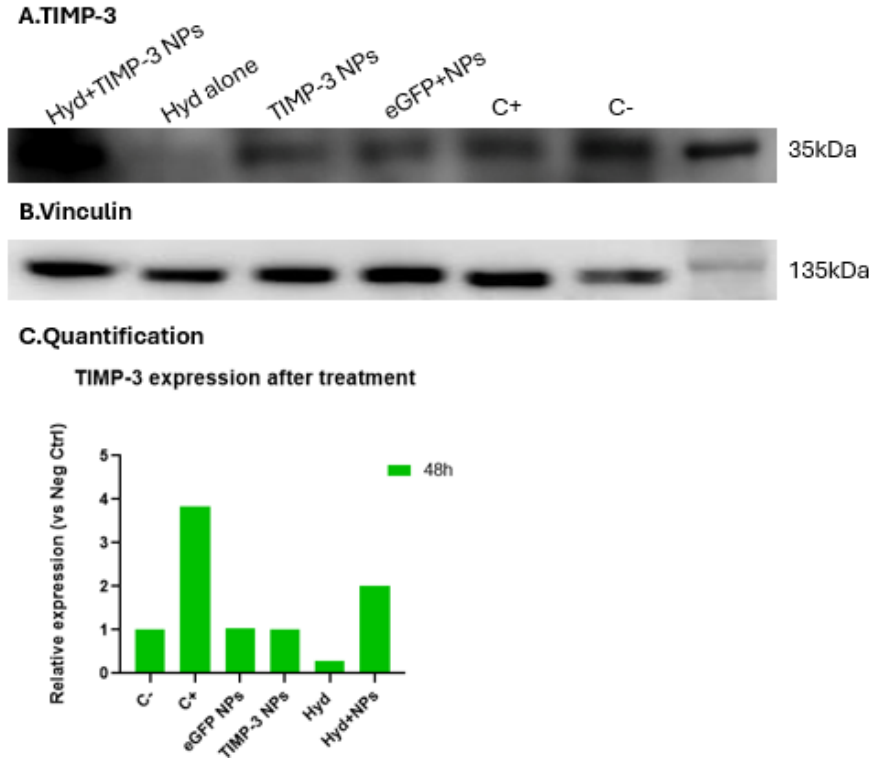


Figure 4.11: Western blot analysis and densitometric quantification of TIMP-3 expression in B16F1 cells after 48 h of treatment. Vinculin was used as the loading control. TIMP-3 expression levels are reported relative to the negative control.

Finally, the Western blot results confirm the findings obtained from the previous release, internalization, and confocal microscopy experiments. The presence of TIMP-3 in the treated samples demonstrates that the nanoparticles released from the system are able to reach the target cells, be internalized, and promote the expression of the protein encoded by the delivered mRNA. Considering the entire experimental workflow, the data suggest that the Hydrogel+NPs system is capable not only of retaining and progressively releasing nanoparticles over time, but also of preserving their biological functionality until the final stage of the process. Protein expression therefore represents the ultimate step required to confirm the effectiveness of the developed delivery platform. Although the magnitude of the response varied among the different experimental conditions and between the two melanoma cell lines analyzed, the overall results support the initial hypothesis underlying this work, demonstrating that the combination of hydrogel and nanoparticles represents a promising platform for local mRNA delivery and for inducing protein expression in melanoma cells [81].

Chapter 5

Conclusions

The results obtained in this work allowed the evaluation and characterization of an innovative local delivery system based on the incorporation of TIMP-3 mRNA-loaded polymeric nanoparticles into a thermoresponsive Poloxamer 407/188 hydrogel for the treatment of metastatic melanoma. The physicochemical characterization of the nanoparticles confirmed properties suitable for gene delivery applications, while flow cytometry studies demonstrated the ability of the Hydrogel+NPs system to provide a gradual and sustained release of biologically active nanoparticles for up to 72 hours. Confocal microscopy analysis also confirmed the internalization of the nanoparticles and the expression of TIMP-3 in the cell and suggests that the protein could be localized in the nucleus. Finally, the expression of TIMP-3 in glycosylated form by Western Blot was confirmed and the mRNA delivered to melanoma cells was a function of the system to induce protein production. As a result, pBAE nanoparticles and thermoresponsive hydrogel are a promising platform for the local and controlled delivery of therapeutic mRNA. By prolonging the release of nanoparticles and enabling expression of the desired protein, this technique could help address some of the problems of the current systemic approach and thus help to retain treatment at the site of the tumor, with the potential to lower systemic side effects.

However, this study presents some limitations. All experiments were performed exclusively *in vitro* and therefore cannot fully reproduce the complexity of the tumor microenvironment and the interactions between tumor cells, the immune system, and the host organism. In addition, although TIMP-3 expression was successfully confirmed, the biological effects resulting from its overexpression, such as its impact on cell proliferation, migration, invasion, angiogenesis, and apoptosis, were not directly investigated. For this reason, future studies should focus on a more comprehensive characterization of both TIMP-3 activity and the developed delivery platform. The unexpected nuclear localization observed by confocal microscopy deserves further investigation to better understand its

biological significance and possible intracellular functions. It will also be important to expand the confocal microscopy experiments by including additional control conditions, such as nanoparticles loaded with non-therapeutic reporter mRNA, for example eGFP. This approach would help distinguish the effects specifically caused by TIMP-3 overexpression from those potentially related to the intrinsic toxicity of the nanoparticles or to the transfection process itself. During the experiments, changes in cell morphology and a reduction in cell density were observed, and these effects could be associated either with TIMP-3 activity or with other experimental factors.

In parallel, secretome analysis may shed some light on changes in the secretion of proteins involved in the remodeling of the ECM, angiogenesis, inflammation, and tumor growth. This would allow for the evaluation of its biological activity. Additional functional assays on cell proliferation, migration, invasion and apoptosis in melanoma would further clarify the therapeutic role of TIMP-3. Like for flow cytometry studies, it would also be interesting to apply the same experimental method as for mCherry-loaded nanoparticles to TIMP-3-loaded nanoparticles. This would give a further insight into the effects of treatment on cell population over time and even more clearly assess the effectiveness of the delivery system and the biological effects of TIMP-3 overexpression.

Although we did a lot of optimization in this work, further Western Blot experiments are needed to make the signal more reproducible and further examine TIMP-3 expression at different time points. A more detailed picture of protein expression kinetics would help us understand the length of the therapeutic effect and the ability of the Hydrogel+NPs system to sustain protein production over time.

Taken together, these future studies could provide more complete validation of the platform and may be an important step towards in vivo studies, where the interactions between tumor cells, the microenvironment and the immune system may enhance the therapeutic effects observed in vitro. Further optimization of the hydrogel formulation and long-term effectiveness may lead to a localized mRNA-based treatment for metastatic melanoma and in general, other diseases with changes in the tumor microenvironment.

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