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**Design of Stimuli-Responsive Nanoplatfoms for Active
Targeting of Breast and Cervical Cancer Cells**

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Resumen

Los nanofármacos aprobados para uso clínico en el tratamiento del cáncer encapsulan y transportan ingredientes farmacéuticos activos (APIs) utilizados en quimioterapia. Estos sistemas, con tamaños entre 1 y 100 nm, buscan mejorar la administración de APIs en el tejido tumoral aprovechando su vasculatura anormal para acumularse pasivamente. Sin embargo, se ha reportado que la dosis de APIs administrada específicamente en las células de cáncer no supera el 1%. Por lo tanto, es imperativo diseñar nanofármacos que se dirijan específicamente a las células cancerosas y potencien las propiedades terapéuticas de los APIs. Este trabajo reporta los resultados de la síntesis y caracterización de nanoestructuras diseñadas para dirigirse específicamente a las células cancerosas y aumentar su temperatura local por métodos hipertérmicos generados mediante estímulos externos.

Combinando la tecnología de despliegue de fagos con acoplamiento molecular, modelamos las interacciones moleculares entre 44 péptidos y nueve proteínas de superficie celular sobre-expresadas en células HeLa de cáncer cervicouterino y células MDA-MB-231 de cáncer de mama. De este modo, seleccionamos los péptidos FKQDAWEAVDIR, TYPYNLQVRHWT y FLSGCGGTAEAT como los mejores candidatos para reconocer selectivamente las células cancerosas. Los péptidos se sintetizaron por medio de síntesis en fase sólida, se purificaron usando HPLC y sus propiedades fisicoquímicas se estudiaron mediante espectroscopia. Se sintetizaron y caracterizaron nanopartículas de oro multiramificadas (MBAuNP) con propiedades fototérmicas. Los conjugados péptido-MBAuNPs se obtuvieron mediante la reacción de adición de Michael. Para explorar su uso potencial como nanofármacos en el tratamiento del cáncer, se estudiaron ampliamente sus propiedades fisicoquímicas mediante SEM, TEM, XRD, UV-Vis, FTIR, entre otros. Paralelamente, se sintetizaron nanoestructuras núcleo-capa con propiedades superparamagnéticas, las cuales demostraron la capacidad de aumentar la temperatura de suspensiones acuosas en aproximadamente 5 °C mediante exposición a un campo magnético alterno de 35 mT.

En suma, este estudio reporta las ventajas del despliegue de fagos asistido por acoplamiento molecular para seleccionar péptidos con alta afinidad a células cancerosas. También contribuye al desarrollo de conjugados péptido-nanopartícula sensibles a estímulos, así como de nanoestructuras núcleo-capa con potencial uso en el tratamiento del cáncer mediante hipertermia localizada, enfatizando la comprensión de sus propiedades fisicoquímicas a fin de garantizar su posterior aplicación como nanofármacos.

Abstract

Nanopharmaceuticals approved for clinical use encapsulate and transport active pharmaceutical ingredients (APIs) used in chemotherapy. These systems, ranging from 1 to 100 nm in size, aim to enhance the delivery of APIs in the target tissue by leveraging the abnormal tumor vasculature to passively accumulate in the tumor microenvironment. However, studies have reported that the dose of APIs delivered to the cell does not exceed 1%. Therefore, it is imperative to design nanopharmaceuticals that specifically target cancer cells and potentiate the therapeutic properties of APIs by increasing cell sensitivity. Herein, we report on the synthesis and characterization of stimuli-responsive nanoplateforms designed to target cancer cells and increase their local temperature via hyperthermal methods.

Using a molecular docking-aided phage display approach, we virtually screened the molecular interactions between 44 phage-displayed peptides with overexpressed cell-surface proteins of HeLa and MDA-MB-231 cancer cells. Phage-display and molecular docking results enabled the selection of FKQDAWEAVDIR, TYPYNLQVRHWT, and FLSGCGGTAEAT as the best dodecapeptide candidates to selectively target cancer cells. Peptides were synthesized by solid-phase peptide synthesis (SPPS), purified using high-performance liquid chromatography (HPLC), and their physicochemical properties were studied using spectroscopic methods. Multibranched gold nanoparticles (MBAuNPs) exhibiting photothermal properties were synthesized and characterized. Peptide-MBAuNPs conjugates were obtained through Michael's addition reaction, which required a meticulous Design of Experiment. To explore their potential use as nanopharmaceuticals in cancer treatment, their physicochemical properties were extensively studied using SEM, TEM, XRD, UV-Vis, IR, among others. In parallel, core-shell nanostructures with superparamagnetic properties were synthesized, demonstrating the ability to increase water suspension temperatures by approximately 5 °C under an alternating magnetic field of 35 mT.

In sum, this study reports on the advantages of a molecular docking-aided phage display approach to select peptides that specifically bind cancer cells. It also contributes to the development of stimuli-responsive peptide-gold nanostar conjugates, as well as core-shell nanostructures with potential use in cancer treatment via site-specific hyperthermia, emphasizing the comprehension of the nanoplateform's physicochemical properties to guarantee further application as nanopharmaceuticals.

Thesis Structure Overview

This thesis consists of eight chapters. The first chapter includes a general introduction that summarizes the background information on nanotechnology-based carriers of active pharmaceutical ingredients (APIs) for cancer treatment, as well as research problems and gaps. It also states the purpose of this research work and the main research questions we aim to address. The subsections of this chapter contain the problem that motivates this research (section 1.1), the research questions and the hypotheses (section 1.2), the justification (section 1.3), the general and specific objectives (section 1.4), and the scope and limitations (section 1.5). Chapter 2 contextualizes the current applications of nanotechnology in cancer treatment. It addresses the questions: What advances have been made in the use of nanotechnology in cancer medicine? And, what remains to be done? Chapter 3 shows the importance of proper nanopharmaceuticals research and development (R+D), placing special emphasis on the Food and Drug Administration (FDA) recommendations and the characterization techniques available to fulfill them. Chapters 4, 5, and 6 report the methodologies, results, and discussions of three research topics related to the three research questions stated in section 1.2. These chapters can be interpreted as a compendium of three research articles: a first article that reports the results of the selection of peptides with high affinity for HeLa and MDA-MB-231 breast cancer cells (MDA) cells through phage display technology supported by molecular docking; a second article that details the synthesis and characterization of multibranched gold nanoparticles (MBAuNPs), and the design of experiment (DoE) for the conjugation of MBAuNPs with cell-related peptides; and a third article that reports the synthesis and characterization of core-shell nanostructures (CSNS), as well as the characterization of their magnetothermal properties. This thesis ends in chapter 7 with the general conclusions, perspectives, and research opportunities identified during the development of this project. Bibliography was inserted at the end of chapter seven, as well as the supporting information and the appendix. Figures and diagrams included in this work that are not referenced were created by the author.

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Acronyms

APIs active pharmaceutical ingredients

EPR enhanced permeability and retention

R+D research and development

DoE design of experiment

FDA Food and Drug Administration

NIR near-infrared radiation

LSPR localized surface plasmon resonance

SPIONs superparamagnetic iron oxide nanoparticles

MDA MDA-MB-231 breast cancer cells

SPPS solid phase peptide synthesis

HPLC high performance liquid chromatography

AuNPs spherical gold nanoparticles

MBAuNPs multibranched gold nanoparticles

CSNS core-shell nanostructures

UV-VIS ultraviolet-visible spectroscopy

FTIR fourier transform infrared spectroscopy

PEG polyethylene glycol

PEG-MAL methoxy polyethylene glycol maleimide

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

EMA European Medicines Agency

MRI magnetic resonance imaging

GNPs gold nanoparticles

MNPs magnetite nanoparticles

SAR specific absorption rate

ICP-OES Inductively Coupled Plasma Optical Emission Spectroscopy

SEM Scanning Electron Microscopy

TEM Transmission Electron Microscopy

XRD X-Ray Diffraction

PPMS Physical Properties Measurement System

DLS Dynamic Light Scattering

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List of Papers

- [1] Quilumba-Dutan V, Carreón-Álvarez C, Sanabria-Ayala V, Hidalgo-Figueroa S, Chakraborty S, Valsami-Jones E, López-Revilla R, Rodríguez-López JL. **Assessment of Phage-Displayed Peptides Targeting Cancer Cell Surface Proteins: A Comprehensive Molecular Docking Study.** *J Pept Sci.* 2025;31(3):e70004. doi:[10.1002/psc.70004](https://doi.org/10.1002/psc.70004)

Chapter 1

General Introduction

Nanotechnology has empowered the development of sophisticated therapeutic methods for cancer through the design of nanopharmaceuticals that encapsulate and transport APIs.¹ Nanopharmaceuticals are small systems ranging from 1 to 100 nm in size in any of their dimensions*, with outstanding physicochemical properties attributed to their small size and high surface-to-volume ratio that differentiate them from bulk materials.^{2,3}

In chemotherapy, nanopharmaceuticals aim to enhance the pharmacokinetics, pharmacodynamics, bioavailability, and stability of APIs, thereby achieving precise delivery of a larger dose of them in the target neoplastic tissue, reducing systemic toxicity.^{1,4,5} First-generation nanopharmaceuticals have focused on the delivery of APIs via passive targeting, which take advantage of the enhanced permeability and retention (EPR) effect caused by the abnormal tumor vasculature that increases the accumulation of the nanopharmaceuticals in the tumor microenvironment.^{1,4} Nevertheless, intrinsic factors of tumor heterogeneity, such as genetic, epigenetic, transcriptomic, and proteomic variations, as well as extrinsic factors, including pH, hypoxia, and cell-to-cell interactions, reduce the effectiveness of passive targeting via the EPR effect.⁶ Therefore, the effectiveness of tumor targeting by nanopharmaceuticals is reduced, leading to increased drug

*According to the FDA, nanopharmaceuticals include nanotechnology-based products that have a size in the range from 1 to 100 nm, but also materials outside of this range and up to 1000 nm, that exhibit related dimension-dependent properties.

release outside the cells and, consequently, the development of multidrug resistance.^{6,7}

A rigorous meta-analysis of 117 reports on nanopharmaceuticals applied to drug delivery showed that 0.7% of the intravenously injected dose was found to accumulate in solid tumors, which means that 99.3% interacts with off-target tissues.⁵ Hence, research must focus on challenging the current paradigm that the nanopharmaceuticals are taken into tumors solely by the EPR effect, and instead explore into active targeting strategies or even multifunctional products to overcome multidrug resistance in chemotherapy.

Active targeting-mediated products, known as second-generation nanopharmaceuticals, may overcome multidrug resistance by site-specific treatment of cancer and secure improved delivery efficiency.^{4,5} Active targeting demands ligands with high affinity to overexpressed cancer cell-surface proteins functionalized to the nanopharmaceutical surface, guaranteeing ligand-mediated binding to cancer cells and facilitating their uptake via receptor-mediated endocytosis, after which the carried APIs can be delivered to exert therapeutic action.^{3,8} Several types of ligands can be used, such as peptides, proteins, aptamers, or antibodies.²

Multifunctional nanopharmaceuticals are categorized as third generation medicinal products that may potentially solve multidrug resistance by combining hyperthermal ablation of tumors via photothermal or magnetothermal methods and active targeting drug delivery.^{9,10} Hyperthermal ablation consists of elevating the temperature of cancer cells between 41- 46°C to destroy them via mitochondrial swelling, protein denaturation, apoptosis, and necrosis.¹⁰ Photothermal ablation leverages the localized surface plasmon resonance (LSPR) effect of metallic nanoparticles, produced when an electromagnetic field induces a resonant coherent oscillation of the conduction band electrons in metallic nanoclusters, which enhance the absorption of the incoming radiation followed by the electron cloud thermalization and the electron-phonon, and phonon-phonon relaxation, leading to heat production.¹¹ Metallic nanoparticles of various shapes, such as gold nanospheres, nanorods, nanostars, nanoshells, among others, absorb electromagnetic radiation in a range from near ultraviolet to near-infrared radiation (NIR), but the absorption of NIR is preferred to guarantee safe phototherapy since it matches the biotransparent window (650-950 nm) where water, blood, skin, and muscles absorb weakly.¹² Moreover, cancer cells have higher sensitivity to heat than healthy ones, thus metallic nanoparticles absorbing NIR have the potential to ablate cancer cells while minimally affecting healthy tissue.¹³ On the other hand, magnetothermal

ablation employs superparamagnetic iron oxide nanoparticles (SPIONs) to produce heat after the application of an alternating magnetic field.¹⁰

In this thesis, we focus on the following research question: Is it possible to combine photothermal and/or magnetothermal properties, as well as active targeting capacity, in a single multifunctional nanoplatform useful for multimodal treatment of cancer? To address this question, we first selected dodecapeptides with high affinity to overexpressed cell-surface proteins of HeLa cervical cancer cells and MDA breast cancer cells using phage display technology supported by molecular docking. Using this novel approach, we selected the best peptide candidates by computing the affinity docking scores and the molecular interactions between 44 dodecapeptides isolated by phage display technology with nine cell-surface proteins identified by a systematic literature review of global HeLa and MDA protein profiles. Then, based on phage display and molecular docking findings, the dodecapeptides FKQDAWEAVDIR, TYPYN-LQVRHWT, and FLSGCGGTAEAT, designated P1, P2, and P3, respectively, were synthesized using microwave-assisted solid phase peptide synthesis (SPPS) and purified using high performance liquid chromatography (HPLC). Then, to prototype an active targeting nanoplatform, we establish a DoE to achieve optimal peptide-nanoparticle conjugation using the Michael's Addition Reaction.¹⁴

To prototype multifunctional nanoplatforms, we synthesized and characterized MBAuNPs that exhibit photothermal properties attributed to their LSPR maximum of absorbance located in the NIR region of the electromagnetic spectrum, as reported in the study conducted by our colleagues.¹⁵ Other three hierarchical CSNS comprising photothermal and/or magnetothermal properties were synthesized and characterized: $\text{Fe}_3\text{O}_4 @ \text{SiO}_2$ (designated CSNS); $\text{Fe}_3\text{O}_4 @ \text{SiO}_2 @ \text{Au}$ (designated CSNS@Au), and $\text{Fe}_3\text{O}_4 @ \text{SiO}_2 @ \text{MBAu}$ (designated CSNS@MBAu). These systems were obtained by taking advantage of the synergetic approach to build in a single nanoplatform the superparamagnetic properties of magnetite nanoparticles (Fe_3O_4 NPs), the high drug loading capacity of silica (SiO_2), and the optical properties of spherical gold nanoparticles (AuNPs) and MBAuNPs.

1.1 Problem Statement

In 2022, 20.0 million new diagnosed cases and 9.7 million cancer deaths were estimated worldwide.¹⁶ Moreover, 28.4 million new cancer cases are projected by 2040.¹⁷ Despite the progress in prevention, diagnosis, and treatment, cancer remains the disease with the highest morbidity and mortality rate.¹⁸ Besides, breast and cervical cancer are the most commonly diagnosed cancers and the leading causes of cancer deaths in women.¹⁶ Scientific reports have confirmed that high morbidity and mortality rates are attributed to cancer stem cells, which lead to cells differing in their tumor-initiating properties.¹⁹ However, cancer stem cells generate genetically related subclones that compete with each other and evolve during tumor progression and treatment, thereby maintaining tumor heterogeneity and tumorigenicity, and ultimately causing metastasis.¹⁹ Likewise, epigenetic factors and the tumor microenvironment might also contribute to tumor heterogeneity.²⁰ These conditions facilitate multidrug resistance, which cannot be overcome by passive targeting-mediated nanopharmaceuticals, which mostly deliver APIs outside cancer cells.^{5,20}

Although passive targeting-mediated nanopharmaceuticals have improved the therapeutic effect of APIs in terms of circulation half-time, stability, bioavailability, solubility, and reduced systemic toxicity, the multidrug resistance caused by the tumor heterogeneity has not been defeated.⁵ The problem becomes critical when results from preclinical studies report that more than 99% of the intravenously injected dose of nanopharmaceuticals interact with off-target tissues.⁵ Therefore, to reduce the chances of multidrug resistance in chemotherapy, research must be directed towards developing products that: 1) actively transport and deliver high loads of APIs to cancer cells; 2) comprise stimuli-responsive properties to enhance the therapeutic effect of the APIs, and 3) have the capability to surpass biological barriers and avoid clearance.²⁰

1.2 Research Questions and Hypotheses

Figure 1.1 details the three research questions addressed in this thesis, as well as, the hypotheses considered for each question. The methodology, and the results and discussion that solve for

hypothesis 1, 2 and 3, are presented in chapters 4, 5, and 6, respectively.

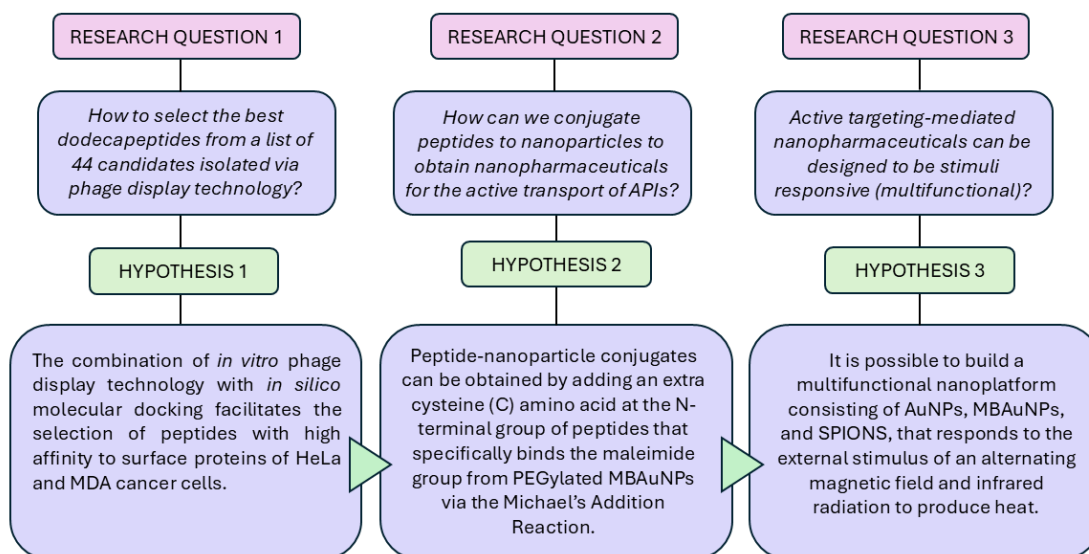


Figure 1.1: Research questions and hypotheses considered in the research thesis.

1.3 Justification

Nanotechnology fast-tracks R+D of nanopharmaceuticals that intent to deliver APIs in the tumor tissue via passive targeting and improve chemotherapy.²⁰ Nanotechnology has enabled nanopharmaceuticals to reach the market for clinical use and remain an exciting topic of research, such that the number of them in advanced-stage clinical trials is continuously growing.^{5,21} However, R+D in this field have to be shifted from passive targeting-mediated monomodal products to active targeting-mediated multifunctional products able to overcome multidrug resistance, a challenge that passive-mediated nanopharmaceuticals have not yet overcome.⁵

Active targeting can be achieved by functionalizing cell-related peptides selected via phage

display technology onto the surface of the nanopharmaceuticals.²²⁻²⁴ Phage display uses libraries of DNA phages whose genome contains many inserts encoding a collection of polypeptides fused to a phage coat protein.²⁵ Polypeptides selected from phage libraries specifically bind cell-surface proteins.²⁶ In this regard, Kalimuthu et al. used a phage library to isolate A20 murine lymphoma cell-specific heptapeptides and functionalize them to AuNPs, prolonging peptide half-life and causing cytotoxic effects towards the target cancer cells.²⁷ Ruks et al. conjugated ultrasmall AuNPs with peptides that selectively bind HeLa cells and facilitate cell membrane penetration and location of the nanoparticles in the cytosol.²⁴

On the other hand, inorganic nanomaterials, such as gold nanoparticles, silica nanoparticles (SiO_2), and SPIONs, have demonstrated to be excellent materials to design stimuli responsive nanoplateforms. For instance, AuNPs exhibit photothermal capacity, which can be leveraged to obtain active targeting-mediated multifunctional nanopharmaceuticals.^{2,28} SiO_2 nanoparticles have demonstrated a high APIs loading capacity, attributed to their high surface-to-volume ratio, and SPIONs allow for magnetothermal ablation and magnetic-mediated accumulation in the tumor.²⁰ Ji et al. synthesized and characterized hybrid nanoplateforms with a SPIO core, coated with a primary and a secondary layer of silica and gold, respectively.²⁹ These hybrid nanoparticles showed efficient photothermal effects when exposed to NIR radiation, which makes it possible to direct them to tumors through the application of an external magnetic field.²⁹ In addition, AuNPs can induce surface heat flux upon absorption of NIR radiation, thanks to their LSPR effect.²⁸ Abdallah and Al-Haddad used this property to reduce the viability of cancer cells by exposing CAL-51 (human breast cancer) and HBL-100 (breast nontumorigenic) cell lines to SPIONs@Au@ SiO_2 CSNS.³⁰ The authors reported 70% of tumor cell death in CAL-51 and 13% in HBL-100 when exposed to 50 $\mu\text{g/mL}$ of CSNS, 70% of tumor cell death in CAL-51 and 20% in HBL-100 under NIR radiation, and 80% in CAL-51 and about 30% in HBL-100 when alternating magnetic fields were used.³⁰ Maximenko et al. encapsulated SPIONs in a continuous SiO_2 shell decorated with AuNPs and immobilized the anticancer drug cisplatin on the surface of the CSNS to test cytotoxicity in two colon cancer cell lines (SW480 and SW620), demonstrating 43-50% cell viability reduction when NIR radiation was used.³¹

1.4 Research Objectives

1.4.1 General Objective

This study aims to develop active targeting-mediated multifunctional nanostructures that specifically target cell surface proteins in HeLa and MDA cancer cells and comprise photothermal and/or magnetothermal properties activated after exposure to NIR radiation and an alternating magnetic field, respectively.

1.4.2 Specific Objectives

- Identification of overexpressed cell-surface proteins in HeLa and MDA via a systematic review of their global protein profiles.
- Molecular docking-based selection of peptides with high affinity to cell-surface proteins of HeLa and MDA cells, from a list of 44 candidates isolated via phage display technology.
- Peptide chemical synthesis using SPPS and purification via HPLC.
- Characterization of the physicochemical properties of the synthesized peptides employing ultraviolet-visible spectroscopy (UV-VIS) spectroscopy and fourier transform infrared spectroscopy (FTIR).
- Chemical synthesis of MBAuNPs and characterization of their physicochemical properties, such as size, shape, crystal structure, hydrodynamic diameter, absorbance, and surface chemistry.
- Implementation of a DoE to achieve the optimal functionalization of the synthesized peptides to the MBAuNPs, to constitute the systems MBAuNPs@P1/P2/P3, and their characterization using UV-VIS and FTIR.
- Implementation of an adequate protocol to synthesize the systems $\text{Fe}_3\text{O}_4@\text{SiO}_2$ (CSNS), $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{Au}$ (CSNS@Au), and $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{MBAu}$ (CSNS@MBAu), and characterization of the physicochemical properties and functionality.

1.5 Scope and Limitations

Diagram 1.2 summarizes the scope and limitations of the thesis by considering each research question and hypothesis. Starting from a list of 44 dodecapeptides identified using phage display technology, we aim to select the best candidates to reduce the required resources during the chemical synthesis of peptides, such as reactants, cost, and time. Due to the difficulties to perform immunochemical assays to select the best peptides, we propose the use of *in silico* molecular docking to support the selection of successful candidates by scoring the binding modes between the peptide sequences and potential targets as function of the number and type of interactions. Another limitation arises during the identification of the potential targets, since one of methods employed to achieve this purpose includes *in vitro* experiments using magnetic beads exposed to cell lysates, followed by characterization with mass spectrometry. However, we circumvent this challenge by performing a systematic literature review of HeLa and MDA global protein profiles to select potential overexpressed receptors to which peptides bound during phage display. Once the successful peptide candidates were identified, they were synthesized using SPPS. However, three major limitations arised during this stage: 1) the limited access to the peptide synthesis equipment available at IPICYT; 2) delays due to strict regulations for the purchase of reagents necessary for the synthesis, but considered dangerous; and 3) the customs procedures that delayed the arrival of synthesized peptides to the laboratory.

While the mentioned limitations were overcome, we decided to move forward with the creation of a DoE to achieve the functionalization of peptides on the surface of nanostructures (e.g. MBAuNPs) to obtain active targeting-mediated nanopharforms. The DoE proposes a Michael's Addition Reaction between methoxy polyethylene glycol maleimide (PEG-MAL) and the cysteine aminoacid inserted in each peptide in presence of the zwitterionic organic chemical buffer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The amino acid cysteine was used to establish the DoE, while peptide arrives to the laboratory.

Finally, once peptide-nanostructure conjugates were achieved, we explored in the desing of multifunctional nanostructures, including AuNPs, MBAuNPs, and CSNS. This stage includes the synthesis and characterization of their physicochemical properties. We did not found technical limitations during the synthesis and characterization since all the resources were available on-

site. Limitations were found during functionality characterization, since there is no equipment available at IPICYT for measuring photothermal and magnetothermal properties. However, these limitations were overcome thanks to the collaboration with Dr. Manuel Cano from the University of Guadalajara, who allowed us to perform calorimetry measurements of magnetic samples.

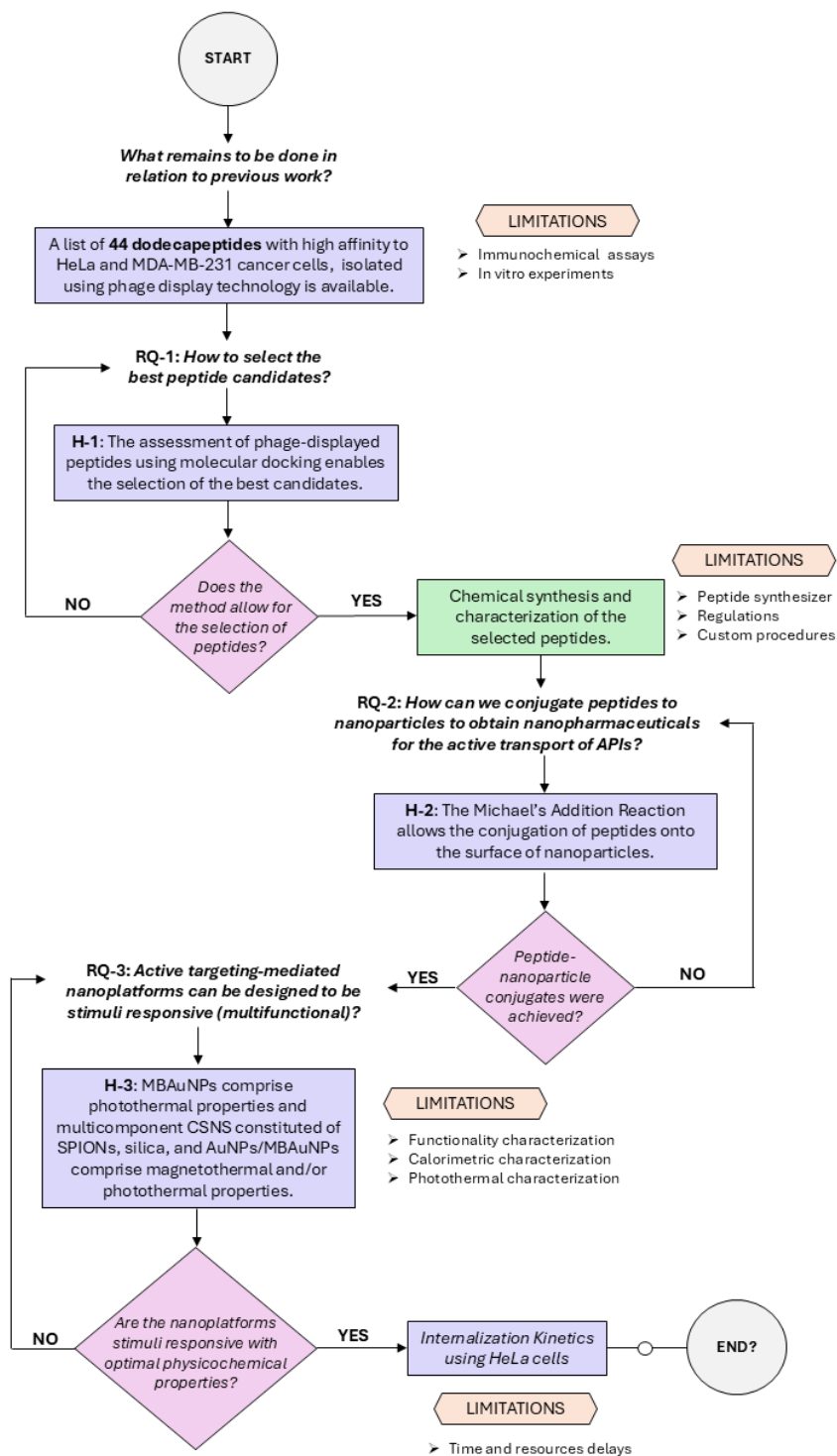


Figure 1.2: Limitations and scope of the project.

Chapter 2

Nanotechnology-based Pharmaceuticals

Nanomedicine, the application of nanotechnology for pharmaceutical and biomedical purposes, has enabled the development of innovative medicinal products for disease diagnostics, treatment, and prevention.^{32–35} According to the European Commission and the FDA, a nanopharmaceutical, nanomedicine, or nanocarrier contains at least one dimension (internal or external) of its components (excipient or active substance) in the nanoscale range of 1 nm to 100 nm (Fig. 2.1 and SI:A.1).^{32,36,37} Nanomedicine covers a broad range of applications including diagnosis (e.g., magnetic resonance imaging (MRI)), ultrasound, and optical (fluorescence) imaging; treatment (e.g., chemotherapy and photodynamic therapy); and prevention (e.g., virosomes and sanitizers).^{38,39}

2.1 Nanopharmaceuticals Approved for Marketing

A literature review of a hundred nanopharmaceuticals approved by the FDA and/or the European Medicines Agency (EMA) revealed that most of the registered nanopharmaceuticals use existing APIs whose pharmacokinetics, pharmacodynamics, biodistribution, safety, and efficacy have been enhanced (SI: A.1).^{32,34,40} Figure 2.2A shows the seven classes of nanopharmaceuticals: liposomes, inorganics, polymers, nanocrystals, micelles, protein-based nanopharmaceuticals,

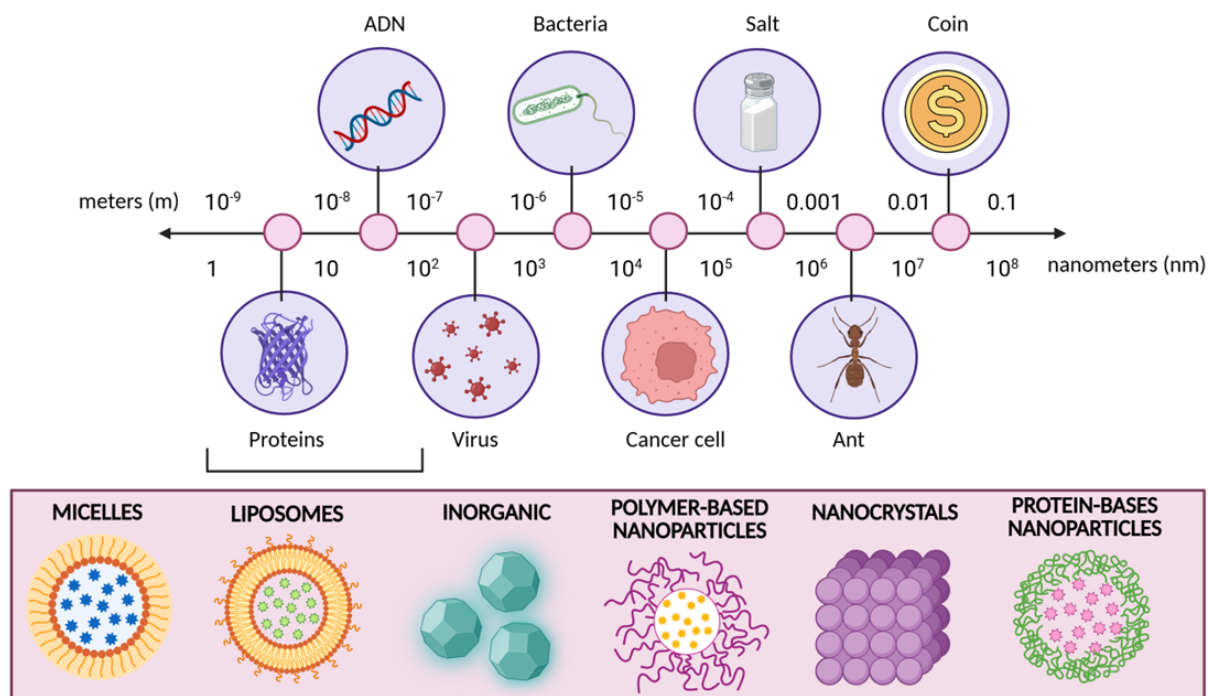


Figure 2.1: Schematic representation of the nanometric scale. Nanopharmaceuticals have a size in the range of 1 nm to 100 nm (or 10^{-9} m to 10^{-8} m)

dendrimers, and microbubbles *.^{34,41} The target disorders to treat include: cancer, chronic kidney diseases, bone diseases, mental disorders, viral infections, and emetic symptoms, among others (Fig. 2.2B).

Nanotechnology has become a growth technology in the pharmaceutical industry.⁴² Over the past few decades, the trend of nanopharmaceuticals approved by the FDA and/or EMA has increased continuously (Fig. 2.2C). Since Diprivan's approval in 1989, more than a hundred nanopharmaceuticals are currently available in the market, and several others are in clinical trials.^{32,43}

*Microbubbles are considered nanopharmaceuticals since their properties fall within the FDA definition for being considered as such: "[...] products outside the nanoscale range of 1-100 nm that can also exhibit similar properties (to nanomaterials) or phenomena attributable to a dimension(s)"

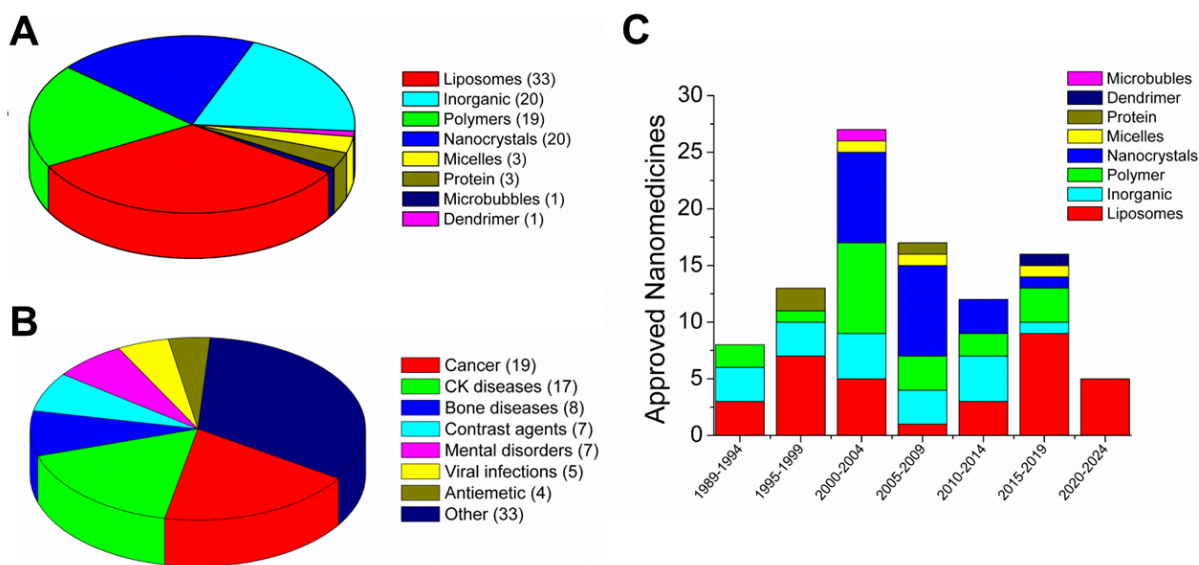


Figure 2.2: Overview of the FDA and EMA approved nanopharmaceuticals. **A.** FDA and EMA-approved nanopharmaceuticals categorized by type. **B.** Major groups of diseases treated by the approved nanopharmaceuticals. **C.** Trends in FDA and EMA nanopharmaceuticals approvals by five-year period, covering from the FDA approval of Doprivan in 1989 to 2024, including the last approvals of the COVID-19 liposomal vaccines in 2021.

2.1.1 Nanopharmaceuticals for Cancer Diagnosis and Treatment in Clinical Use

The International Agency for Research on Cancer estimated that nearly 20 million new cases of cancer, alongside 9.7 million deaths, occurred in 2022.¹⁶ Cancer treatments include surgery complemented by chemotherapy and/or radiotherapy.⁴⁴ However, surgery is contraindicated to remove small tumours, radiotherapy can damage healthy cells, organs, and tissues, and chemotherapy uses highly toxic non-selective small molecules with poor drug accumulation in the tumor tissue, low bioavailability, and short circulation time, causing systemic toxicity and multidrug resistance.^{9,18,35}

Cancer encompasses a wide variety of diseases, all of which are characterized by the abnormal and uncontrolled growth of heterogeneous populations of cells, involving genetic and metabolic complexities, tumor microenvironment changes, and different sensitivity to treatment.^{2,3,44} Nan-

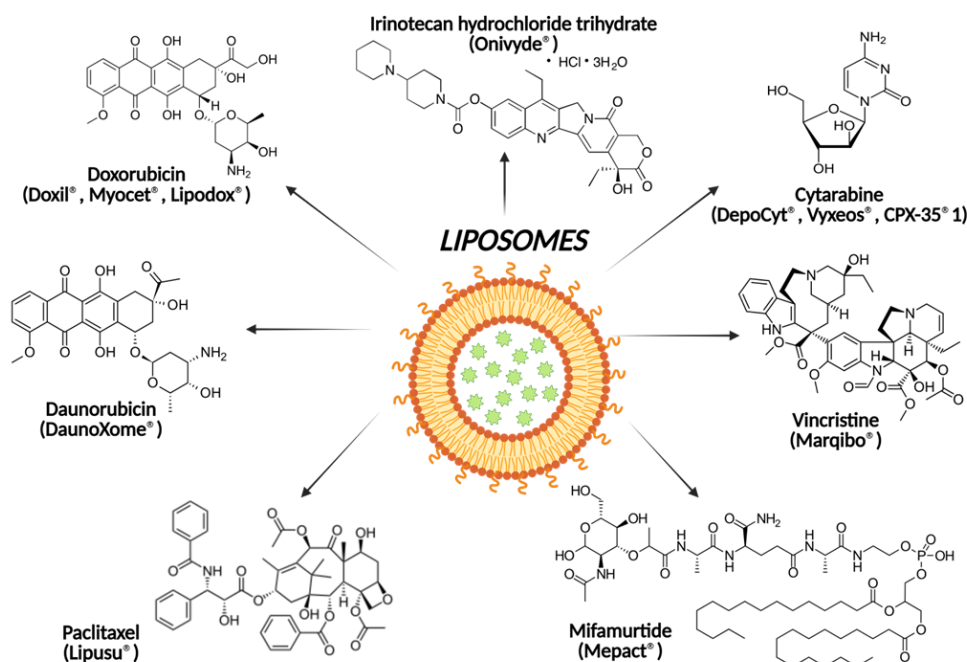


Figure 2.3: Schematic representation of approved nanopharmaceuticals with liposomal formulations for cancer treatment. The APIs are represented as green stars enclosed in the liposome.

otechnology has offered the possibility of modifying the release mechanism of existing APIs with poor solubility, high toxicity, and low absorption, to confer them with improved site-specificity, circulation time, bioavailability, and sustained release.^{35,44,45} For example, the anticancer drug Doxorubicin (Fig. 2.3) was engineered into the liposomal nanoformulated form Doxil[®] (also known as Caelyx[®], Evacet[®], and Lipodox[®]) and approved by the FDA for the treatment of Kaposi's sarcoma (1995), ovarian cancer (1999), and multiple myeloma (2007).^{38,42} Due to the conjugation of the 90 nm-liposome surface with polyethylene glycol polymer (PEGylation), the circulation half-life was increased, thus improving bioavailability and reducing the side effects when compared with the non-encapsulated drug.^{1,42}

Daunorubicin, another anticancer drug used for the treatment of HIV-associated Kaposi's Sarcoma, was encapsulated in a 45 nm-liposome and commercialized as DaunoXome after FDA approval in 1996. The liposomal form of Daunorubicin shows fewer side effects, attributed to the

increased concentration of the drug in tumors.^{38,42} The anticancer drugs Paclitaxel, Irinotecan, Cytarabine, Vincristine, and Mifamurtide were also engineered into the liposomal nanoformulated forms Lipusu® (FDA-2016), Onivyde® (FDA-2015), DepoCyt® (FDA-2007), Marqibo® (FDA-2012), and Mepact® (EMA-2009), respectively, to improve circulation time, drug biodistribution, and increase passive delivery to tumors.^{38,42}

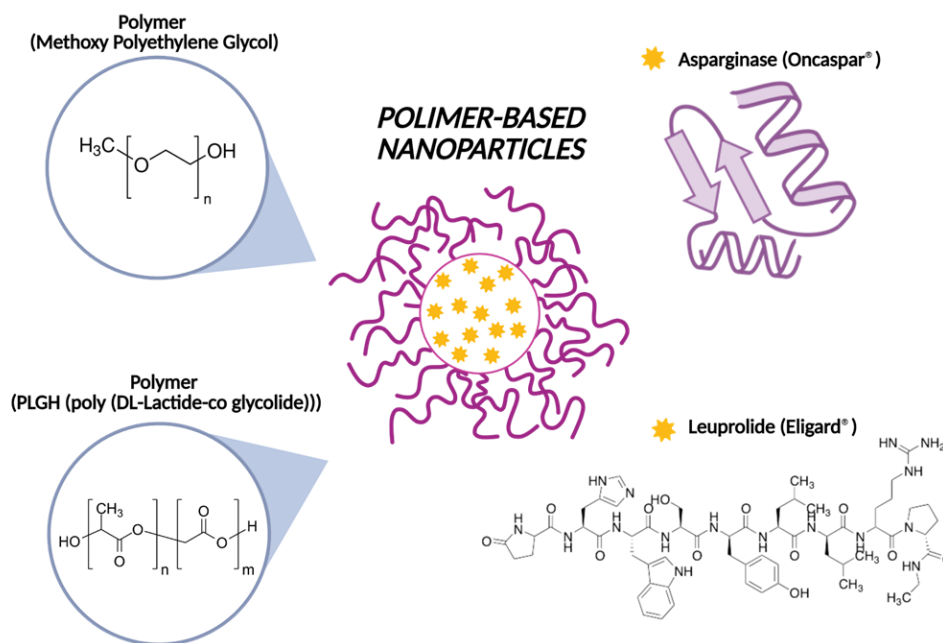


Figure 2.4: Schematic representation of FDA-approved polymer-based nanopharmaceuticals for the treatment of cancer.

Polymer-based nanopharmaceuticals incorporate APIs into their inner hydrophobic core.³⁵ The synthetic polymer polyethylene glycol is extensively used to conjugate APIs to its polymeric chains (Fig. 2.4).⁴² Oncaspar® (FDA-1994) consists of PEGylated-L-asparaginase and is employed in the treatment of acute lymphoblastic leukemia and chronic myelogenous leukemia.⁴² Due to PEGylation, L-asparaginase exhibited a longer half-life, which enables dose reduction.⁴² The nanopharmaceutical Eligard® (FDA-2002) was designed using the polymer poly(DL-lactide-co-glycolide) conjugated with the palliative agent leuprolide acetate for the treatment of prostate cancer.⁴²

The binding of proteins to APIs has been used as a strategy to mitigate their toxic effects.⁴² A good example of this type of nanoformulation is the protein-based nanopharmaceutical Abraxane® (Fig. 2.5), which consists of albumin nanoparticles bound to paclitaxel and was approved by the FDA for the treatment of breast cancer (2005), lung cancer (2012), and pancreatic adenocarcinoma (2013).⁴² Abraxane exhibits higher response rates, longer time to tumor progression, and lower incidence of neutropenia and sensory neuropathy compared to Taxol, a pharmaceutical containing lipophilic paclitaxel that requires the emulsifier and solubilizer Cremophor EL[†] to make it suitable for injection.⁴²

Polymeric micelles are CSNS composed of amphiphilic block copolymers to encapsulate APIs with poor solubility.³⁵ Genexol-PM® (FDA-2007) was designed to deliver paclitaxel for the treatment of breast cancer, non-small-cell lung cancer, and ovarian cancer.^{35,46} The encapsulation of paclitaxel in a polymeric micelle (Fig. 2.5) reduces the toxicity associated with Cremophor EL.³⁵

Utilizing the high atomic number (Z) of certain inorganic nanoparticles, innovative methods have been developed for oncological applications.⁴⁶ For example, the high-Z element hafnium (Z=72) was used to develop Hensify® , a radiosensitizer administered via direct intratumoral injection, which releases energy when exposed to electromagnetic radiation and increases the efficacy of radiotherapy.⁴⁶ Resovist/Cliavist (EU-2001), Feridex/Endorem (FDA-1996), Gastro-MARK/Umirem (FDA-2001), and Ferumoxtran-10 (Nederland-2013) are SPIONs approved for contrast-enhanced MRI.⁴⁷

2.1.2 Novel Applications of Nanomedicine

Cancer Detection and Diagnosis

Successful treatment of cancer requires effective primary detection and diagnosis.⁴⁸ Cancer nanomedicine enables the improvement of imaging methods, such as optical coherence tomography, MRI, or photoacoustic imaging, as well as the design of highly sensitive and selective

[†] polyoxyethylated castor oil

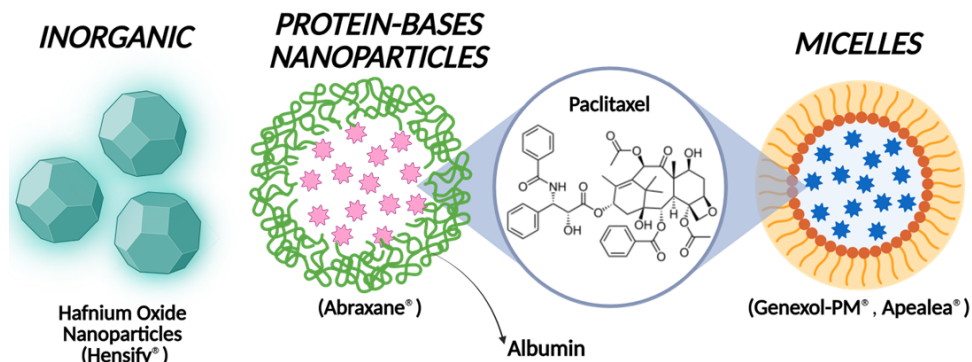


Figure 2.5: Micellar, inorganic, and protein-based nanopharmaceuticals approved by the FDA for cancer treatment.

biosensors.^{48,49}

Optical coherence tomography is a non-invasive and non-contact imaging method that measures optical path length differences from backscattered light of tissue layers (interferometric detection).⁵⁰ In cancer detection, optical coherence tomography enables visualization of tissue structural abnormalities during real-time biopsy and surgical procedures; however, it has limited imaging depth (1-2 mm) attributed to the high scattering of NIR light in biological tissues and limited specificity in differentiating between dysplastic lesions and tumor tissues, or highly proliferative and necrotic areas.⁵⁰ Nanomedicine promises to overcome these limitations by integrating metallic nanoparticles conjugated with ligands targeting surface proteins of cancer cells as contrast agents to enhance the homogeneous distribution of nanoparticles in the tumor, discriminating healthy tissues, and thus addressing the lack of site-specific imaging and the limited penetration depth.^{49,50}

MRI measures the magnetization of hydrogen atoms in the tissue's water molecules, providing an anatomical image based on changes in proton magnetization.⁴⁹ MRI leverages SPIONs as contrast agents to retain them in the tumor microenvironment because of defective lymphatic drainage.⁵¹ However, image visibility is reduced contrast between the biological background and the cancer tissue. Similar to optical coherence tomography, proper conjugation of the nanoparticles with ligands targeting cell surface proteins enhances nanoparticle localization in

tumor, increasing SPIONs accumulation and improving imaging contrast.

In photoacoustic imaging, tissue molecules are excited after energy absorption from a pulsed laser irradiation, and return to the basal state (ground state) when the stimulus is turned off.⁵² During its return to the basal state, molecules release energy in the form of heat, which induces thermoelastic expansion of the surrounding tissues, producing acoustic waves that are captured by an ultrasound transducer.⁵² Photoacoustic imaging has the potential to enable lymphatic imaging, but observing the lymph nodes and lymphatic circulation remains challenging due to the lack of endogenous contrast agents with NIR absorption.⁵² Quantum dots, polymer-based nanoparticles, and metallic nanoparticles demonstrate excellent NIR absorption capabilities for effective photoacoustic imaging.⁴⁸

Nanotechnology has also empowered the design of highly sensitive and selective nanochips to detect biomarkers of cancers.^{48,51} The small size and high surface area-to-volume ratio of nanochips enhance their sensitivity, enabling the detection of low concentrations of biomarkers and even their real-time monitoring to evaluate disease progression and therapeutic effectiveness.⁴⁸

Cancer Treatment

Besides improving the delivery of traditional anticancer APIs, nanomedicine facilitates the translation of innovative therapies into clinical and preclinical use (Fig.2.6).⁴⁶ Nucleic acid therapy, including siRNA[‡], mRNA[§], and gene delivery, leverages nanomedicine through nanocarriers to overcome transfection, immune recognition, and degradation by stabilizing and delivering nucleic acids into cells.^{53,54}

Novel nanotechnology-based therapies, such as hyperthermic (heat) or hypothermic (cold) thermal ablation of tumors, e.g. photothermal therapy, cryosurgery, magnetic hyperthermia, and photodynamic therapy, offer new opportunities for precision medicine, allowing localized cancer

[‡]Small interfering ribonucleic acid inhibits gene expression by silencing genes.

[§]Messenger ribonucleic acid carries genetic information from DNA to the ribosomes, where proteins are synthesized.

treatment.^{2,9} Cryosurgery is a hypothermic method that kills cells by forming ice crystals during cooling.⁹ Photodynamic therapy is a non-invasive technique that utilizes photosensitizers[¶] to generate reactive oxygen species, which in turn kill cancer cells.⁵⁵ However, cryosurgery fails to distribute the ice crystals to cover all the tumor tissue due to the irregular tumor shape, and photosensitizers lack specificity.^{49,55} In this regard, nanoparticles can be exploited in both techniques to overcome these challenges; cryosurgery may employ them to increase thermal conductivity in peripheral areas, and photodynamic therapy may take advantage of the poor lymphatic drainage in the tumor microenvironment, which causes the long-term and targeted accumulation of the nanoparticles at the tumor.^{49,55} Photothermal therapy and magnetic hyperthermia are novel hyperthermic methods for cancer treatment that employ nanoparticles to cause tumor cell damage.² Although innovative, the main challenge in the clinical translation of these techniques is the lack of tumor selectivity, which limits their effectiveness.⁵⁵

Cancer Prevention

Cancer immunotherapy is used to treat cancer and prevent metastasis and recurrence by delivering tumor-specific antigens to immune cells to recognize cancer cells, thereby generating an immune response to destroy them and achieve a long-lasting therapeutic effect.^{18,51,54} To induce anti-cancer immunity, tumor antigens must be effectively delivered to lymph nodes, specifically to antigen-presenting cells, which produce tumor-specific antigens, also called neo-antigens.¹⁸ However, the side effects of immunotherapeutics, such as high blood pressure, nausea, diarrhea, and fatigue, hinder the success of immunotherapy.⁵⁵ Nanoparticles enable the delivery of immunomodulatory agents to the lymph nodes and protect tumor antigens from degradative enzymes, thus improving therapeutic efficacy and minimizing dose-dependent systemic toxicity.⁵⁵

[¶]A photosensitizer is an agent that after being excited by light at a specific wavelength, reacts with the molecular oxygen to create reactive oxygen species (ROS) in the target tissue, resulting in cell death.

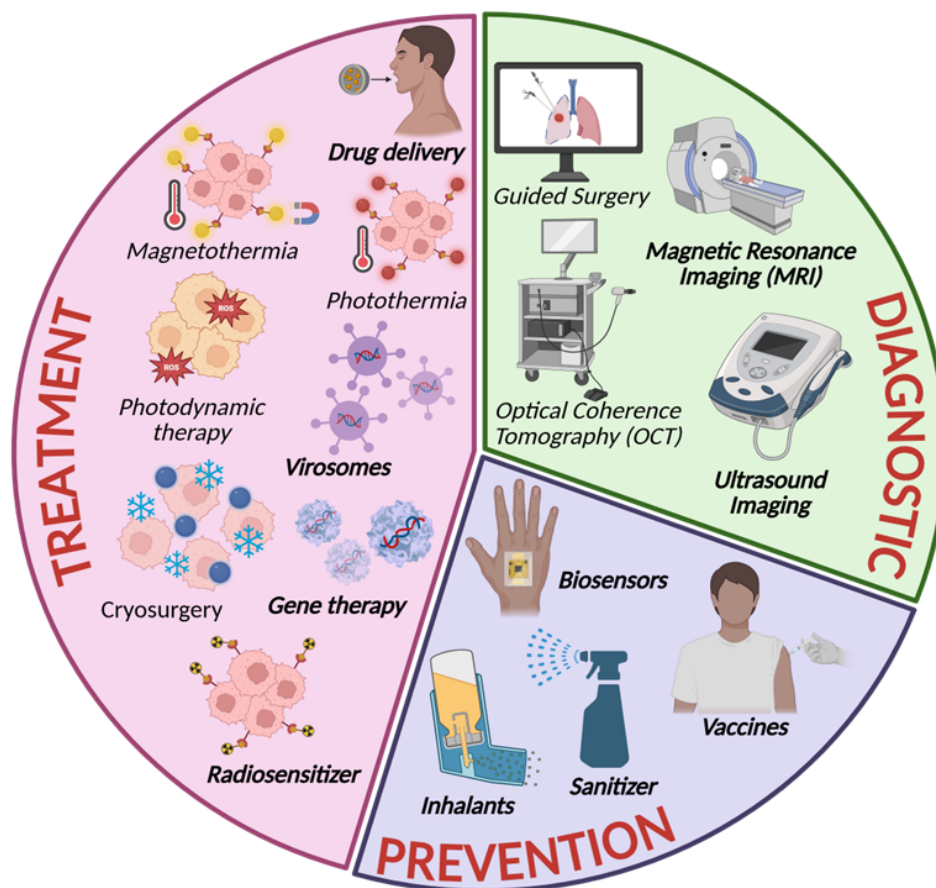


Figure 2.6: Cancer nanomedicine applied in cancer diagnosis (imaging and guided surgery), prevention (vaccines, inhalants, biosensors, and sanitizers), and treatment (drug delivery, magnetothermia, photothermia, photodynamic therapy, gene therapy, radiotherapy, and cryosurgery). Nanotechnology- based treatment methods shown in **bold** are currently available for clinical use.

2.2 Targeting Mechanisms of Nanopharmaceuticals

Instead of reaching a site-specific accumulation in tumor tissue, small molecular APIs distribute throughout the body without discriminating between healthy and tumor tissue, causing severe side effects. In contrast, nanoparticles benefit from the properties of the tumor microenvironment to accumulate specifically in the tumor by means of passive or active targeting.⁵⁶ Thanks to passive transport of drugs, nanopharmaceuticals have obtained marketing approval, since they

have demonstrated improved therapeutic profile and reduced systemic toxicity compared to the therapeutic profile of non-encapsulated APIs.

2.2.1 Passive Targeting Mechanism

Passive targeting is the cornerstone for the development of cancer nanopharmaceuticals.^{57,58} It leverages the EPR effect and the conditions of tumor microenvironment, which enable passive accumulation of nanopharmaceuticals at the tumor site (Fig. 2.7).^{1,56,57} In contrast to conventional small molecular APIs, which diffuse freely into healthy tissues and organs, causing systemic toxicity, nanopharmaceuticals accumulate within tumor tissue due to the lack of lymphatic drainage and the disrupted dynamic transport of interstitial fluid.^{49,55–58} The targeting mechanism of most FDA or EMA-approved nanopharmaceuticals relies on the EPR and tumor microenvironment-mediated passive tumor targeting.^{56,58} Doxil®, DaunoXome®, Onivyde®, DepoCyt®, and Abraxane®, are some examples.⁴²

Passive targeting, mediated by the EPR effect, enhances the effectiveness of nanopharmaceuticals by increasing site specificity and minimizing systemic toxicity.⁵⁹ However, the EPR effect has heterogeneous pathophysiological features among patients and tumor types, in terms of tumor blood flow, type, hypoxic areas, vascular permeability, location, size, stage, microenvironment, vascular density, and status of blood perfusion, which limits precision cancer treatment.^{56,58,59} Moreover, the physicochemical properties of the nanopharmaceuticals, such as charge, size, and surface modifications, play a critical role in leveraging EPR-mediated passive targeting.⁵⁸ Therefore, nanopharmaceuticals design must effectively address site-specific accumulation, cellular uptake, and subcellular localization.⁵⁶

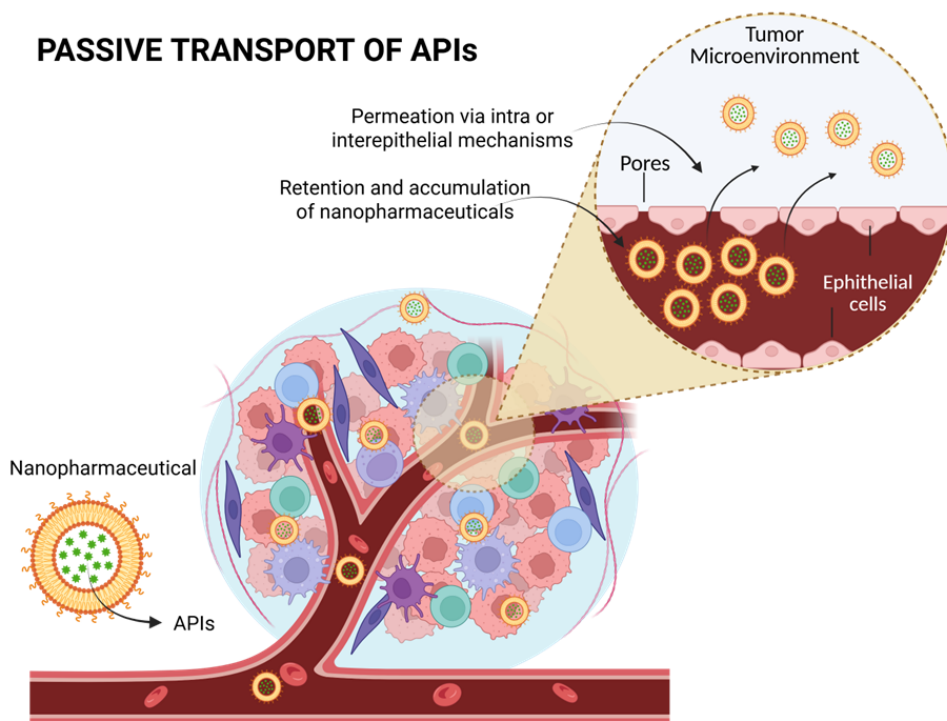


Figure 2.7: Passive transport of nanopharmaceuticals.

2.2.2 Active Targeting Mechanism

Active targeting relies on selective binding of nanopharmaceutical to tumor, thereby sparing healthy tissues.⁴⁸ Selective binding is achieved by functionalizing the nanopharmaceuticals with targeting ligands, such as antibodies, peptides, aptamers, proteins, carbohydrates, and small molecules, that selectively bind to overexpressed proteins at the cell surface, usually resulting in receptor-mediated endocytosis, thus enhancing tumor tissue retention, cellular uptake, and internalization of the nanopharmaceutical (Fig. 2.8).^{51,56,59}

Currently, there are no FDA or EMA nanopharmaceuticals based on active targeting.⁴⁷ Nevertheless, some liposomes, polymer-based nanoparticles, and protein-based nanoparticles using active targeting have entered the clinical stage; liposomal C225-ILS-DOX (FDA-Phase I) was de-

signed to target the overexpressed endothelial growth factor receptor (EGFR) in tumors; polymer-based CT-322 (FDA-CS) targets the vascular endothelial growth factor receptor 2 (VEGFR-2), and CALAA01 (FDA-Phase I) targets human ribonucleotide reductase M2 (RRM2).⁴⁷

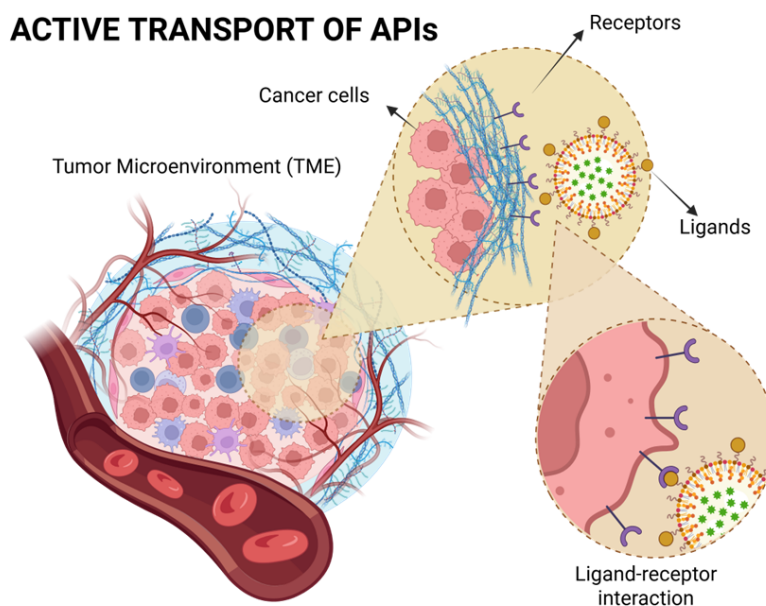


Figure 2.8: Active transport of nanopharmaceuticals.

2.3 Challenges and Research Opportunities

So far, nanotechnology-based medicinal products on the market or in clinical trials have demonstrated advantages in diagnosis, treatment, and prevention of diseases, particularly cancer. In diagnosis, nanoformulations have improved imaging signals and enabled the development of new imaging and detection technologies.⁵¹ In treatment, nanoformulations have overcome the limitations of conventional drugs, such as low aqueous solubility, rapid clearance by the liver and kidneys, and drug resistance.⁵¹ Moreover, nanotechnology has enabled the development of innovative methods of cancer treatment, such as photothermal and magnetothermal therapy, and targeted drug delivery.⁵⁴

Despite these advances in nanotechnology-supported cancer treatment, challenges remain. Monomodal cancer treatments are unable to eliminate the entire tumor and prevent metastasis due to the heterogeneous tumor microenvironment, as demonstrated by long-term use of anticancer drugs, which induce multidrug resistance.⁵⁴ Multimodal therapy addresses these challenges, leading to more efficacious anticancer treatment through the design of single nanoplateforms comprising multiple functionalities.⁵⁴ The multifunctional mechanism of action may begin with effective binding of the nanoplateform to overexpressed proteins on the surface of cancer cells (active targeting) to proceed with imaging of the tumor environment (e.g. contrast agents), and finally apply an external stimulus (e.g. NIR radiation) to increase the sensitivity of cancer cells for subsequent delivery of the anticancer drugs (Fig. 2.9).⁵⁴

Although multifunctional nanoplateforms appear promising, their design involve complex nanostructured systems that affects large-scale production and complicates clinical translation.⁴⁷ Therefore, the design of multifunctional nanoplateforms should follow a Quality by Design (QbD) approach as encouraged by the FDA and the International Conference on Harmonization.⁴⁷

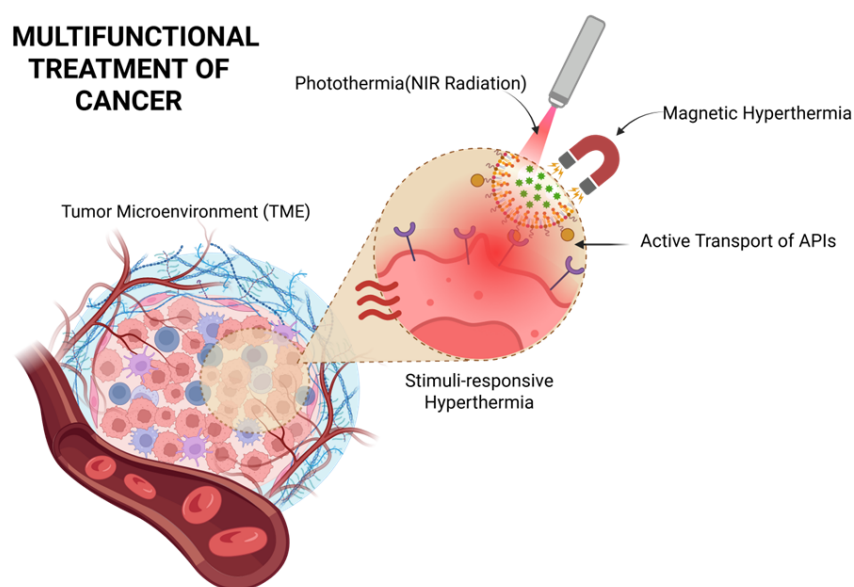


Figure 2.9: Multifunctional treatment of cancer.

Chapter 3

Design of Nanopharmaceuticals

For a nanopharmaceutical to be approved by the FDA or EMA, it must demonstrate optimized formulation of APIs that have already been approved, which implies a meticulous and expensive process of R+D^{32,36} As shown in Diagram 3.1, the FDA process for a new drug to be approved includes the following steps: 1) basic research to study and prototype the nanoformulation with optimal physicochemical properties confirmed by standardized characterization methods and required biological functions established via in vitro studies; 2) via preclinical testing in an animal model, the pharmacokinetic and pharmacodynamic profile, the deposition, exposure, dose range, toxicity, and immunogenicity are evaluated to establish the therapeutic efficacy and potential toxicities; 3) FDA regulatory submission for clinical development, which requires detailed documentation of previous research; 4) assessment of the dosing, toxicity, and excretion in a small group (phase I); 5) evaluation of the safety and efficacy in a larger group (phase II); and 6) multi-center, randomized and placebo trials in diverse populations (phase III). Following a regulatory review of the clinical results, a decision is communicated regarding approval, rejection, or requests for additional information. An additional Phase IV includes post-marketing changes.^{36,60} The scope of this study covers the design of multifunctional active targeting-mediated nanoplateforms (step 1), so we will emphasize on the synthesis and characterization of their physicochemical properties and functionalities.

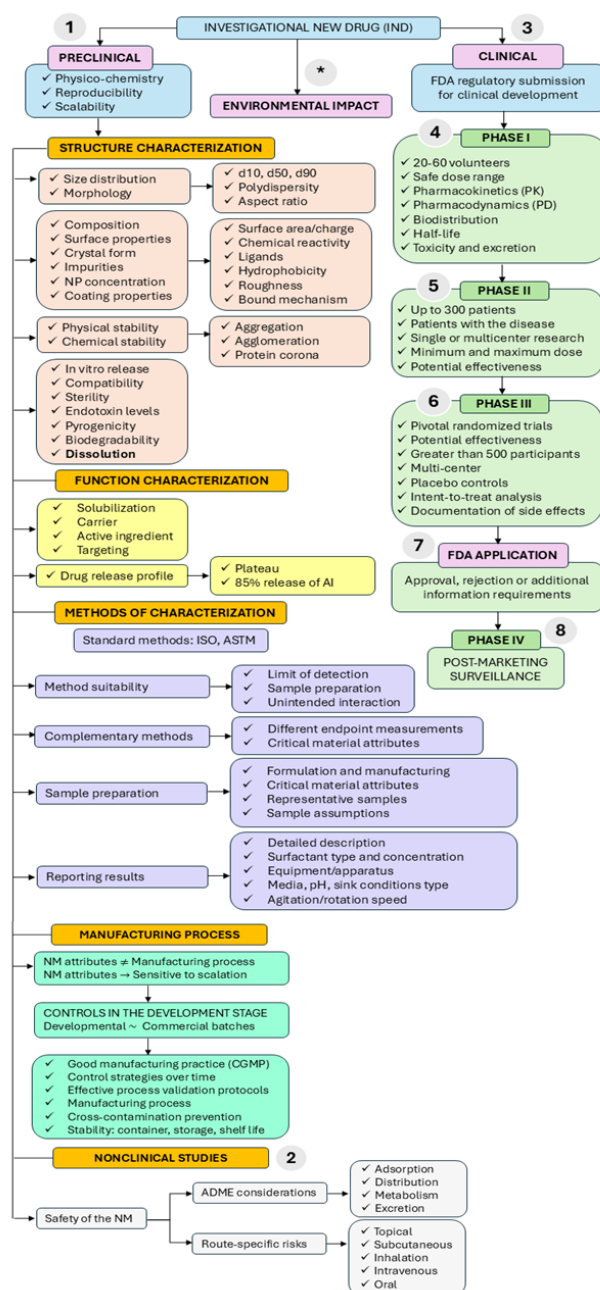


Figure 3.1: The FDA premarketed recommendations to register a nanomaterial-based Investigational New Drug (IND). The process includes the preclinical (steps 1 and 2), clinical (steps 4 to 6), and the post-marketing phases. Environmental impact studies are included in the diagram. The diagram was designed by the author based on information from the references 34, 58 and 59.^{36,60,61}

3.1 Mechanism of Intratumoral Targeting

To achieve intratumoral delivery of anticancer APIs, it is essential to have full control over the nanopharmaceutical transport in the body.³² For this purpose, it is important to understand the fundamental concepts of how nanoparticles interact with biological systems after systemic administration and the barriers that prevent them from reaching the target sites.⁶² Nanopharmaceuticals may enter the body by oral administration, inhalation, intravenous, intradermal, and intramuscular injection, after which they will traverse biological barriers during their journey to the target site.⁶³ Most of the FDA and EMA-approved nanopharmaceuticals are administered via intravenous injection, so only the biological barriers encountered in this route will be considered.⁴²

3.1.1 Endocytosis via Mononuclear Phagocytic System

The mononuclear phagocyte system is the first barrier that nanopharmaceuticals encounter following intravenous injection.⁶⁴ The system is constituted by macrophages located in different organs, primarily in liver and spleen, along with blood monocytes and dendritic cells.⁶⁴ Upon administration, nanopharmaceuticals interact with serum proteins*, particularly albumin, immunoglobulin, fibrinogen, and opsonin proteins, acquiring a protein corona that alters their physicochemical properties and endows the nanopharmaceutical with a new biological identity, replacing the synthetic identity and determining their interaction with the biological system (Fig. 3.2).^{32,62,65} The protein corona increases the hydrodynamic diameter of the nanopharmaceuticals, inducing aggregation and generating immune responses that promote cellular phagocytosis, which consists in the uptake and internalization of particles larger than 0.5 μm by phagocytic cells, particularly Kupffer cells and hepatocytes.^{62,63,66} Phagocytosis is the consequence of an immune response to clear pathogens, diseased cells, and foreign materials unrecognized by the body.⁶³ The protein corona consists of phagocyte cell surface receptors that will enable cellular uptake, followed by entrapment within phagocyte vesicles, and clearance of the nanopharmaceuticals. Phagocytosis of nanopharmaceuticals is mainly mediated by macrophages of the mononuclear phagocyte sys-

*Proteins found in the serum, which is the liquid part of the blood.

tem, which promotes accumulation in the liver and spleen and the subsequent biliary elimination via the stools.⁶⁷

3.1.2 Renal Clearance Pathway

Similar to APIs, nanopharmaceuticals must be metabolized or cleared from the body to prevent accumulation and systemic toxicity.⁶⁷ Compared with the mononuclear phagocyte system, the renal clearance pathway reduces the toxicity of nondegradable nanoparticles, such as noble metal nanoparticles or quantum dots.⁶⁸ The process of renal clearance of nanopharmaceuticals is carried in the kidney, specifically in their basic and structural unit called cortical nephrons, which are constituted by the renal corpuscle, proximal tubule, loop of Henle, distal tubule, collecting ducts, and peritubular capillaries. The process of filtration consists on the transport of blood into the renal corpuscle to the glomerulus, which possesses high blood pressure that filter the nanopharmaceuticals into the Bowman's space.⁶⁸ The filtered nanopharmaceuticals flows from the Bowman's space to the proximal tubules where they are finally eliminated through urinary excretion.^{68,69}

3.1.3 Extravasation to the Tumor Microenvironment

Nanopharmaceuticals that escape endocytosis and avoid renal clearance will pass from the systemic circulation to the tumor vascular system.^{32,70} Due to the high abnormality, increased vascular density, deformities, and irregular branching of the tumor vascular system, nanopharmaceuticals move slowly and even stay stagnant (EPR effect), having enough time to diffuse out of the vessels and cross into the extracellular matrix of the tumor via interendothelial or transendothelial extravasation.^{70,71}

Interendothelial extravasation consists of the transport of the nanopharmaceutical from the tumor vessels into the extracellular matrix through pores or open fenestrations between adjacent endothelial cells.^{32,72} Whereas transendothelial extravasation uses macropinocytosis, clathrin-mediated endocytosis, and the caveolae pathway to translocate the nanopharmaceuticals into the

extracellular matrix using vesiculo-vacuolar mediated transportation.^{72,73}

3.1.4 Intratumoral Targeting

Once the nanopharmaceuticals have crossed the tumor vascular barrier, they have to navigate through the tumor microenvironment to target cancer cells.³² Nanopharmaceuticals have to face the complex stroma (non-malignant cells) and parenchyma (malignant cells) components of the tumor microenvironment, as well as the elevated pressure gradients that affect their flow to the extracellular matrix. The physicochemical properties of the nanopharmaceuticals influence the interaction with malignant cells. In this regard, active targeting may promote the binding of nanopharmaceuticals to cancer cells.

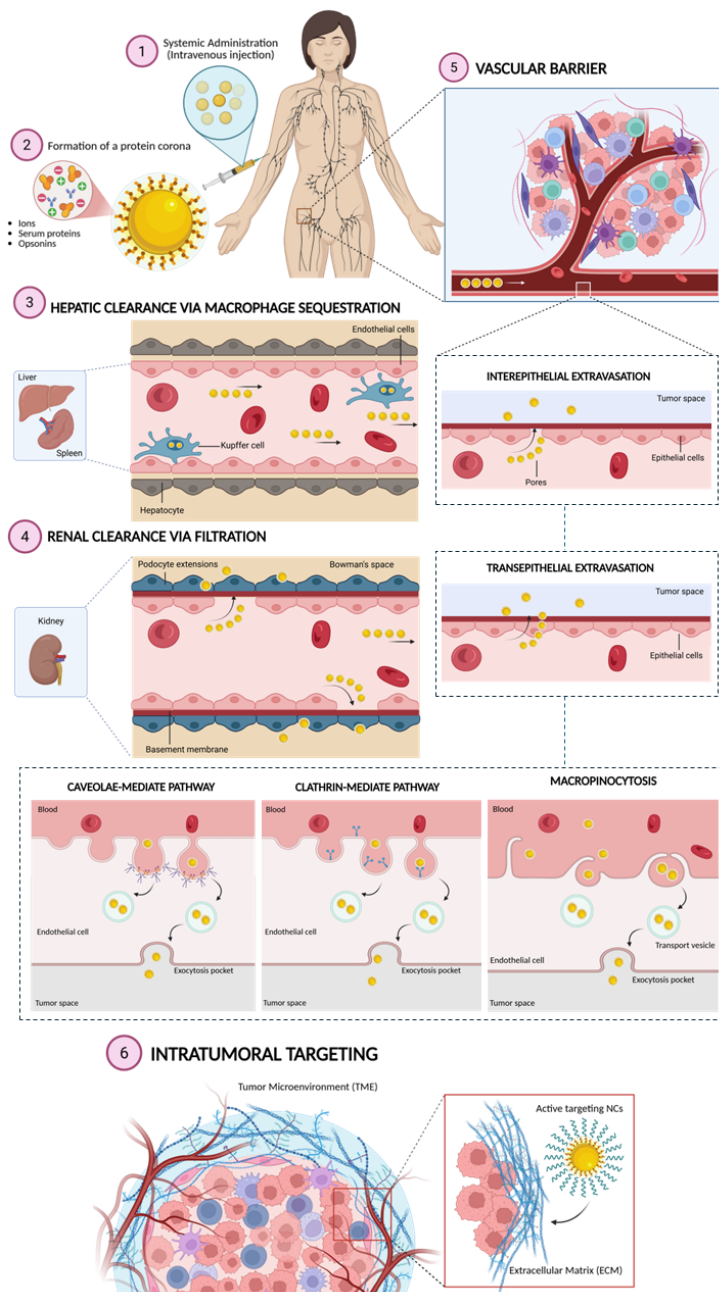


Figure 3.2: Mechanism of intratumoral targeting after intravenous injection of nanopharmaceuticals, including their biological barriers. After intravenous injection (1), a protein corona is formed on nanopharmaceuticals due to the absorption of serum proteins (2). This new biological identity will promote hepatic (3) and renal clearance (4). Nanopharmaceuticals that overcome clearance have to face the vascular barrier (6) to extravasate the tumor microenvironment via interepithelial or transepithelial mechanism. The last barrier consists of the intratumoral targeting (7).

3.2 Physicochemical Properties of Nanopharmaceuticals

Based on the biological barriers previously detailed, the factors that affect the nanopharmaceutical's journey in the body include the biological environment and its biochemistry, and the physicochemical properties of the nanopharmaceutical. In this regard, attributes of nanopharmaceutical that influence intratumoral targeting are detailed below.

3.2.1 Morphological Properties

Size

The morphological characteristics of nanopharmaceuticals, such as size and shape, influence half-life in blood, uptake by immune cells (hepatic clearance), metabolism in the kidneys (renal clearance), escape from the reticuloendothelial system, vascular extravasation, and intratumoral targeting.⁷⁴

Larger nanopharmaceuticals are cleared more rapidly than smaller ones due to macrophage uptake.³² To overcome renal clearance, nanopharmaceuticals must traverse three glomerular layers with a size-dependent filtration selectivity: fenestrated endothelium (70-90 nm); the glomerular basement membrane composed of pores (2-8 nm), and the filtration slits (4-11 nm) bridged by slits diaphragm (43 nm), and finally being reincorporated into the vascular circulation.^{63,67} Combining each layer of the glomerular capillary wall, the pore size is reduced to 4.5 to 5 nm in diameter, known as the kidney filtration threshold.³² Thus, to overcome glomerular filtration, nanopharmaceuticals should be smaller than this threshold.⁶⁹ On the other hand, nanopharmaceuticals with a size in the range of 10 to 20 nm may undergo rapid liver uptake.⁶⁹ Modifying the size may enhance the intratumoral targeting of cancer cells. For example, Setyawati et al. showed that AuNPs of sizes between 10 to 30 nm induce micrometer-sized gaps between endothelial cells, which enable their migration along these interendothelial gaps, avoiding endocytic-independent ways.⁷¹

In general, smaller nanopharmaceuticals have a higher probability of being taken up by the

cells and crossing the vascular barrier after escaping renal and hepatic clearance.⁷¹ However, the interaction of small nanopharmaceuticals with surrounding biomolecules is enhanced, which translates into greater toxicity.⁶³ For example, Chen et al. reported the toxic effect of silver nanoparticles on fish red blood cells and found that 15 nm nanoparticles induce higher oxidative stress and membrane damage due to an increased interaction with blood cells when compared with 50 and 100 nm nanoparticle.⁷⁵

Shape

Nanopharmaceuticals can be designed as nanospheres, nanorods, nanostars, nanowires, nanosheets, nanotubes, nanocubes, etc., which influences their molecular dynamics, hepatic and renal clearance, cellular uptake, and intracellular transport.⁷⁴ Macropinocytosis-mediated cellular uptake has a geometry-dependent correlation in cancer and phagocytic cells. Moreover, it has been proposed that cells possess mechanosensitive processes that sense the nanopharmaceutical aspect ratio and adapt their cellular uptake response. A change in shape from a spherical to anisotropic structure increases the contact area and, consequently, the adhesion to the endothelial wall.⁷⁶

3.2.2 Surface Properties

Surface charge

Due to the adsorption of serum proteins onto the surface of nanopharmaceuticals to form the protein corona, their charge may be normalized to an overall negative potential (-5 mV to -10 mV), which affects their cellular uptake, internalization, and cytotoxicity, since positively charged nanopharmaceuticals are more electrostatically attractive to negatively charged cell membranes.^{62,63} Moreover, surface charge attributed to protein corona indirectly influences the glomerular capillary filtration since the hydrodynamic diameter is increased by 15 nm, reducing glomerular filtration.⁶⁹ On the other hand, charge-selective glomerular filtration is attributed to fixed negative charges within the capillary wall; thus, filtration is increased for cationic molecules.⁶⁹ However, after filtration, cationic nanopharmaceuticals may undergo clearance due to their recognition and

ingestion by macrophages. Surface charge influences intratumoral targeting. Cationic nanopharmaceuticals extravasate the endothelial wall mainly by clathrin-mediated endocytosis, whereas anionic nanopharmaceuticals do so by caveolin-mediated endocytosis.⁶³

Surface chemistry

Protein corona modifies the interaction of nanopharmaceuticals with the biological environment. It promotes nanopharmaceutical recognition by phagocytes, activating clearance mechanisms and decreasing blood circulation times. Surface modifications to prevent serum protein adsorption and reduce phagocyte recognition involve the use of antifouling and hydrophilic polymers, such as polyethylene glycol and zwitterionic polymers.⁵ Intratumoral targeting may be enhanced by ligands on the surface of nanopharmaceuticals, referred to as active targeting. Targeting ligands include peptides, proteins, antibodies, antibody fragments, and nucleic acids. These biomolecules mediate cellular uptake and internalization. Cationic nanopharmaceuticals have more interaction with the endothelium, compared to negatively or neutrally charged particles.⁷⁶

3.3 Characterization Methods

The FDA has established a guide of recommendations for the nanomedicine industry to follow a risk-based evaluation of nanopharmaceuticals, considering a list of factors to ensure their safety and effectiveness (Fig. 3.3).^{61,77} Primordially, these factors guarantee adequate characterization of the structure and function of the nanomaterial, sufficient understanding of its intended application, and the relation between the nanomaterial attributes and its quality, safety, and efficacy.⁶¹ From this perspective, we have placed special emphasis on the characterization of the nanostructures synthesized in this study. For this purpose, we have used different characterization techniques to address the physicochemical properties of the nanostructures.

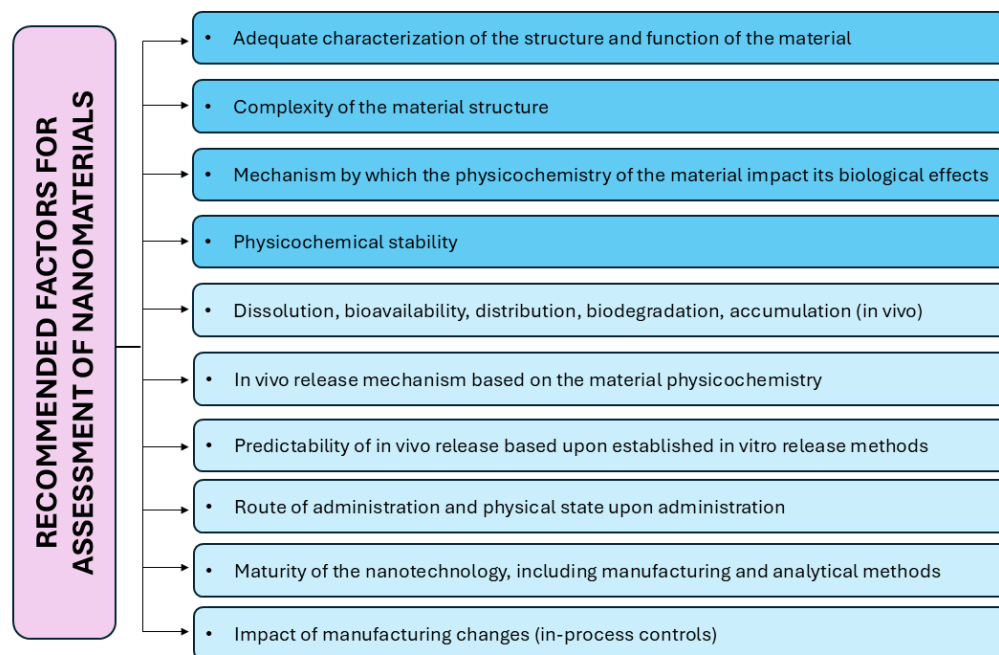


Figure 3.3: Recommended factors for assessment of nanomaterials. This thesis focus on factors highlighted in blue for adequate synthesis and characterization of nanoplateforms.

Characterization technologies allow for the study of the physicochemical properties of nanomaterials.⁷⁸ From the results obtained after characterization, we can correlate the physicochemical parameters of nanomaterials with their further performance as nanotechnology-based pharmaceuticals.⁷⁹ Fig. 3.4 summarizes the information about nanomaterials provided by the different characterization technologies includes: 1) size distribution and morphology, which influence their reactivity, stability, and bioavailability; 2) surface chemistry and charge, which dictate their interaction with biological environments; 3) optical and magnetic properties, which determine their functionalities in sensors, photothermal and magnetothermal therapies; 4) crystalline properties and purity, which influence their mechanical, electrical and thermal properties; and 5) chemical composition and stability in biological fluids to ensure long-lasting physicochemical properties.⁷⁹ In addition, characterization technologies help to optimize synthetic protocols, ensure reproducibility, establish specific applications, and comply with regulatory standards and recommendations.⁷⁹ In this work, we take advantage of the following characterization techniques

to study the physicochemical properties of the nanoplateforms: UV-VIS and FTIR Spectroscopies, Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), Scanning Electrom Microscopy (SEM), Transmission Electrom Microscopy (TEM), X-Ray Diffraction (XRD), and Physical Properties Measurement System (PPMS). The basic principles and mechanisms of the techniques are discussed in the supporting information section SI:A.2.

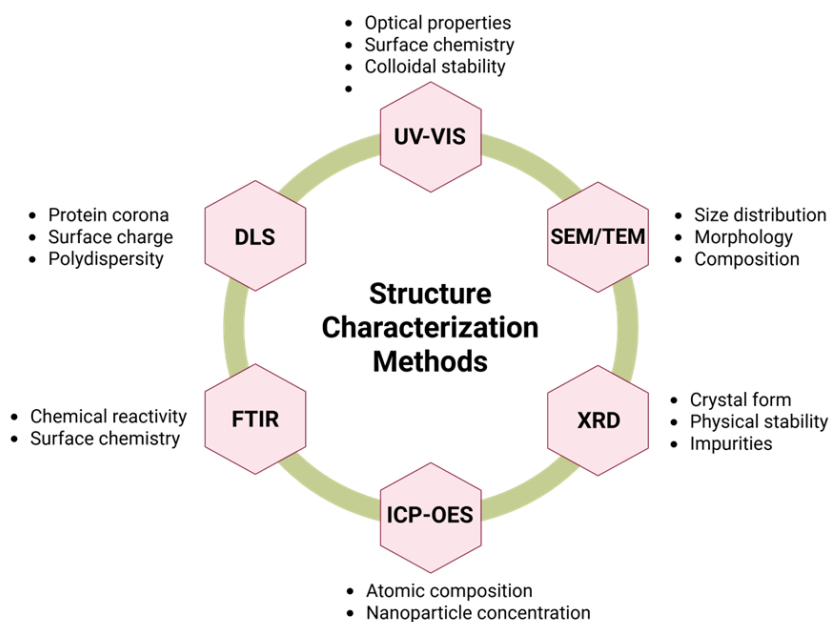


Figure 3.4: Structure characterization methods.

Chapter 4

Phage Display Technology Aided by Molecular Docking

4.1 Introduction

Current research aims to identify ligands targeting cancer-specific biomarkers to improve therapeutic selectivity for efficient and safer cancer treatments.^{80,81} Targeted ligands such as antibodies, aptamers, proteins, and peptides are potential tools to enhance the therapeutic selectivity and efficacy of chemotherapeutic drugs.⁸² Among these ligands, peptides stand out as candidates for targeted therapy due to their high specificity and ability to bind to sites where small molecules cannot.^{83–85} Moreover, they are attractive due to their small size (< 30 amino acids), low immunogenicity, rapid tissue penetration, stability at room temperature, ease of synthesis, and lower manufacturing costs than larger molecules such as antibodies.⁸⁶ Targeted peptides can be applied as peptide-drug conjugates or imaging agents for cancer treatment.^{25,35,87}

Phage display, the most frequently used technique to isolate peptides with high affinity for cell surface components, uses libraries of DNA phages whose genome contains many inserts encoding a collection of peptides fused to a phage coat protein.^{25,88–90} Numerous studies have successfully applied the phage display technique to isolate peptides targeting surface proteins

of cancer cells. The peptide P7 with inhibitory activity of the basic fibroblast growth factor which plays an important role in tumor angiogenesis and the progression of numerous cancers, such as ovarian, breast, colon, and leukemia, was isolated from a phage display library.^{91–94} The peptide AP8 that binds the acidic fibroblast growth factor and avoids its interaction with fibroblast growth factor receptors to prevent the stage and grade of breast cancer was identified using phage display.⁹⁵ The peptide sp22 was designed from a set of eight peptides identified using phage display technology and showed a significant level of inhibition of the protein CD59 overexpressed in tumor cells from the intestine, ovary and prostate.⁹⁶

Phage display is usually performed on whole cells or target proteins to obtain a large set of potent high-affinity peptide sequences.⁹⁷ To decide on peptides with the highest affinity, phage clones are selected randomly after three to five biopanning rounds, and an immunochemical assay is performed to reduce the number of candidate peptides.^{25,91,95,98} Molecular docking has also been employed to support the selection of successful candidates by scoring the binding modes between the peptide sequences and potential targets as function of the number and type of interactions.⁹⁹ Using phage display aided by molecular docking, the peptides PRWAVSP and DTFNSFGRVRIE were identified to specifically bind proteins TIMP-1 and PAI1, respectively, both biomarkers related to MDA breast carcinoma cell line.¹⁰⁰ Jin et al. identified the CSB1 dodecapeptide targeting the breast cancer cell line MCF-7 and used molecular docking tools to explore the interaction between CSB1 and the 5-lipoxygenase-activating protein involved in breast cancer progression and prognosis.²⁵ In related studies, Li et al. identified the CSP3 dodecapeptide targeting SiHa cervical cancer cells, and Kwak et al. the L20 dodecapeptide specific for five colon cancer cells (LoVo, HCT116, HT29, SW480, and DLD-1) complemented with molecular docking analysis to explore how the L20 α -helix structure is linked to strong cell attachment.^{98,101}

Herein we report a novel approach to identify and select peptides with high affinity to cell-surface proteins of HeLa cervical cancer and MDA breast cancer cell culture monolayers, by combining the phage display technique with in-silico molecular docking. After systematically searching the plasma membrane proteins overexpressed in human breast and cervical cancer cells, we assessed and scored their interaction with each high-affinity peptide through molecular docking. This approach could help to adequately select peptide ligands with high affinity to cell surface proteins that might be conjugated with drugs, imaging agents, and nanoparticles targeting

breast and cervical cancer cells for diagnosis and treatment.

4.2 Methodology

4.2.1 Phage Display Experiments

In a previous study carried out by our colleagues Carreón et al¹⁰², 44 dodecapeptide sequences were isolated using phage display technology. Briefly, MDA and HeLa cell lines were cultured in Petri dishes using DMEM and Leibovitz's L-15 media, respectively, supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. A Ph.D.-12 M13KE phage library displaying random 12-mer peptides was screened against confluent HeLa and MDA cells through four rounds of binding and amplification, using increasing concentrations of Tween 20 and alternating BSA to minimize non-specific binding. Phages were eluted with glycine buffer, neutralized, amplified in *E. coli* ER2738, and purified via PEG/NaCl precipitation. Titration involved serial dilution, infection of *E. coli*, and staining with IPTG/X-Gal to identify clones expressing peptide inserts. Final clones were directly titrated, stored, and amplified for further analysis. DNA from selected phage clones was extracted using lysis and precipitation methods, amplified by PCR with specific primers flanking the 336-bp region containing the 12-mer insert, and sequenced to infer the encoded peptide sequences.¹⁰²

4.2.2 Selection of Potential Protein Biomarkers

A systematic review was carried out by searching articles in the Scopus database (www.scopus.com). The keywords *proteomics*, *global mapping*, and *protein expression profile* were used to obtain the related articles. The criteria for selecting the searched articles included year of publication (2000-2023), English full-text articles, and articles or reviews related to HeLa and MDA cell proteomics. Protein profiles of HeLa and MDA cells were then obtained and organized in tables containing thousands of proteins from subcellular units such as endoplasmic reticulum, mitochondria, and plasma membrane. By limiting the search to plasma membrane proteins, the

number of potential targets was reduced to 166 for HeLa cells and 205 for MDA cells. With the search criteria *biomarkers*, *receptors*, and *function in cancer cells*, priorities were assigned to the most frequently reported proteins.

4.2.3 Molecular Docking Calculations

Linear amino acid sequences of the 44 cloned phage dodecapeptides were modeled with the Molecular Operating Environment program (MOE 2019, www.chemcomp.com) and structurally optimized under a force field potential OPLS-AA.¹⁰³ Three-dimensional structures of HeLa and MDA plasma membrane protein biomarkers were obtained from the Protein Data Bank (PDB, www.rcsb.org), including the extracellular and transmembrane domain interfaces in Epidermal Growth Factor Receptor signaling (PDBid: 3NJP), the hyaluronan binding domain of human CD44 (PDBid: 1UUH), the CD146/AA98 Fab complex (PDBid: 6LYN), the crystal structure of the extracellular segment of integrin AVB3 in complex with the Arg-Gly-Asp ligand (PDBid: 1L5G), and the crystal structure of p11-Annexin A2 (N-terminal) fusion protein in complex with AHNAK1 peptide (PDBid: 4HRG), corresponding to the EGFR, CD44, CD146, Integrin, and Annexin A2 biomarkers in HeLa cells, respectively; the human Vimentin coil 2B fragment (PDBid: 1GK4), the recombinant human placental Annexin V complexed with K-201 (PDBid: 1HAK), the human Galectin-1 in complex with Lactulose (PDBid: 6B94), and the X-ray structure of full-length Annexin A1 (PDBid: 1HM6), corresponding to the Vimentin, Annexin A5, Galectin-1, and Annexin A1 biomarkers in MDA cells, respectively.

Calculations of molecular docking between the dodecapeptides and the protein biomarkers were performed with the HDock server (<http://hdock.phys.hust.edu.cn>), and a ranking of the best ten conformational models of each peptide-protein complex was obtained. Visualization of the modeling results was performed in PyMOL (<https://pymol.org>) using the PDB files of the peptide-protein complexes. The score obtained from the conformational model 1 for each complex was considered for affinity analysis. Protein-peptide interaction, in terms of polar contacts, was analyzed in five (models 1, 3, 5, 8, and 10) of ten models to determine which amino acid residues were repeatedly involved. Conformation model 1 was used to analyze the interacting regions and residues in the protein biomarkers.

4.3 Results and Discussion

The generation of ligands targeting overexpressed cancer biomarkers could deeply improve therapeutic selectivity for efficient and safer cancer treatments.¹⁰⁴ Peptide ligands are promising candidates for developing therapeutic and diagnostic tools delivered to specific tumor biomarkers such as those of cervical and breast cancers.⁸³ The isolation of peptides with high affinity to overexpressed cancer biomarkers can be performed by phage display where a large set of peptide sequences is generated, and further synthesis of the displayed peptides requires a careful selection of the best clones.⁹⁷ In this study, we used a novel approach to select peptides with high affinity to cell-surface proteins of the HeLa cervical cancer and MDA breast cancer cell lines by supporting the phage display technique with *in silico* molecular docking to simplify the selection of peptides targeting cancer cells.^{89,100}

4.3.1 Phage clones with high affinity to the surface of HeLa and MDA cells

Biopanning is the process by which peptides with high affinity to target cells are identified.^{105,106} To isolate high-affinity peptides, a phage display peptide library consisting of a collection of nucleotide triplet sequences inserted in the terminal end of a phage coat protein is incubated with an immobilized target, followed by extensive washing to remove non-bound phages, eluting the binders and enriching them by amplification in host bacteria. After three to five biopanning rounds the peptide sequences fused to the coat protein of phage clones are inferred by DNA sequencing of the insert amplification products.¹⁰⁶ Starting with a 12-mer M13KE phage library Carreon et al. carried out four cycles of screening/amplification on HeLa and MDA monolayer cell cultures and isolated 44 phage clones with high affinity to the surface of both cell monolayers (Table 4.1): 21 targeting HeLa cells (designated H1-H21), and 23 targeting MDA cells (designated M1-M23).

Table 4.1: Amino acid sequences of the dodecapeptides displayed by the phage clones obtained after four biopanning rounds on HeLa and MDA cell culture monolayers.

HeLa cells		MDA-MB-231 cells	
Name	Sequence	Name	Sequence
H1	DSSPRMWPNRIT	M1	SITTKGWIGPGM
H2	FFNSSNVNVNND	M2	ILGRLSLVEYRH
H3	VPFNVVYPRVIE	M3	FKQDAWEAVDIR
H4	TMQDWNENKSSF	M4	SVDHKWKTGNNG
H5	FKQDAWEAVDIR	M5	RNYTPVDHSGGQ
H6	NIVSWYDSQRPR	M6	WTQTTWINMSPHM
H7	NESGITRIALQD	M7	SLQTLRLGHNTS
H8	MPLRYPTQVTLD	M8	SNARLLTASNVY
H9	FNQDAWEAGDIR	M9	LPVNNYGHSGTT
H10	HNRKGWKASEIK	M10	GTEKVNVAKWTR
H11	LSSEFDTKQSGQ	M11	NLNFQKYNKLNM
H12	GEWRARIDQDVS	M12	TGDLSLFLRKLN
H13	SVDQDIRARWEG	M13	QEPHSIFGLQIN
H14	SLQTLRLGHNTS	M14	EGLTEHGYAYPT
H15	VDIKSHFSHTGK	M15	ADSGRTTSKTSY
H16	QAQDKRVPRDIW	M16	DFGGFSRNRMMT
H17	WIDRPVRKDQAQ	M17	DSSPRMWPNRIT
H18	FLSGCGGTAEAT	M18	DGGSGFSWQKYG
H19	TYPYNLQVRHWT	M19	NFGQIKFSQYRH
H20	DQQDAWEAKDIR	M20	SLGMKTHQISLR
H21	TMNDWNTNKSSF	M21	QMYMKDNSRNVP
		M22	WHFDKSRTLNTN
		M23	VDIKSHFSHTGK

4.3.2 Selection of potential plasma membrane protein targets

A systematic review of the HeLa and MDA protein profiles identified 44 potential plasma membrane protein targets: 32 in HeLa cells (Table A.2), and 12 in MDA cells (Table A.1). Among them, Annexin A2 (P07355), EGFR (P00533), CD44 (P16070), CD146 (P43121), and Integrin alpha V (P06756) were selected as potential targets in HeLa cells, and Vimentin (P08670), Annexin A1 (P04083), Annexin A5 (P08758), and Galectin-1 (P09382) were selected in MDA cells (Fig. 4.1). These proteins belong to the ErbB (EGFR), immunoglobulin-like (CD146, CD44), integrin (Integrin), intermediate filament (vimentin), calcium-dependent membrane-binding (Annexin A1, A2, and A5), and lectin (Galectin) families of proteins, and have been reported as over-expressed biomarkers in HeLa and MDA cells.^{89,107–110} Altered or mutant expression of these biomarkers accounts for cancer cells' pathogenesis and progression, leading to cell proliferation, angiogenesis, invasion, metastasis, and inhibition of apoptosis.^{107,108,111–115}

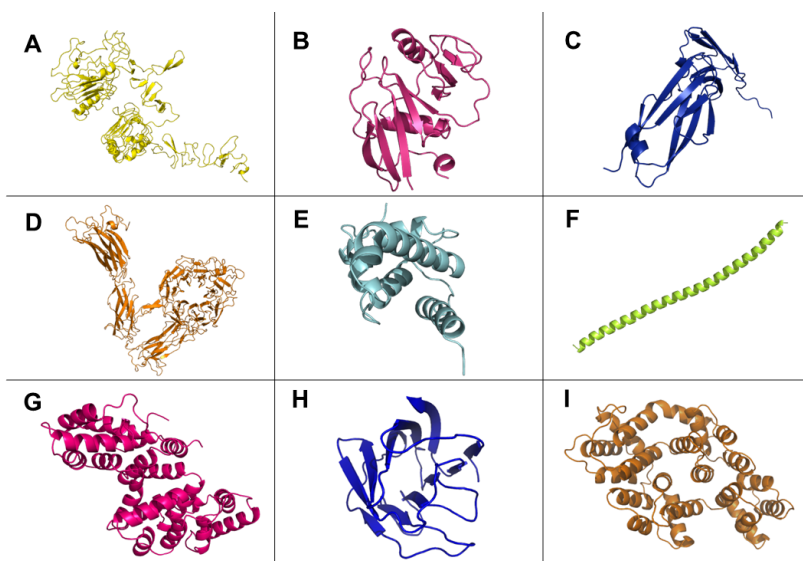


Figure 4.1: The three-dimensional structures of the active sites of biomarkers were obtained from the PDB database. For HeLa: (A) EGFR (PDBid: 3NJP), (B) CD44 (PDBid: 1UUH), (C) CD146 (PDBid: 6LYN), (D) Integrin (PDBid: 1L5G), and (E) Annexin A2 (PDBid: 4HRG). For MDA: Vimentin (PDBid: 1GK4), Annexin A5 (PDBid: 1HAK), Galectin-1 (PDBid: 6B94), and Annexin A1 (PDBid: 1HM6)

4.3.3 Molecular docking of the dodecapeptides to their potential protein targets

Selection of the best peptides from the pool of candidates isolated from phage display may be simplified using molecular docking.¹¹⁶ The purpose of a molecular docking calculation is to predict via computational simulations the pose with the lowest energy as the predicted binding mode between two molecules.^{117–119} Modelling the interaction between peptides and a set of potential protein targets, the pool may be reduced by selecting the peptides that demonstrate the lowest binding energies.⁸⁴ Following this approach, Nobrega et al. used the ClusPro molecular docking tool for computer-aided phage display to select the peptides PRWAVSP and DTFNS-FGRVRIE from a pool of peptide sequences obtained by screening 7-mer and 12-mer M13KE phage libraries on MDA cells and proposed the proteins TIMP-1 and PAI1 as their potential targets, respectively.¹⁰⁰ In another study conducted by Yu et al., β -Lactoglobulin (β -Lg) was used as target to screen a 12-mer phage library and isolate eleven peptides (designated P1 to P11) which were then modeled and docked using AutoDock CrankPep to predict the binding energy and the molecular interactions with β -Lg.⁹⁰

The identification of accurate binding orientations obtained after the molecular docking simulations requires energy-based scoring functions, used by most docking tools such as ClusPro, RosettaDock, HADDOCK, AutoDock, HDOCK, among others.^{120–122} In the particular case of the HDOCK used in this work for molecular docking studies, once the structures of the peptide and the protein molecules were uploaded to the server, sampling process of putative binding modes through a fast Fourier transform (FFT)-based global search method was executed, and the energy score for each ligand-receptor pose was evaluated with a knowledge-based scoring function. Specific binding site information can also be provided by the user prior docking calculation.^{118,121} From the total energy scores, we obtained a ranking (models 1 to 10) of different binding modes of each peptide-protein complex.^{121,123}

4.3.4 Docking scores

The docking scores of the 44 phage-displayed peptides to HeLa and MDA plasma protein biomarkers were assessed using molecular docking (Tables A.3 and A.4). Figures 4.2A and 4.2B show the peptides with the highest energy scores for HeLa and MDA plasma protein biomarkers, respectively, obtained from conformational model 1. Peptides H1 (DSSPRMWPNRIT), H6 (NIVSWYDSQRPR), and H19 (TYPYNLQVRHWT) had the highest total energy scores for HeLa protein biomarkers, with values of -916.32, -979.21, and -1093.24, respectively (Tables A.3), and peptides M6 (WTQTTWINMSPHM), M16 (DFGGFSRNRMMT), and M19 (NFGQIKFSQYRH) had the highest total scores on the MDA protein biomarkers, with total scores of -732.21, -745.5, and -739.64, respectively (Tables A.4). In contrast, peptides H18 (FLSGCGGTAEAT) and M3 (FKQDAWEAVDIR) showed the lowest scores with values of -678.17 and -551.26, respectively.

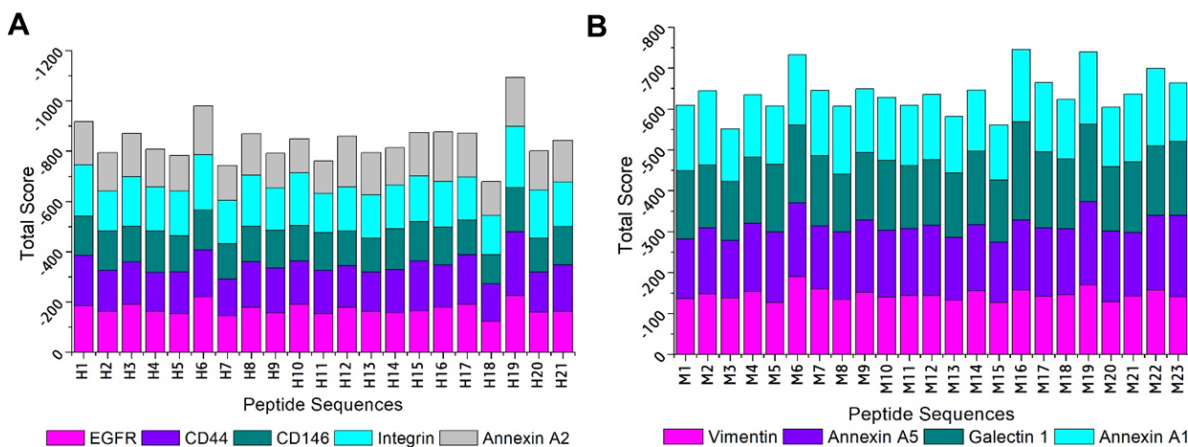


Figure 4.2: Molecular docking scores for each peptide-protein complex between the H and M dodecapeptides with each potential biomarker in HeLa (A) and MDA (B) cell lines, respectively. The total score represents the sum of the energies obtained from conformational model 1 of each peptide-protein complex.

The total scores of peptide-protein complexes suggest that the poses obtained between the H peptides and HeLa biomarkers are more viable to occur than those between the M peptides and MDA biomarkers. This appears to be confirmed by comparing the total score of the interaction

of peptide M3 (-551.26), designated as H5 (-783.28) in HeLa, with all the biomarkers in both cell lines. Moreover, although M3 shares the same QDAWEAxDIR motif (9/12 common residues) with the H9 (FNQDAWEAGDIR) and H20 (DQQDAWEAKDIR) sequences, there is a significant difference in the score of the binding modes of M3 with all MDA biomarkers. In contrast, the scores for H5 (-783.28), H9 (-790.73) and H20 (-800.18) peptides are similar, suggesting that the interaction poses are enhanced by the biomarker type.

4.3.5 Post-docking ligand-receptor interaction analysis

A post-docking interaction analysis of the peptide-protein complexes can also be helpful to gain insights into the elements influencing the docking scores, such as specific interacting regions and residues in the receptor, chemical characteristics of the interacting amino acid side chains in the ligand and the receptor, and polarity of the peptides, guiding the improvement of the energy scores and supporting the rational peptide design.^{81,84,124}

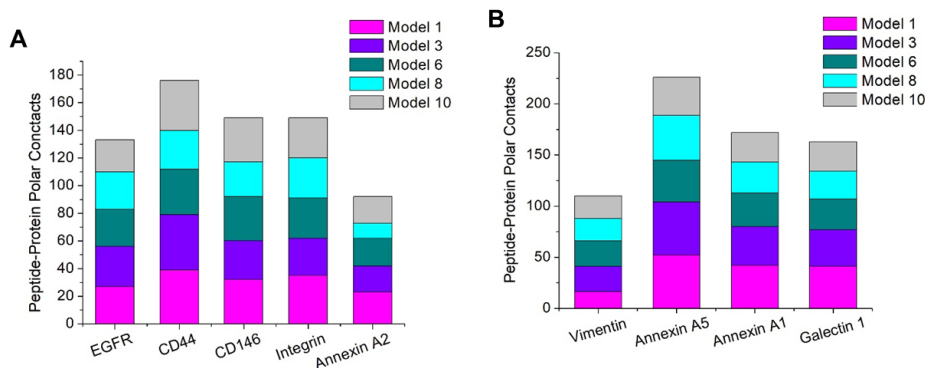


Figure 4.3: The total number of peptide-protein polar contacts in HeLa (A), and MDA (B). The interactions were analyzed using conformational models 1, 3, 6, 8, and 10.

Visualization of five of the 10 peptide-protein complex models to analyze the interaction of H and M peptides with HeLa and MDA plasma membrane biomarkers showed that multiple residues of each peptide sequence appeared to be involved in polar contacts (Fig. 4.4). A larger number of polar contacts were noted in the complexes established between H peptides and EGFR, CD44, CD146, and Integrin Alpha V, in contrast with Annexin A2 (Fig. 4.3A). Similarly, the

interaction of M peptide sequences with MDA biomarkers showed more polar contacts formed with Galectin, Annexin A1, and Annexin A5, contrasting with the results obtained for Vimentin (Fig. 4.3B). The phage display results suggest that the H5/M3 and H1/M17 peptide sequences bind with high affinity to common receptors overexpressed on the cell surface of HeLa and MDA targets, consistent with the molecular docking results that H5/M3 and H1/M17 peptides interact with greater affinity to CD44 and Annexin A5, overexpressed on the surface of both cell lines. This finding suggests that these biomarkers are potential receptors in both cell lines.

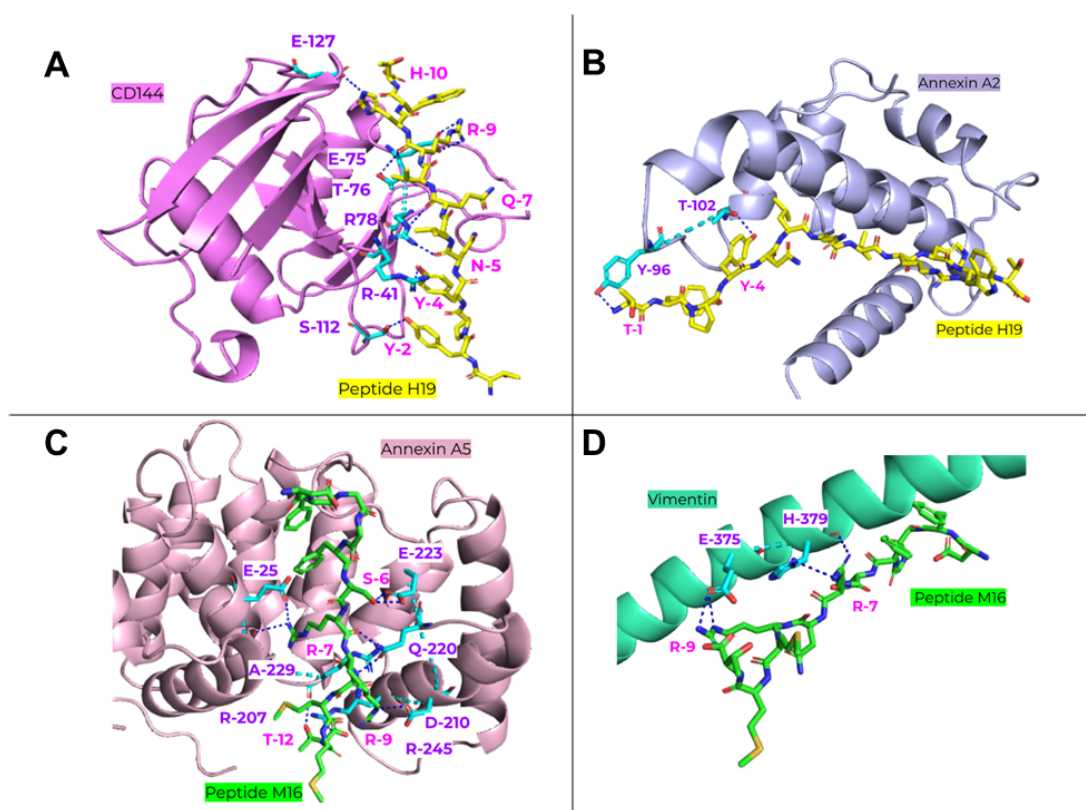


Figure 4.4: Visualization of the interaction between peptide H19 and two protein biomarkers in HeLa cells: (A) CD44 (the highest docking score) and (B) Annexin A2 (the least docking score), and interaction between peptide M16 and two protein biomarkers in MDA cells: (C) Annexin A5 (the highest docking score) and (D) Vimentin (the least docking score). Protein residues are represented in blue sky color and labeled in purple; residues in peptide sequences are labeled in magenta.

Specific interacting regions and residues in HeLa and MDA biomarkers were identified after

visualization of model 1 of each peptide-protein complex (Tables 4.2 and 4.3). For HeLa biomarkers (Table 4.2), it was found that the EGFR interacted with H peptides through the extracellular domains (ED) I (residues 7-200) and III (residues 317-470), both regions known to incorporate ligands.^{125,126} The interacting region in CD44 included the ED I (residues 1-133) through residues that are considered "hot spots" and are essential for protein dimerization in CD44, such as C77, R78, Y79, R90.¹²⁷ In CD146, the interacting region comprised two of the three extracellular immunoglobulin-like constant domains C2-2 (residues 304-404) and C2-3 (405-505).¹²⁸ Integrin alpha V interacted through residues in the extracellular $\alpha_V\beta$ -propeller domain (residues 1-450) which is responsible for the recognition of ligands, such as RGD motifs on the fibronectin and fibrinogen.¹²⁹ In Annexin A2, the N-terminal domain was forming a complex with protein S100-A10, so peptides interacted the most with residues (3-83) from S100 and residues T102 and S111 from Annexin A2.^{110,130,131} For MDA residues (Table 4.3), Vimentin interacted with M peptides through the central α -helical rod domain (residues 269-407), specifically, the region from R364 to Q384.¹³² In contrast to Annexin A2, the biomarkers Annexin A5 and A1 formed polar contacts with peptides through the N-terminal residue Q3 and Y21, respectively, and numerous residues in the C-terminal domains.^{110,133} Galectin-1 bound peptides via interaction of the carbohydrate-recognition domain (CRD, residues 44-73).¹³⁴

Specific residues in the interacting regions of the biomarkers were found to participate recurrently in the formation of the complexes with several peptide sequences. The recurrent interacting residues in HeLa biomarkers were Q8 and T15 in EGFR; E75, T76, C77, R78, Y79, and S109 in CD44; R406 and R419 in CD146; Y18, D219, and Y221 in Integrin alpha-V; and D57 and T96 in Annexin A2. The residues in MDA biomarkers were R207, D211, and Q220 in Annexin A5; R48 and H52 in Galectin-1; and R292 in Annexin A1. Classifying amino acids by the chemical characteristics of the side chain as follows: aliphatic (Alanine (A), Valine (V), Isoleucine (I), and Leucine (L)), sulfur (Cysteine (C) and Methionine (M)); hydroxyl (Serine (S) and Threonine (T)); acidic (Aspartic acid (D) and Glutamic acid (E)); amide (Asparagine (N) and Glutamine (Q)); basic (Histidine (H), Lysine (K) and Arginine (R)); aromatic (Tyrosine (Y), Tryptophan (W) and Phenylalanine (F)); and excluding Glycine (G) due to its simplest structure and Proline (P) due to its non-reactive side chain, results suggest that the acidic D residue, the amide Q residue, the aromatic Y residue, and the basic R residue bind most frequently to peptides.¹³⁵

The proportion of polar and charged residues in a structure contributes to the binding affinity of ligand-receptor interactions.¹³⁶ To analyze how the peptide properties improved the docking scores, we analyzed the polar, non-polar, charged and uncharged character of residues in H and M sequences according to the following classification: polar: H, N, Q, S, T, Y, E, D, K, R; non-polar: W, A, F, G, I, V, L, M, P, C; charged: H, E, D, K, R; uncharged: T, S, Q, N; and compared the character of the peptides with the total scores.^{135,137,138} The results confirmed that total scores of the interactions between H and M sequences with HeLa and MDA biomarkers, respectively, were highest for peptide sequences containing more polar and charged residues than non-polar residues. Moreover, for future in vitro experiments, the presence of polar and charged residues on the peptide sequences may be helpful to stabilize them in an aqueous medium due to the formation of a hydration layer, thus improving water stability.¹³⁶

The total scores and modes of interaction between the H1, H6, and H19 sequences with all the surface biomarkers defined for HeLa cells suggest once again that the formation of peptide-protein complexes is determined by the presence of one or more specific polar residues, such as Y, Q, N, and R, in the peptide sequence. Binding occurred through the aromatic (Y), acidic (Q and N), and basic (R) functional groups of the lateral chains of these residues, such as the guanidino group of the R residue, the phenolic -OH group of the Y residue, and the amide groups -NH₂ of the Q and N residues. Particularly, the cationic guanidinium moiety, formed by three amino groups conjugated to a central carbon atom, imparts a strong capacity for electrostatic interactions and the formation of bidentate hydrogen bonds in three directions. At physiological pH, the protonated R residue facilitates the interaction with protein surfaces.¹³⁹ On the other hand, the phenolic -OH group of tyrosine can mediate proton-coupled electron transfer processes in biological systems at most pH values, thus undergoing hydrogen bonding with HeLa cell biomarkers.¹¹⁶ Likewise, the amide group of Q and N residues exhibits both donor and acceptor H-bonding sites, which mediate interaction with all biomarkers.¹⁴⁰ Additionally, although the binding scores between the M peptide sequences and the selected MDA biomarkers were lower than the ligand-receptor binding energies in HeLa analyses, the presence of the guanidino and amide functional groups appeared to facilitate binding.

Although insights into the peptide-protein interactions can be inferred via post-docking interaction analysis, it fails to capture the conformational changes and flexibility of the docked

biomolecules in real time.¹⁴¹ Prediction accuracy of the best peptide sequences from the pool of 44 candidates can be advanced by integrating molecular dynamics (MD) simulations.¹²⁰ To fortify this study, MD have to be considered for the refinement of post-docking results and analysis, since it explores the energy profile of peptide-protein complexes considering their conformational changes.¹⁴¹

Table 4.2: Residues in the interacting region of HeLa protein biomarkers. Residues marked in bold showed recurrent interaction with several peptide sequences.

Biomarker	Interacting Residues	Interacting Region
HeLa		
EGFR	V6, Q8 , G9, S11, N12, T15 , Q16 L17, G18, S26, N32 G315, I316, H346, R353, G354, D355, S356, K465	ED I ED III
CD144	N25, T27, C28, E37, R41, Y42, E75 , T76 , C77 , R78 , Y79 , R90, N94, I96, T108, S109 , S112, Y114, E127, R150	ED I
CD146	S344, D345, V346, E354 R406 , R419 , T420, Q421, V423, N424, H457, R459, T469, S471, E472, D474, P477, D504	ED C2-2 ED C2-3
Integrin alpha-V	Y18 , K42, S97, V98, R122, Y178, W179, D218, D219 , Y221 , Y224, R245, R248, R339, Y364, S399	$\alpha_V\beta$ -propeller
Annexin A2	Q3, D21, K56, D57 , Q69, F72, T96 S73 , T79, N83, Y92 T102, S111	Protein S100-A10 N-terminal domain

Table 4.3: Residues in the interacting region of MDA protein biomarkers. Residues marked in bold showed recurrent interaction with several peptide sequences.

Biomarker	Interacting Residues	Interacting Region
MDA-MB-231		
Vimentin	R364, Q370, E374, E375, M376, H379, R381, E382, Y383, Q384	α -helical rod domain
Annexin A5	Q3 A99, T101, E106, T118, E120, R123, D133, L156, D164, S204, R207 , F210, D211 , Q220 , E222, E223, R227, E228, K242, R245, A249, E253, Y256, Y257, M259, K260, 261, S275, D280, E287, K290, N291, R292, D303	N-terminal domain C-terminal domain
Galectin-1	H44, N46, R48 , H52 , D54, C60, N61, K63, T70, E71, R73	Carbohydrate-recognition domain
Annexin A1	Y21 K81, D93, K97, A99, T101, G102, H103, E105, E106, K113, D121, A125, K128, D133, D135, L160, D259, E280, Q284, A285, I289, G290, T291, R292 , H293, K294, Y319	N-terminal domain C-terminal domain

4.4 Conclusions

In this chapter the following question was solved: How to select the best dodecapeptides from a list of 44 candidates isolated via phage display technology? To solve this question, we hypothesized that by supporting the phage display technology with molecular docking, the selection of the best peptide candidates may be simplified. This hypothesis was confirmed after performing molecular docking calculations to model the interaction between 44 peptides with a set of potential protein

targets (Annexin A2, EGFR, CD44, CD146, Integrin alpha V, Vimentin, Annexin A1, Annexin A5, and Galectin-1) and gain insights into the factors strengthening the peptide-protein binding docking scores. We stabilised the factor that influence the peptide-protein interaction. Factor included the specific interacting regions, particularly the extracellular domains in the protein targets; the chemical characteristics of the interacting amino acid side chains in the peptides and protein targets; and the polarity of the peptides. To fortify the findings reported in this study, in vitro experiments to validate the specific protein targets followed by refinement of the post-docking results through molecular dynamic simulations will be further pursued.

Chapter 5

Peptide-Gold Nanoparticle Conjugates

5.1 Introduction

Marketed liposomes Myocet® (doxorubicin; EMA-2000) and Lipusu® (paclitaxel; FDA-2016), protein-based Abraxane® (paclitaxel; FDA-2005), and the micellar Genexol-PM® (paclitaxel; FDA-2016) and Apealea® (Paclitaxel; EMA-2018) were designed to treat breast and cervical cancer.^{38,42,46} Despite the advantages of these innovative nanopharmaceuticals carrying doxorubicin and paclitaxel, such as reduced adverse side effects, increased the response rates, and improved site-specificity, breast and cervical cancer continue having high incidence and mortality rates, so that these affections are the most commonly diagnosed cancers and the major cause of cancer deaths in women.^{9,17,87}

Wilhelm and colleagues reported that 99.3% of the intravenously injected dose of nanopharmaceuticals interacts with off-target tissues.⁵ This high percentage reaching healthy tissue promotes multidrug resistance, which contributes to the global crisis of cancer mortality.^{6,20} It is imperative to develop nanopharmaceuticals that specifically recognize neoplastic tissue and trigger the release of encapsulated drugs to enhance the therapeutic effect of the delivered drug.¹¹ Neoplastic tissue exposed to heat exhibits increased sensitivity to chemotherapeutic drugs due to enhanced blood vessel permeability, allowing more drugs to reach and accumulate at the tumor

site.^{11,142} In this regard, gold nanoparticles (GNPs) have been extensively investigated for their ability to convert NIR radiation into heat.^{18,54,143}

GNPs exhibit LSPR attributed to the oscillation frequency of free electrons resonating with the frequency of the incident radiation, resulting in a plasmon band.^{49,144,145} The absorbed radiation translates into nonradiative energy dissipation (heat) that can be used to increase the temperature of neoplastic tissue (hyperthermia).^{146,147} Modifying the morphology of GNPs, from spherical (AuNPs) to multibranched (MBAuNPs), shifts the LSPR band from the visible to the NIR region of the electromagnetic spectrum, as a function of the width, length, or number of branches. This tunability expands the use of non-ionizing NIR radiation as an external stimulus to induce energy dissipation by GNPs.¹⁴²

Several studies have demonstrated the capacity of GNPs to convert NIR radiation into heat. Carreon et al. reported a temperature increase of around 5 °C generated by MBAuNPs (LSPR = 775 nm) in phantom gels using an 808 nm NIR laser with a radiation power of 1 W/cm².²⁸ Espinosa et al. investigated the effect of the nanostar size (25-150 nm), and the LSPR region (500-1000 nm), on heating efficiency of nanostars in water suspension, and found a dependency on particle size and excitation laser wavelength.¹⁴² Besides, GNPs have also demonstrated efficient radio-sensitizing capacity attributed to their high atomic number (Z) and ability to absorb X-rays, making them promising candidates as tumor radiosensitizers in radiotherapy, and as contrast agents in computed tomography. Therefore, GNPs can be engineered to serve as multimodal nanopharmaceuticals.¹¹

Active targeting of neoplastic tissue can be achieved by the attachment of targeting ligands to GNPs. Harrison et al. demonstrated that co-functionalizing pegylated AuNPs with a Receptor-Mediated Endocytosis peptide enhances cellular internalization in MDA and MCF-10A cell lines.¹⁴⁸ Rusk et al. conjugated ultrasmall AuNPs with peptides that selectively target the model protein hPin1, via the thiol group of a terminal cysteine, and found that the nanoparticles assist the peptide to cross the cell membrane and locate in the cytosol of HeLa cells.²⁴

In this context, we hypothesized that photothermal MBAuNPs conjugated with cell-related peptides would specifically bind to HeLa and MDA cancer cells and cause cell damage via tumor ablation. We employed the peptides FKQDAWEAVDIR (designated as P1), TYPYNLQVRHWT

(designated as P2), and FLSGCGGTAEAT (designated as P3), selected from Chap. 4, as peptides with the highest affinity to HeLa and MDA cancer cells. P1, P2, and P3 were synthesized using microwave-assisted solid-phase peptide synthesis (SPPS) and purified using High Performance Liquid Chromatography (HPLC). In this chapter, we also present a DoE to functionalize P1, P2, and P3 to the surface of PEGylated MBAuNPs using the Michael's addition reaction, to constitute the systems MBAuNPs@P1, MBAuNPs@P2, and MBAuNPs@P3.

5.2 Methodology

5.2.1 Reagents

Gold (III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) 99.99% trace metals basis, trisodium citrate dihydrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$) $\geq 99\%$ FC, silver nitrate (AgNO_3) ACS reagent $\geq 99.0\%$, L-ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) ACS reagent $\geq 99.0\%$, sodium hydroxide (NaOH) ACS reagent $\geq 97\%$, hydrochloric acid (HCl), HEPES sodium salt ($\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$) $\geq 99.0\%$ (titration), and methoxypolyethylene glycol maleimide (PEG-MAL, $\text{C}_9\text{H}_{16}\text{N}_2\text{O}_4$, $M_w = 5000$) $\geq 90\%$ (NMR), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol 96% was purchased from REPROQUIFIN (Estado de México, México). All samples were prepared using Milli Q water (18 M Ω).

For peptide synthesis: N, N, Dimethyl Formamide (DMF, $\text{C}_3\text{H}_7\text{NO}$), Piperidine ($\text{C}_5\text{H}_{11}\text{N}$), 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, $\text{C}_{11}\text{H}_{16}\text{F}_6\text{N}_5\text{OP}$), N-Methyl-2-pyrrolidone (NMP, $\text{C}_5\text{H}_9\text{NO}$), 1-Hydroxybenzotriazole (HOBt, $\text{C}_6\text{H}_5\text{N}_3\text{O}$), Rink amide 4-methylbenzhydrylamine (MBHA) resin ($\text{C}_{47}\text{H}_{44}\text{N}_2\text{O}_6$), N, N-diisopropylethylamine (DIPEA, $\text{C}_8\text{H}_{19}\text{N}$), dimethyl sulfoxide (DMSO, $\text{C}_2\text{H}_6\text{OS}$), Acetic Anhydride ($\text{C}_4\text{H}_6\text{O}_3$), Diethyl ether (Et_2O , $\text{C}_4\text{H}_{10}\text{O}$), Trifluoroacetic acid (TFA, $\text{C}_2\text{HF}_3\text{O}_2$), and amino acids: Fmoc-Cys(Trt)-OH, Fmoc-Phe-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Val-OH, Fmoc-L-Ala-OH·H₂O, Fmoc-L-Trp(Boc)-OH, Fmoc-L-Glu(OtBu)-OH·H₂O, Fmoc-Asp(OtBu)-OH, Fmoc-L-Ile-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-Thr(tBu)-OH, Fmoc-L-Pro-OH·H₂O, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Leu-OH, Fmoc-L-Met-OH, and Fmoc-Gly-OH were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.2.2 Seed-mediated synthesis of MBaunPs

AunPs seeds were synthesized using the inverse Turkevich method.¹⁴⁴ Briefly, a 25 mM HAuCl₄ · 3H₂O aqueous solution was injected into a 1 wt.% trisodium citrate solution at 100 °C, under vigorous magnetic stirring.¹⁴⁴ For the growth of branches on the AunPs seeds, 40 mL of a 0.25 mM HAuCl₄ · 3H₂O aqueous solution (0.01 mmol) was placed in a 50 mL beaker, followed by the addition of 40 µL of 1M HCl (10 mL, 0.01 mol) solution and 400 µL of the as-synthesized seeds. Immediately, and simultaneously, 400 µL of a 3 mM silver nitrate (1 mL, 0.003 mmol) solution and 200 µL of a 0.1M ascorbic acid (10 mL, 0.001 mol) solution were added. A change in the color of the solution, from colorless to blue-gray, was noted immediately, so the mixture was removed from the plate and stored at 4 °C.¹⁴⁹

5.2.3 Synthesis and characterization of P1, P2, and P3

Peptide microwave synthesis using Liberty Blue

The synthesis of peptides P1, P2, and P3 was based on the peptide microwave synthesis using a Liberty Blue peptide synthesizer. A careful cleaning process of the Liberty Blue system was carried out, including: amino acid and reagent bottles (performed twice), reaction vessels (performed twice), fluidics (performed once), and deprotection (performed once). Once the system was cleaned, a method for the synthesis of the peptide sequence P1 (CFKQDAWEAVDIRG) was programed in the Liberty Blue software. The sequence was typed from the N-terminus to the C-terminus. Note that the amino acid cysteine (C) was added at the N-terminus for future peptide-nanoparticle conjugation experiments. The amino acid glycine (G) was added since an amide functional groups is required for resin coupling. Due to the application of peptides for further *in vivo* experiments, the N-terminus and C-terminus were acetylated and amidated, respectively. Table 5.1 shows the parameters used for peptide synthesis.

After the method parameters were set, solutions were prepared according to the mass (g) and volume (mL) of amino acids required by the Liberty Blue software. The Fmoc-amino acids were completely dissolved in DMF to obtain 0.2M amino acid solutions. For amino acids having

solubility problem like histidine (H), 20% DMSO in DMF was used. The amounts of the activator base, activator, and deprotector were calculated using the reagent calculator of the Liberty Blue software: HBTU (0.5M) in DMF was used as activator, 2M DIEA in NMP was used as activator base, and a solution of 20% Piperidine in DMF mixed with HOBt was used for deprotection. The resin Rink Amide MBHA was pre-swelled for 30 minutes in DMF before starting the synthesis. All amino acid, reagent, and swelled resin solutions bottles were placed into the correct position on the Liberty Blue synthesizer and the synthesis was initiated. The synthesis was repeated for peptides P2 (CTYPYNLQVRHWTG), and P3 (CFLSGCGGTAEAT).

Table 5.1: Parameters used during Fmoc Solid Phase Peptide Synthesis using Liberty Blue

Parameter	Value
Resin	
Name	Rink Amide MBHA
Scale	0.25 mmol
Loading	0.78 mmol/g
Type	High Swelling
Preloaded	No
C-terminus	Amide
Mass required	0.321 g
Resin Cycle	
Resin	0.25-Resin Swelling (HS)
Final Deprotection	0.25-Final Deprotection (HS)

Capping, Cleavage, and Purification

For capping, the resin was removed from the reaction vessel, placed in a Falcon tube, and swelled in 10 mL DMF for 20 minutes, using a shaker. DMF was filtered using a vacuum pump, and 20 mL of a capping solution containing 20% Acetic Anhydride (4 mL), and 20% DIEA (4 mL) in DMF (12 mL), was added to the Falcon tube. The mixture containing the resin and the capping solution was shaken for 25 minutes. The capping solution was filtered and the resin was washed

three times with 7 mL DCM and three times with 7 mL Et₂O. Resin was let to air dry for 1 hour using the vacuum pump.

For cleavage, a 20 mL TFA cleavage solution, containing 20% TFA in DCM, was added to the resin and the mixture was shaken under N₂ for 2 hours. The mixture was filtered under vacuum to eliminate the resin. For peptide precipitation, the filtered TFA peptide solution was dropwise to cold Et₂O and then freeze for 30 minutes. The mixture was centrifuged for 30 minutes at 4400 rpm and the supernatant was decanted. 20 mL cold Et₂O was added to the crude peptide, vortexed, and centrifuged 30 minutes at 4400 rpm to remove by-products. Crude peptides P1, P2, and P3 were purified using reverse HPLC along with mass spectrometry to ensure $\geq 95\%$ purity (Figs. A.8, A.9 and A.10). Purified peptides were lyophilized and stored at 4°C.

Characterization of P1, P2, and P3

Physical and chemical parameters of peptides P1, P2, and P3 were studied using the ProtParam module of the ExPASy website (www.web.expasy.org), ProtPi website (www.protpi.ch), and the PepDraw website (<http://pepdraw.com/>). The computed parameters include the molecular weight, theoretical pI, amino acid composition, and hydrophobicity.

Peptides P1, P2, and P3 were characterized by UV-VIS and FTIR. For this purpose, peptide solutions were prepared by dissolving 0.5 mg of each peptide in HEPES aqueous solution (1 mL, 50 mM, pH = 7.4). UV-VIS spectra were recorded on a Cary 60 UV-VIS spectrophotometer with a 4 mL-quartz cuvette of 10 mm optical path length to analyze peptide absorbance. FTIR spectra were recorded in an IRTracer-100 Fourier Transform Infrared Spectrometer (Shimadzu) equipped with an attenuated total reflection (ATR) diamond accessory to register transmittance over the wavenumber (ν) interval of $4000 > \nu > 400 \text{ cm}^{-1}$. A total of 50 scans were averaged to produce the spectrum. Measurements were obtained in triplicate to ensure reproducibility. The interferometer was scanned at 4 resolution, and spectra were obtained by performing a Fourier transform of the interferogram after apodization with a Happ-Genzel.

5.2.4 Design of Experiment for Peptide-MBAuNPs Conjugation

Study of the hydrolysis of the PEG-MAL polymer

An aqueous solution of HEPES (100 mL, 50 mM) was prepared. 10 mL of this solution was placed in four 15 mL-Falcon tubes, and the pH of each sample was reduced with HCl, from 10 to 5.10, 6.40, 7.40, and 8.40, respectively. 1 mL of each pH-modified HEPES stock solutions were deposited in 2 mL-Eppendorf tubes, and 0.35 mg of PEG-MAL powder was added. Mixtures were agitated manually until the PEG-MAL was completely dissolved. To analyze the hydrolysis of the maleimide ring of PEG-MAL, its optical properties were monitored using UV-VIS spectroscopy in three different periods: day one (immediately after preparation), day two, and day three, as well as FTIR spectroscopy on days one and three. Samples were stored at 4°C during stability studies.

Conjugation of PEG-MAL and L-Cysteine

The conjugation between PEG-MAL and L-Cysteine (L-Cys) was based on the Michael's addition reaction for two different molar ratios, 1:10 and 1:17 (PEG-Mal: L-Cys). 1 mg of PEG-MAL was deposited in two Eppendorf tubes, followed by the addition of 1 mL 50 mM HEPES buffer (pH = 6.40) to each tube. In the same way, 0.050 mmol (sample A) and 0.087 mmol (sample B) of L-Cys were mixed with 1 mL of HEPES buffer in two independent Eppendorf tubes. Samples A and B were mixed with the PEG-MAL solutions and sonicated in a water bath at room temperature for 10 minutes. Samples were stored at 4°C and analyzed by UV-VIS and FTIR spectroscopies.

Study of the stability of MBAuNPs at a variable pH scale

The pH of four suspensions of as-synthesized MBAuNPs (Section 5.2.2) was modified from 2.6 to 6.0, 7.0, and 8.0, using a NaOH aqueous solution (30 mM) and maintaining the suspensions under vigorous magnetic stirring. Each sample was sonicated during 5 minutes and stored at 4°C. The optical properties of the four suspensions were analyzed using UV-VIS spectroscopy.

For this purpose, 2 mL of each sample were placed in a 4 mL-quartz cuvette. Measurements were carried out in days 1, 2, and 10.

PEGylation of the MBAuNPs (MBAuNPs@PEG-MAL)

1 mg (5.02 mM), 2.5 mg (12.55 mM), 5 mg (25 mM), and 7.5 mg (50 mM) of PEG-MAL were deposited in different Eppendorf tubes containing 1 mL of an aqueous solution of HEPES 50 mM (pH = 7.4). Each mixture was sonicated for five minutes, and added to four Falcon tubes containing 10 mL of MBAuNPs suspensions (pH = 7.4). The mixtures were sonicated for one hour, maintaining the temperature of the ultrasonic bath below 35°C. Afterwards, the samples were centrifuged for 30 min at 6500 rpm, and resuspended in 6 mL HEPES (pH = 7.4) to proceed with the UV-VIS analysis. The samples were stored at 4°C for future experiments. The four samples obtained were designated as MBAuNPs@PEG-Mal-5, MBAuNPs@PEG-Mal-12 mM, MBAuNPs@PEG-Mal-25, and MBAuNPs@PEG-Mal-50.

Conjugation of the MBAuNPs with Peptides P1, P2, and P3

0.5 mg of P1, P2, and P3 were deposited in independent 2 mL-Eppendorf tubes containing 0.5 mL of an aqueous solution of HEPES 50 mM (pH = 7.4). The mixtures were sonicated for 10 min, maintaining the temperature of the ultrasonic bath below 35 °C. Three samples of MBAuNPs@PEG-Mal-5 suspended in 1 mL of HEPES (50 mM, pH = 7.4) were mixed with each peptide solution and sonicated for one hour, maintaining the temperature of the ultrasonic bath below 35 °C. The samples were centrifuged for 30 min at 3500 rpm, and resuspended in 1 mL of water.

5.2.5 Formation of protein corona onto MBAuNPs

1 mL of complete medium (DMEM + FBS 10%) was deposited in a 15 mL-Falcon tube and mixed with 2 mL of PEGylated MBAuNPs. The sample was sonicated in a water bath at room

temperature for 10 minutes, and centrifuged for 20 minutes at 5000 rpm at 20 °C. The volume was made up to 2 mL using deionized water, sonicated in a water bath at room temperature for 10 minutes, and stored at 4 °C for UV-VIS, Dynamic Light Scattering (DLS), and TEM characterization. Another sample was prepared in the same way using non-PEGylated MBAuNPs.

5.2.6 Characterization of pure and functionalized MBAuNPs

UV-VIS and DLS measurements

UV-Vis spectra of AuNPs, MBAuNPs were recorded on a Cary 60 UV-Vis spectrophotometer with a 4 mL-quartz cuvette of 10-mm optical path length to analyze their absorbance in the range 200-1100 nm. DLS measurements were recorded using a Zetasizer Nano Series. For hydrodynamic size determination, 1.5 ml of sample solution was measured in a disposable cuvette. For zeta potential, samples were analyzed using a standard zeta cuvette. The samples were analyzed three times at 25 °C.

FTIR Spectroscopy

FTIR analysis was carried out to gain insights into the chemical functional groups on the surface of nanomaterials to confirm the PEG-MAL hydrolysis, PEG-MAL:L-Cysteine conjugation, MBAuNPs PEGylation, and peptide-MBAuNPs conjugation. Measurements were performed in an IRTracer-100 Fourier Transform Infrared Spectrometer (Shimadzu) equipped with an attenuated total reflection (ATR) diamond accessory to register transmittance over the wavenumber (ν) interval of $4000 < \nu < 400 \text{ cm}^{-1}$. A total of 50 scans were averaged to produce the spectrum and the experiments were performed in triplicate to ensure reproducibility. The interferometer was scanned at 4 resolution, and spectra were obtained by performing a Fourier transform of the interferogram after apodization with a Happ-Genzel.

Scanning (SEM) and Transmission (TEM) Electron Microscopy

To study protein corona formation, TEM analyses were performed using a JEOL 1400 microscope operating at 80 kV. Before placing the samples onto the TEM grids, they were washed according to the following protocol: 500 μ L of as-synthesized MBAuNPs were placed in an Eppendorf tube and the volume was made up to 1.5 mL using absolute ethanol. The sample was sonicated in a water bath at room temperature for five minutes, centrifuged for 10 minutes at 14000 rpm and 20 °C, and the supernatant was discarded. The volume was made up to 1.5 mL with absolute ethanol and the washing protocol was repeated three times. Then, a drop of the sample was deposited onto carbon-coated copper grids and ethanol was evaporated at room temperature for 24 hours. The same procedure was carried out with samples MBAuNPs@PEG-MAL, MBAuNPs@CM, and MBAuNPs@PEG-MAL@CM.

The morphological characteristics of the AuNPs and MBAuNPs were analyzed with a Dual Beam Scanning Electron Microscope FEI-Helios Nanolab 600 from which micrographs were obtained at acceleration voltages of 8 kV and 10 kV, current of 86 pA, and 4 mm working distance, with a secondary electrons (SE) detector and a backscattered electrons (BSE) detector. Prior to the characterization, samples were washed and prepared as follows: 200 μ L of each sample was deposited in 2 mL microcentrifuge tubes and the volume was made up to 1 mL with ethanol (MBAuNPs) or distilled water (AuNPs). The tubes were immersed in an ultrasonic bath for 5 min and centrifuged for 10 min at 13500 rpm. The supernatant was removed by a careful aspiration to conserve the pellet, the volume was made up to 1 mL with ethanol, and the process of ultrasonication/centrifugation was repeated two more times. After this, a drop of each sample was deposited on aluminum sample holders using capillary tubes (CAT. NO. 2502, Kimble-Chase, USA). Samples were allowed to dry overnight. ImageJ software (imagej.net) was used to calculate the size distribution of nanostructures randomly selected from SEM micrographs.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

To determine the concentration of gold atoms in MBAuNPs, 1 mL of the sample was digested with HCl: HNO₃ (3:1). Inductively coupled plasma atomic emission spectroscopy (ICP-OES)

of samples was performed in a Perkin Elmer ICP-OES instrument.

X-Ray Diffraction (XRD)

XRD analysis was performed in a Bruker D8 Advance Equipment with Cu K α radiation (1.5418 Å) generated at 40 kV and 30 mA. The signal was measured in a 2θ range of 10 to 80 degrees, with steps of 0.01 degrees. To prepare the samples, MBAuNPs and AuNPs were deposited on glass sample holders and dried in an oven at °C overnight.

5.3 Results and Discussion

5.3.1 Physicochemical properties of MBAuNPs

Morphological and dimensional analysis

MBAuNPs were synthesized using the seed-mediated method at room temperature. This method required the preformation of spherical gold nanoparticles (AuNPs) acting as nucleation points for heterogeneous growth.¹⁵⁰ The multibranched morphology was obtained using ascorbic acid as a reducing agent and silver nitrate as a shape controller and branch yielder.^{143,151} The micrographs in Fig. 5.1 show AuNPs with spherical shape and average diameter of 12.45 ± 0.02 nm (Fig. 5.1A) and MBAuNPs with a mean tip-to-tip length of 78.49 ± 2.12 nm (Fig. 5.1B). The size distribution histograms for AuNPs and MBAuNPs fit well with a Gaussian distribution, supported by R-Square values of 0.99 and 0.83, respectively.

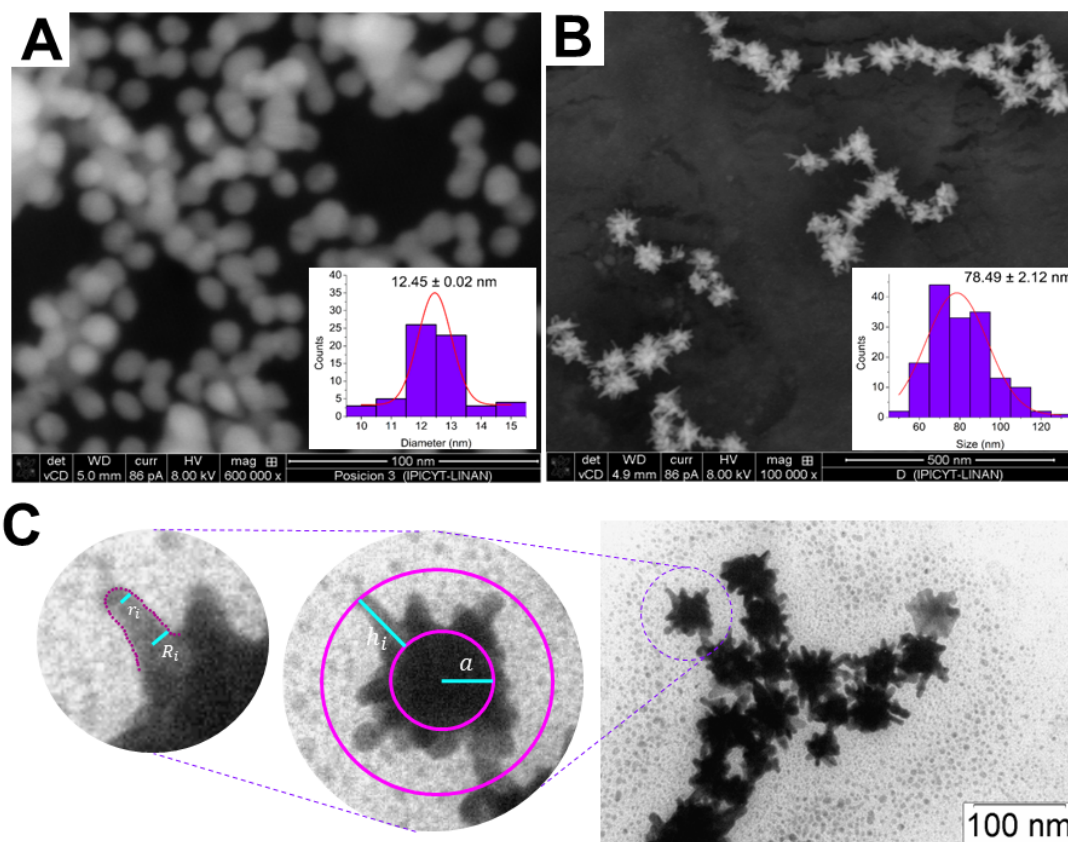


Figure 5.1: Morphological properties of AuNPs and MBAuNPs and size distribution histograms (insets). **A.** SEM micrograph for spherical AuNPs with a mean diameter of 12.45 ± 0.02 nm. The total number of AuNPs counted was 64. **B.** SEM micrograph for gold nanostars (MBAuNPs) with a mean length of 78.49 ± 2.12 nm. The total number of MBAuNPs counted was 158. **C.** TEM micrograph of MBAuNPs and the parameters used to calculate the area and volume of a single MBAuNPs; average core radius $a = 16.08 \pm 3.01$ nm; average tip length $h_i = 24.85 \pm 5.24$ nm; average radius of a branch $r_i = 3.31 \pm 0.54$ nm; and average radius at the base of a branch $R_i = 6.55 \pm 0.94$ nm.

The area and volume of a single MBAuNP were calculated using the equations reported by Tsoulos et al (SI: Calculations A.4.1).¹⁵² The parameters used to calculate the area and volume are indicated in Fig. 5.1C and were determined by measuring 158 nanoparticles in SEM micrographs with the ImageJ software (imagej.net). The parameters obtained were: average core radius $a = 16.08 \pm 3.01$ nm; average tip length $h_i = 24.85 \pm 5.24$ nm; average radius of a branch $r_i = 3.31$

± 0.54 nm; and average radius at the base of a branch $R_i = 6.55 \pm 0.94$ nm, consistent with the dimensions and studies of reproducibility of the seed-mediated Yuan's protocol, reported by Ramsey et al.¹⁵³ The number of branches was considered to be 18 according to the statistical analysis of Tsoulos et al.¹⁵², but calculations for 8, 12, 16, 17, and 20 tips are provided in the supporting information section. The average volume of one MBAuNP with 18 branches was 1.09×10^5 nm³ and the area was 1.59×10^4 nm². The average area, 4.87×10^2 nm², and volume, 1.01×10^3 nm³, of a single AuNP were calculated using the formulas for a sphere of radius 6.225 ± 0.01 nm.

Table 5.2: Dimensions of spherical (AuNPs) and multibranched (MBAuNPs) gold nanoparticles obtained using TEM micrographs.

Name	Size (nm)	Volume (nm ³)	Area nm ²
AuNPs	12.45 ± 0.02	1.01×10^3	4.87×10^2
MBAuNPs	78.49 ± 2.12	1.09×10^5	1.59×10^4

Crystalline properties

The crystalline properties of AuNPs and MBAuNPs were studied by XRD. The diffraction pattern for AuNPs and MBAuNPs is shown in Fig. 5.2. Spherical and anisotropic gold showed four peaks located around 37.9° , 44.12° (43.77° for MBAuNPs), 64.54° , and 77.48° assigned to (111), (200), (220), (311) lattice planes indexed to the face-centered cubic (Space Group Fm-3m No. 225) crystalline structure according with the data reported in the crystallographic card PDF No. 00-004-0784 and literature.^{31,154} The crystallite sizes, obtained via the Scherrer equation (SI: Calculations A.4.2), were calculated from the Full Width at Half Maximum (FWHM) of the most intense peak assigned to the lattice plane 111 for AuNPs and MBAuNPs. The crystallite sizes obtained were 11.86 nm for AuNPs and 10.70 nm for MBAuNPs (Table 5.3).¹⁵⁵ These results revealed that spherical AuNPs are single-crystal nanoparticles since the size is approximately similar to the average diameter of 12.45 ± 0.02 nm obtained from SEM micrographs, unlike MBAuNPs, whose reported size in SEM is about eight times larger (78.49 ± 2.12 nm), indicating nanoparticle polycrystallinity.^{155–157}

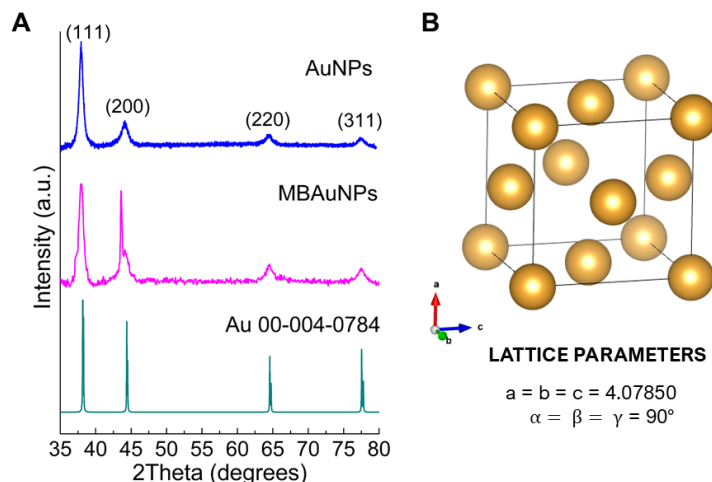


Figure 5.2: Diffraction pattern of the AuNPs and MBAuNPs. The four characteristic peaks for both nanomaterials are compared with those reported in the crystallographic card PDF No. 00-004-0784 for gold (Au). Inset indicates the crystalline structure of Au for single-crystal AuNPs and polycrystalline MBAuNPs.

Table 5.3: Crystalline parameters of AuNPs and MBAuNPs

Sample ID	2θ (°)	Lattice Plane	FWHM (°)	D (nm)
AuNPs	37.94	111	0.74	11.86
MBAuNPs	37.95	111	0.82	10.70

Concentration of AuNPs and MBAuNPs in suspension

The content of Au ions in the suspensions of AuNPs and MBAuNPs was quantified using the ICP-OES analytical technique. The concentration of ions in AuNPs and MBAuNPs ranges from 0.708 to 1.07 mM and 0.164 to 0.257 mM, respectively (SI: Table A.7). The number of AuNPs per liter of suspension was estimated by dividing the total number of atoms per liter of the sample (5.41×10^{20} Au atoms/L), calculated from the ICP-OES results, and the average number of ions in a single AuNP (59612.66 Au ions).^{28,152,158} As result, the concentration of AuNPs per liter of solution was around 9.08×10^{15} AuNPs/L (SI: Calculations A.4.4). For the calculation

of the number of MBAuNPs per liter of suspension, the unit cell edge length of 0.408 nm was considered as reported in the crystallographic card PDF No. 00-004-0784 and confirmed by our experimental results. The volume of the unit cell was calculated as a perfect cube of 0.0679 nm^3 . Therefore, in a single MBAuNP of volume 1.09×10^5 (Table 5.2), there are 1.61×10^6 unit cells. In the fcc unit cell of Au, there are 4 atoms. Thus, in 1.61×10^6 unit cells of Au there are 6.44×10^6 Au atoms, equivalent to a density of 59.08 Au atoms per nm^3 for the overall MBAuNP, which is in good agreement with literature.¹⁵² Using the concentration of Au atoms obtained by ICP-OES analysis of the as-synthesized MBAuNPs (43.8 mg/L), the number of Au atoms per liter was 1.34×10^{20} Au atoms/L and the number of MBAuNPs per liter of solution was 2.08×10^{13} MBAuNPs/L.

Table 5.4: ICP-OES results and concentration of Au ions and NPs per liter of solution.

Designation	Au ions (ICP-OES)	Au atoms per liter	Nanoparticles per liter
	mM	Au atoms/L	NPs/L
AuNPs	0.708 - 1.07	5.41×10^{20}	9.08×10^{15}
MBAuNPs	0.164 - 0.257	1.34×10^{20}	2.08×10^{13}

Optical properties of the MBAuNPs

Metallic NPs show optical properties derived from the collective oscillations of free electrons due to the influence of an oscillating external electromagnetic field.^{156,159} This effect is called the localized surface plasmon resonance effect (LSPR) and occurs when the frequency of incident light matches the frequency of surface electrons (resonance), causing a maximum absorbance in the visible region of the electromagnetic spectrum.^{159–161} This plasmon can be shifted to longer wavelengths as a function of the size, shape, solvent dielectric, or functionalization of the metallic nanoparticle surface with ligands.^{160,162} Herein, the multibranched shape of gold NPs was studied to take advantage of their plasmonic properties, which cause an extinction maximum in the near-infrared (NIR) region of the spectrum, as a tool for the photothermal ablation of cancer cells.^{28,149} Prima facie, as-synthesized AuNPs and MBAuNPs suspended in water showed plasmons at $519.68 \pm 1.98 \text{ nm}$ and $780.61 \pm 31.47 \text{ nm}$, respectively (Fig. 5.3A). Due to the

high sensitivity of the LSPR to changes in size and shape of the structures, it may be difficult to achieve MBAuNPs with a homogeneous geometry, which affects the reproducibility of the LSPR position.¹⁵³ This was evidenced by a standard deviation of 31.47 nm, obtained for MBAuNPs LSPR from independent synthetic procedures under the same experimental conditions. However, a strategy to improve LSPR reproducibility may include the reduction of the synthetic temperature to delay the reduction of gold ions onto the AuNPs seeds to control the homogeneous growth of the branches.¹⁵³

In this study, we found that the position of their LSPR was affected by other factors arising from the synthetic procedure, such as unreduced gold ions in the solution, the presence of ligands on the surface of the NPs, or the solvent dielectric in which they are suspended.¹⁶³ Therefore, determining the precise position and absorption of the LSPR is essential for accurately determining plasmon-dependent parameters such as the molar concentration of NPs or the molar extinction coefficients.¹⁶³ In this regard, the influence of pH, unreduced gold ions, surface chemistry, and solvent was studied to approximate the accurate LSPR position and absorption of MBAuNPs. The analysis of the optical properties of AuNPs under the influence of the mentioned factors has already been studied by other authors and is reported in a vast amount of literature.^{163,164}

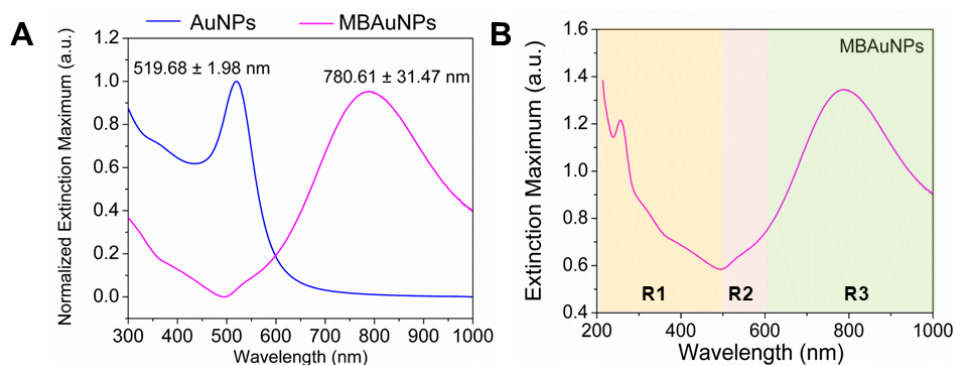
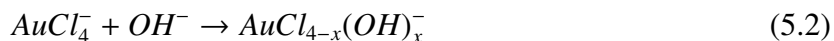
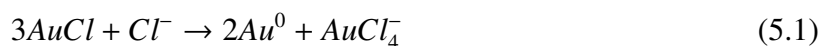


Figure 5.3: UV-Vis spectrum of AuNPs and MBAuNPs. **A.** LSPR of as-synthesized AuNPs and MBAuNPs suspended in water, located at 519.68 ± 1.98 nm and 780.61 ± 31.47 nm, respectively. The absorbance was normalized from 0 to 1 to focus on the LSPR position. **B.** Regions of the UV-Vis spectrum of MBAuNPs at a concentration of 10^{20} MBAuNPs/L (non-normalized absorbance); the region attributed to unreduced gold ions species and organic substances acquired after surface functionalization (R1: 200 - 480 nm), the plasmonic signal caused by the core of the MBAuNPs (R2: 480 - 600 nm), and the region attributed to the hybridization of the plasmon from each branch of the MBAuNPs (R3: 600 - 1000 nm).

Influence of the pH and unreduced gold ions

Fig. 5.3B shows the optical properties of MBAuNPs in the UV-Vis-NIR region (200-1000 nm) of the electromagnetic spectrum. To analyze the influence of pH and unreduced gold ions, the spectrum was divided into three regions: the region attributed to unreduced gold ions species and organic substances acquired after surface functionalization (R1: 200-480 nm),¹⁶³ the plasmonic signal caused by the core of the MBAuNPs (R2: 480-600 nm),¹⁶³ and the region attributed to the hybridization of the plasmon from each branch of the MBAuNPs (R3: 600-1000 nm).^{144,149,165}



In aqueous solutions, tetrachloroauric (III) acid ($HAuCl_4$) dissociates (Eq. 5.1), generating $AuCl_4^-$ ions that absorb in the region from 200 to 400 nm, and this absorption decreases as the pH increases.^{163,166} The pH of the suspension of as-synthesized MBAuNPs was around 2.6 (Fig. 5.4) due to the presence of almost only $AuCl_4^-$ ions, so NaOH (30 mM) was used to increase the pH of the as-synthesized suspensions and decrease the concentration of the $AuCl_4^-$ ions.¹⁶³ As evidenced in Fig. 5.4A, the absorption intensity of the peak at 242 nm (region R1) decreases as the pH increases from 2.6 to 8.0. These changes were attributed to the substitution of Cl^- to form hydroxyl containing gold complexes, like $AuCl_{4-x}(OH)_x^-$ (Eq. 5.2).^{163,166} The position of the LSPR in the NIR region of the electromagnetic spectrum was blue-shifted over time and/or due to pH modification. After increasing the pH to 6.0, 7.0, and 8.0, the absorption attributed to ionic species (R1) reduced significantly, causing a blue-shift of the LSPR of 57 nm, 70 nm, and 62 nm immediately after the synthesis, respectively (Fig. 5.4B-inset).

The increment of the pH increases the concentration of $AuCl_{4-x}(OH)_x^-$ species, in particular $AuCl_3(OH)^-$, stabilizing the LSPR of the MBAuNPs suspension over time as evidenced in Fig. 5.4C. At pH 2.6, the LSPR in R3 was blue-shifted by 59 nm in two days and 75 nm in ten days. Modifying the pH to 6.0, 7.0, and 8.0 reduced the LSPR blue shift after ten days post-synthesis

from 75 nm at pH 2.6 to 64 nm, 30 nm, and 38 nm, respectively. These results support the use of MBAuNPs at physiological pH, which is essential for biofunctionalization.

The pH modification of the MBAuNPs suspension and the change in the ionic strength induced nanoparticle aggregation over time.¹⁶⁷ This effect was evidenced by the reduction of the extinction maximum intensity (%) of the MBAuNPs suspensions with an as-synthesized concentration of 6.03 pM (Fig. 5.4D). The ionic strength at pH 2.6, caused by the presence of $AuCl_4^-$ ions, seems to prevent the aggregation of MBAuNPs over time since the reduction of the extinction was ~5% two days post-synthesis and ~10% after 10 days. In contrast, the increment of the pH to 6.0 caused a reduction of the intensity of the extinction maximum of ~11% two days and ~50% ten days post-synthesis. At pH 7.0, the reduction of the extinction after two days was ~5% and ~35% ten days post-synthesis. The largest decrease in the extinction intensity was evidenced at pH 8.0 since the reduction two days and ten days post-synthesis was ~48% and ~55%, respectively. Moreover, it was experimentally confirmed that even centrifugation of MBAuNPs post-synthesis caused their flocculation (aggregation and agglomeration) and complicated their resuspension.¹⁵³ To achieve nanoparticle resuspension and improve stability, PEGylation of the MBAuNPs was considered as an alternative.

Influence of PEGylation of MBAuNPs

PEGylation is the formation of covalent bonds of polyethylene glycol (PEG) derivatives on the surface of nanoparticles or other compounds.^{168,169} To investigate the influence of the surface modification of MBAuNPs on their optical properties, we employed the polymer polyethylene glycol (PEG), commonly used to improve the water solubility, stability, and biocompatibility of the NPs.^{148,170–172} PEGylation of NPs also improves their biostability and circulation time by reducing nonspecific interactions with serum proteins, forming a protein corona, avoiding uptake by the reticular endothelial system, and reducing phagocytosis. It enables the chemical conjugation of small biomolecules on the surface of NPs, such as peptides, and protects the ligands by reducing the steric hindrance.^{169,173,174}

We considered the use of polyethylene glycol maleimide (PEG-MAL), which has been used as a linking intermediate to conjugate therapeutic proteins or peptides to nanoparticles through

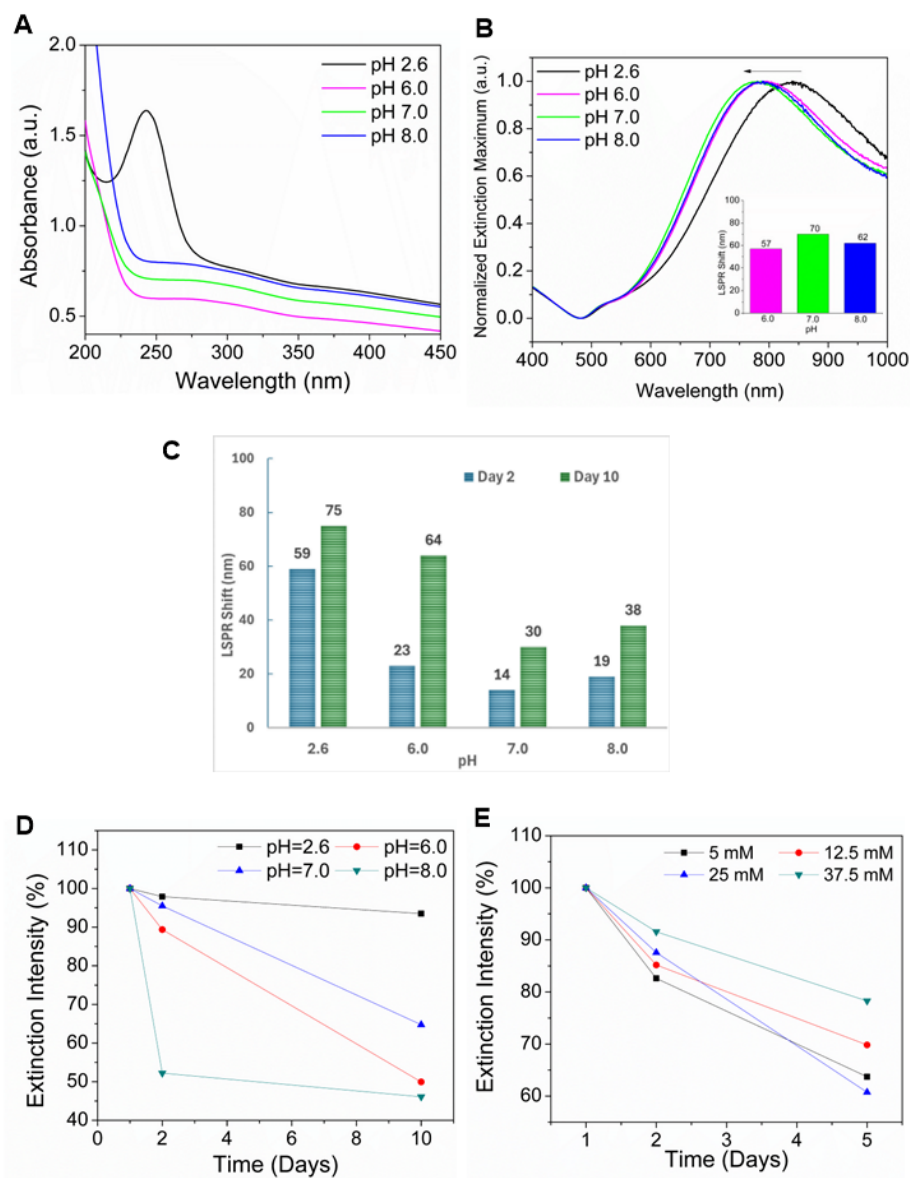


Figure 5.4: Influence of the pH on the optical properties of MBAuNP suspensions. **A.** Decrease of the maximum absorbance intensity attributed to unreduced gold ions (region R1) after pH modification. **B.** LSRP blue-shift (region R3) after pH modification. Inset indicates the blue shift in nm measured during day 1. **C.** Stability of MBAuNP suspensions over time (days 2 and 10) at pH=2.6 (as-synthesized), 6.0, 7.0, and 8.0. The increase in pH decreases the amount of unreduced gold ions and stabilizes the position of the LSPR peak. Reduction (in %) of the extinction maximum intensity after pH modification of **D.** as-synthesized MBAuNPs (6.03 pM) and **E.** PEGylated MBAuNPs.

Michael's addition reaction of thiol to maleimide double bond to form a stable thioether linkage.¹⁷⁰ The maleimide reactive group possesses fast reaction kinetics and high specificity at physiological pH, room temperature, and aqueous buffers.¹⁷⁵ To evaluate the influence of PEGylation on the optical properties of MBAuNPs, different amounts of PEG-MAL were added to MBAuNPs suspended in HEPES (pH = 7.4). The samples were sonicated, centrifuged for 1 hour, and resuspended in 3 mL of HEPES to reach a final concentration of 111.16 pM. Fig. 5.5A shows the optical properties of as-synthesized MBAuNPs measured immediately after synthesis.

All the samples prepared in triplicate (samples A, B, and C) exhibited the LSPR peak at 817 nm, attributed to the star-shaped morphology of MBAuNPs, and a peak at 242 nm attributed to unreduced gold ions. After the pH modification from 2.6 to 7.1 of all triplicates (Fig. 5.5B), the LSPR was blue-shifted 72 nm, locating the plasmon at 735.67 ± 8.14 nm and confirming the effect of the pH on the plasmonic properties of the MBAuNPs.

PEG-MAL lacks absorbance in the visible or NIR region, and the intensity of the peaks at 231 nm and 300 nm located in the ultraviolet region increased with increasing concentration (SI: Figure A.11). After the PEGylation of MBAuNPs (Fig. 5.5C-F), the LSPR (region R3) of all the samples was blue-shifted to ~ 700 nm, and the stability of the plasmon was maintained in time. A broadening effect of the LSPR was evidenced as the concentration of PEG-MAL increased.¹⁶³ This broadening may be attributed to the increase in the local refractive index of the MBAuNPs' chemical surface.¹⁶⁷ Regarding the optical properties enclosed in the region R1, a broad maximum of absorbance around 265 nm indicated the presence of PEG-MAL molecules on the surface of the MBAuNPs to constitute the PEGylated system MBAuNPs@PEG-MAL_x, where x is the concentration of PEG-MAL used during the synthetic protocol (x = 5, 12.5, 25, or 37.6 mM).

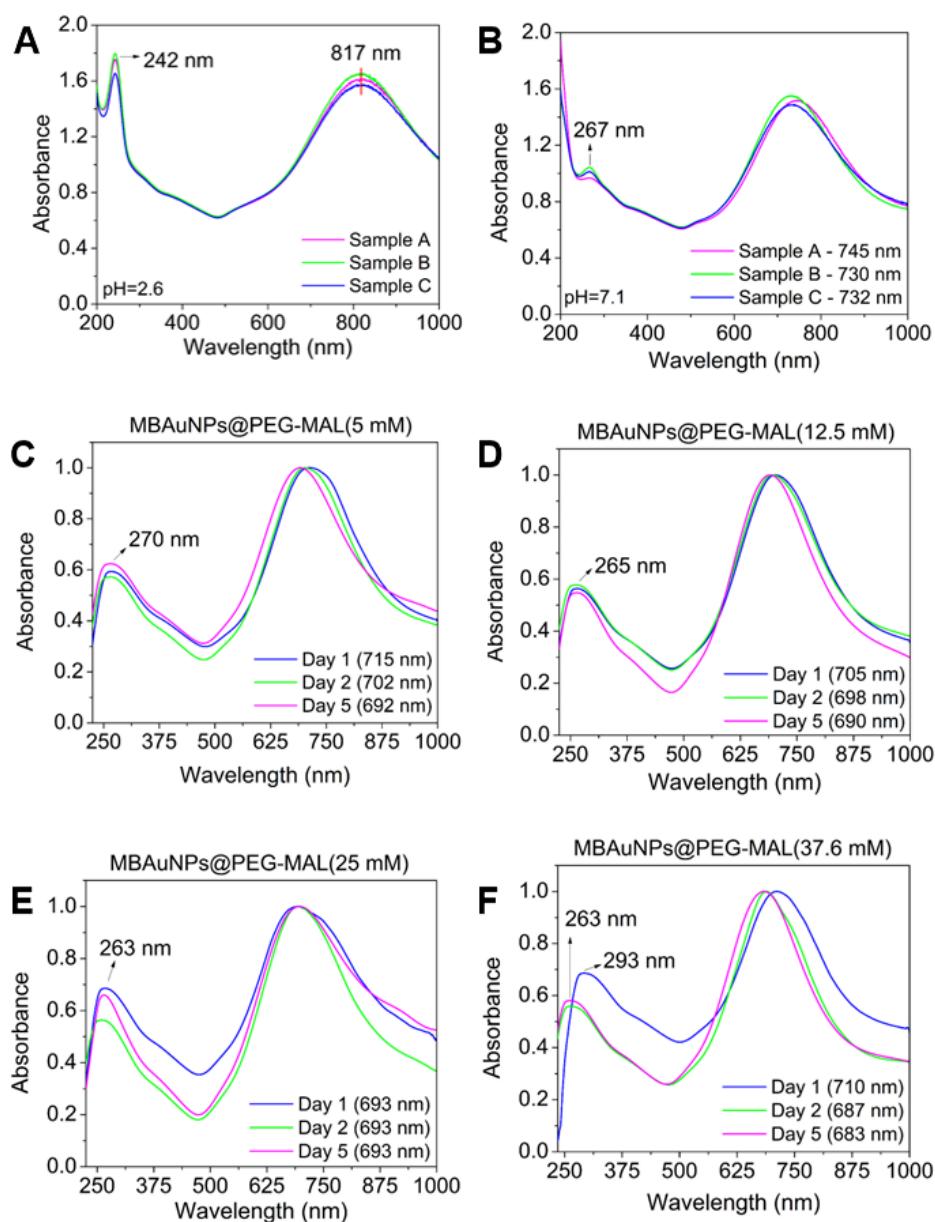


Figure 5.5: Influence of PEGylation on the optical properties of MBAuNP suspensions.

Molar extinction coefficient of AuNPs and MBAuNPs

The molar extinction coefficient (ϵ) of light is the net effect of scattering and absorption and describes the interaction between radiation and matter.¹⁷⁶ It allows the facile estimation of nanoparticle concentration in suspension, which is essential for developing their applications in biomedicine, therapy, imaging, and sensing.^{177,178} The extinction coefficient can be calculated from Beer-Lambert's law, using the molar concentration of nanoparticles in suspension, the extinction maximum at the LSPR obtained via UV-Vis spectroscopy, and the cell path length (1 cm) of the spectrophotometer.¹⁵²

Various studies assume the total reduction of the Au ions added to the reaction for the synthesis of AuNPs; however, this assumption leads to an erroneous estimation of the molar concentration of NPs in the sample, and consequently, the extinction coefficient is miscalculated.¹⁷⁸ Herein, the yield of the Au ions reduction to synthesize AuNPs and MBAuNPs was estimated by considering the number of ions in suspension, calculated from the ICP-OES results, and the total amount of ions used during the synthesis (Table 5.5).

Following other studies reported in the literature, the inverse Turkevich method to synthesize AuNPs and a seed-mediated protocol to synthesize MBAuNPs produced yields of 89.85% and 85.92%, respectively.^{153,157,178,179} A strategy to increase the yield of Au reduction to above 90% during MBAuNPs synthesis includes the synthesis at low temperatures (5 °C) or the replacement of the citrate-capped AuNP seeds by surfactant-stabilized seeds, however, this is not favorable for biomedical applications.^{153,157} The molar ratio for the stoichiometric reduction of Au^{3+} to Au^0 with ascorbic acid used in this work was 1:2, resulting in 4% of Au ions that appear to be wasteful, as evidenced in Fig. 5.4. The molar concentrations (C_M) of AuNPs and MBAuNPs in solution were calculated as the ratio between the total atomic gold concentration added to the reaction volume and the previously determined number of atoms per AuNPs or MBAuNP as determined via ICP-OES.^{152,158,180} The molar concentration (C_M) of AuNPs and MBAuNPs were 1.509×10^{-8} mol/L (0.015 pM) and 3.335×10^{-11} (33.35 pM), respectively.

The extinction maximum of the LSPR was registered and plotted versus the molar concentration of the suspension (Fig. 5.6B and D), and the molar extinction coefficients for AuNPs and MBAuNPs were then obtained from the slope of the linear region as stated in Beer-Lambert's law

(SI: Calculations A.4.6).¹⁸⁰ The values obtained were $(2.35 \pm 0.02) \times 10^8$ for AuNPs and $(4.80 \pm 0.02) \times 10^{10}$ for MBAuNPs, supported by R-Square values of 0.999 and 0.989, respectively. These values are consistent with those reported in literature (Table A.4.7)

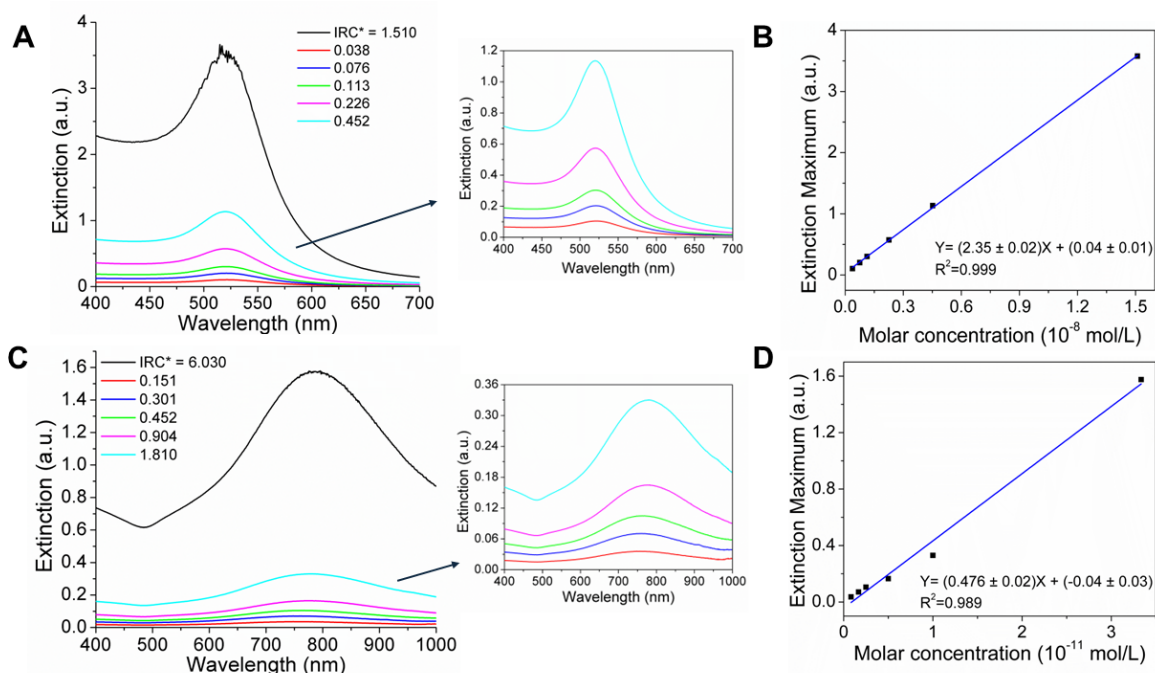


Figure 5.6: Extinction spectra of AuNPs and MBAuNPs. **A.** Extinction spectra of AuNPs and **C.** MBAuNPs at different relative concentrations. Insets indicate an amplified view of each extinction spectrum. Figures **B.** and **D.** show the experimental data of maximum extinction at the surface plasmon resonance band (LSPR) vs. corresponding molar concentration and linear fitting curves for AuNPs and MBAuNPs, respectively. The extinction coefficients (ϵ) were obtained from the slope of the linear region of the plots in B and D, resulting in values of $\epsilon = (2.35 \pm 0.02) \times 10^8$ for AuNPs and $\epsilon = (4.80 \pm 0.01) \times 10^{10}$ for MBAuNPs. R-Square values for the linear fitting were 0.999 and 0.989, respectively. *IRC = initial relative concentration.

Table 5.5: Yield of the Turkevich method to synthesize AuNPs and the seed-mediated method to synthesize MBAuNPs. Molar concentration and extinction coefficients of AuNPs and MBAuNPs

Designation	Yield of reaction	Molar concentration	Extinction coefficient
	%	mol/L	$\text{M}^{-1}\text{cm}^{-1}$
AuNPs	89.85	1.509×10^{-8}	$(2.35 \pm 0.02) \times 10^8$
MBAuNPs	85.92	3.335×10^{-11}	$(4.80 \pm 0.02) \times 10^{10}$

5.3.2 Protein corona formation

Nanoparticles entering the body contact biological fluids, such as plasma, and acquire a protein corona that will determine the biostability of the system in vivo. Moreover, the formation of this corona may compromise the function of ligands on the surface of nanoparticles, such as peptides or antibodies.¹⁸¹ Therefore, evaluating the adhesion of biomolecules onto nanoparticles is of prime importance in nanoparticle design for biomedical applications.

We evaluated the formation of protein corona by exposing PEGylated and non-PEGylated MBAuNPs to complete media (DMEM + FBS 10%). Measurements of the hydrodynamic diameter (H_D) and ζ -potential obtained via DLS characterization correlated with the optical response. The hydrodynamic size of non-PEGylated MBAuNPs increased by 31.94 ± 1.12 nm when exposed to the complete medium. For PEGylated MBAuNPs, the increase in size was even larger 75.13 ± 2.32 nm. Therefore, results suggest that the exposure of MBAuNPs to the complete medium increases the hydrodynamic size of the MBAuNPs due to the adsorption of biomolecules in the complete medium onto their surface, and that PEGylation with PEG-MAL promotes this adsorption. Moreover, the surface charge of MBAuNPs and MBAuNPs@PEG-MAL could have had an important role in the formation of a protein corona since electrostatic forces influence protein-nanoparticle interactions.¹⁸¹ In this aspect, the surface charge of non-PEGylated MBAuNPs was slightly more negative when exposed to the complete medium, changing from -9.41 ± 1.04 mV in water to -13.90 ± 0.26 mV. In the same way, the surface charge of PEGylated MBAuNPs changed from -21.03 ± 0.75 mV to -22.50 ± 0.56 mV, confirming the influence of PEG-MAL on protein corona formation.

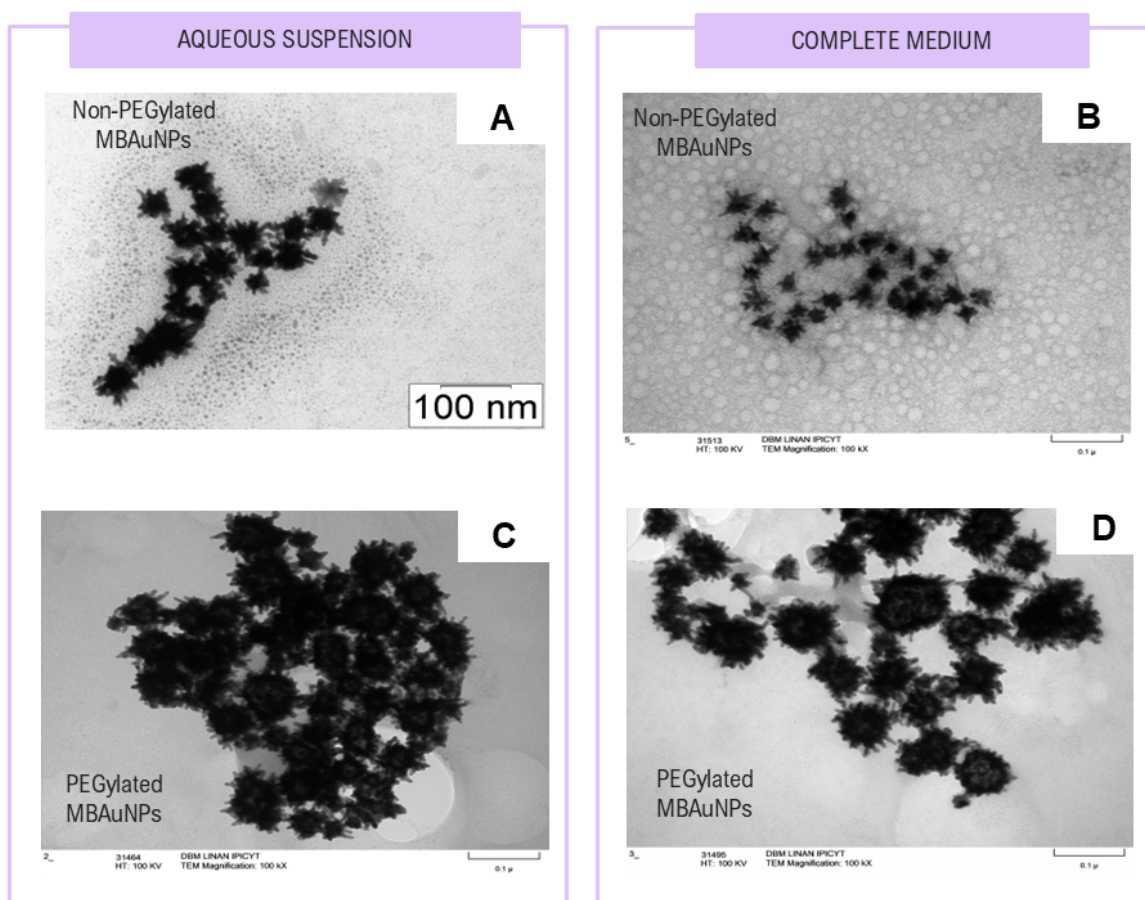


Figure 5.7: Protein corona formation for non-PEGylated MBAuNPs suspended in water (A) and complete medium (B). Protein corona formation for PEGylated MBAuNPs suspended in water (C) and complete medium (D).

5.3.3 Physicochemical properties of P1, P2, and P3

In Chap. 4, we performed a virtual screening analysis to explore the interaction between 44 phage displayed peptides, including P1, P2, and P3, with overexpressed proteins on the surface of HeLa and MDA cells. Results suggested that the presence of specific polar residues in peptides, such as tyrosine (Y), glutamine (Q), asparagine (N), and glutamine (Q), facilitates peptide-protein interactions. However, conformational modes between peptides and proteins were

performed with protonated peptides at physiological conditions (pH=7.4) and without considering the presence of solvents. In this regard, the study of the physicochemical properties of peptides, such as amino acid composition, isoelectric point (pI), net charge, hydrophobicity, and acid/basic character, under acid and neutral conditions allows us to gain insight into their influence on peptide-protein interactions in healthy and tumor tissue. In addition, these parameters play an important role in the formation of aggregates, which reduce the physical stability of the peptide, affects peptide bioactivity, and increase chemical degradation, toxicity, and immunogenicity.¹⁸² Therefore, comprehension of the physicochemical properties of peptides enables the rational design and structure optimization of peptides for suitable peptide synthesis, peptide-nanoparticle conjugation experiments, and therapeutic application.¹⁸³

Table 5.6: Physicochemical parameters of original and modified P1, P2, and P3.

Peptide sequence	pI	Character	Molecular Weight	Charge	Hydrophobicity
	Theoretical		g/mol	pH = 7	kcal/mol
FKQDAWEAVDIR (C ₆₇ H ₁₀₀ N ₁₈ O ₂₀)	4.26	Acid	1517.76	-1	19.81
TYPYNLQVRHWT (C ₇₄ H ₁₀₄ N ₂₀ O ₁₉)	10.07	Basic	1617.8	+1	9.08
FLSGCGGTAEAT (C ₄₆ H ₇₂ N ₁₂ O ₁₈ S ₁)	4.0	Acid	1153.5	-1	13.96
C FKQDAWEAVDIR G (C ₇₂ H ₁₀₈ N ₂₀ O ₂₂ S ₁)	4.26	Acid	1677.79	-1	20.94
C TYPYNLQVRHWT G (C ₇₉ H ₁₁₂ N ₂₂ O ₂₁ S ₁)	9.02	Basic	1777.83	+1	10.21
C FLSGCGGTAEAT (C ₄₉ H ₇₇ N ₁₃ O ₁₉ S ₂)	4.0	Acid	1256.51	-1	13.94

Amino acid composition

At first sight, the addition of the amino acid cysteine (C) and glycine (G) did not impact the physicochemical properties of peptides (Table 5.6). However, the addition of extra residues should be carefully considered, since it can influence chemical degradation and bioactivity of the peptides at specific pH ranges. For instance, at $\text{pH} < 3$, residues glutamine (Q) and asparagine (N) may undergo decomposition by the hydrolysis of their amide groups (deamidation). At $\text{pH} > 6$, amino acids N and Q may undergo intramolecular cyclization to form carboxylates aspartic acid (D) or glutamic acid (E), respectively. At $\text{pH} > 6$, histidine (H), tryptophan (W), tyrosine (Y), methionine (M), and cysteine (C) may oxidize and degrade.¹⁸² Although we considered the addition of the amino acid C to achieve the conjugation of P1, P2 and P3 to PEGylated MBAuNPs via thiol linkage, this amino acid usually forms dithiol bonds with other C amino acids, which requires cleavage to make the -SH functional groups available for conjugation. The formation of dithiol bonds enables peptide cyclization forming a secondary structure without being linked to other peptides. Secondary structures increase proteolytic stability and cell permeability of the peptides.¹⁸⁴

Chemical synthesized peptides carry charged amino and carboxyl termini.¹⁸⁵ N-acetylation (-COCH₃) and C-amidation (-CONH₂) are the most common termini modifications.¹⁸³ Modified peptides mimic natural ones due to the absence of charged termini. They also possess increased cellular uptake and permeability, and enhanced proteolytic stability.¹⁸⁵ As shown in Figs. 5.8, 5.9, and 5.10, P1, P2, and P3 were synthesized with these modifications to potentiate in vivo applications.¹⁸³

To analyze how the type of amino acids influence the physicochemical properties of P1, P2, and P3, we classified them by the type of side chain, polarity, charge, and hydrophobicity (Fig. 5.8, 5.9, and 5.10). Regarding the side chain functional groups, peptide P1 and P2 contain a wide variety of amino acids in their sequence. P1 is constituted by aromatic (W, F), aliphatic (A, V, I), sulfur (C), acidic (D, E), amide (Q), and basic (K, R); P2 contains aromatic (Y, W), aliphatic (V, L), sulfur (C), hydroxyl (T), amide (N, Q), and basic (H, R); and P3 contains aromatic (F), aliphatic (A, L), sulfur (C), hydroxyl (S, T), and acidic (E).

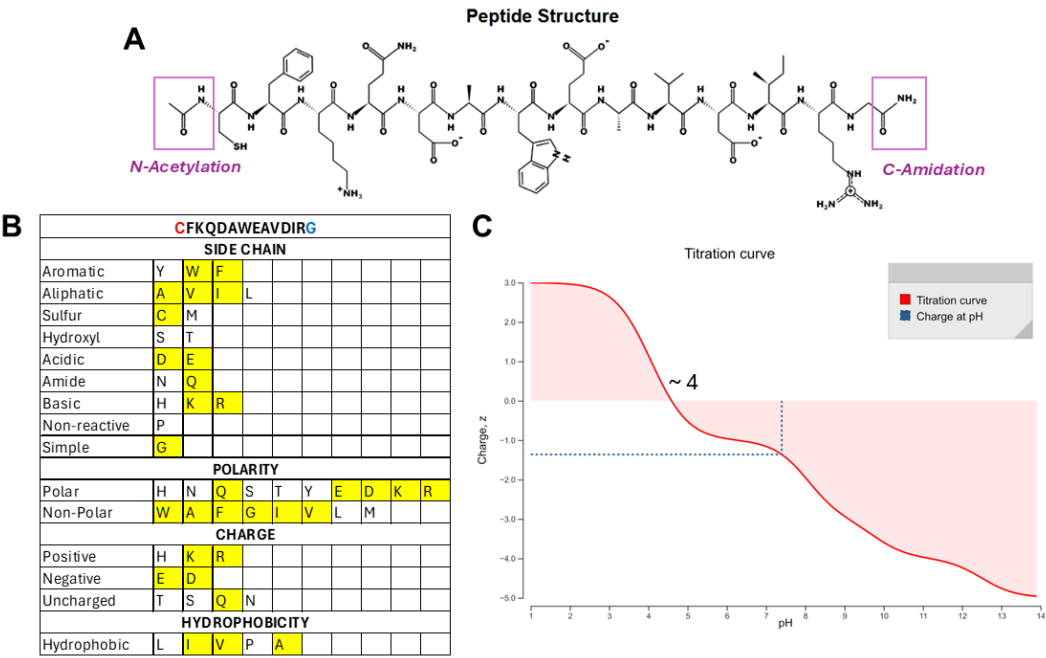


Figure 5.8: **A.** Chemical structure of P1, including N-termini acetylation and C-amidation. **B.** Physicochemical parameters of P1. **C.** Titration curve and isoelectric point of P1.

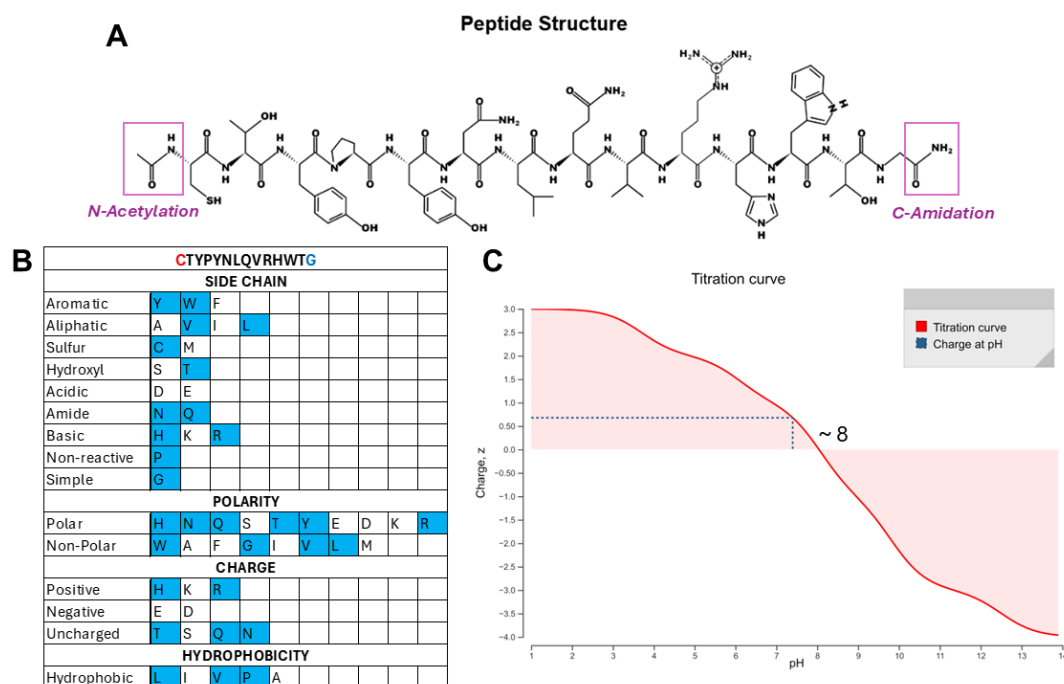


Figure 5.9: **A.** Chemical structure of P2, including N-termini acetylation and C-amidation. **B.** Physicochemical parameters of P2. **C.** Titration curve and isoelectric point of P2.

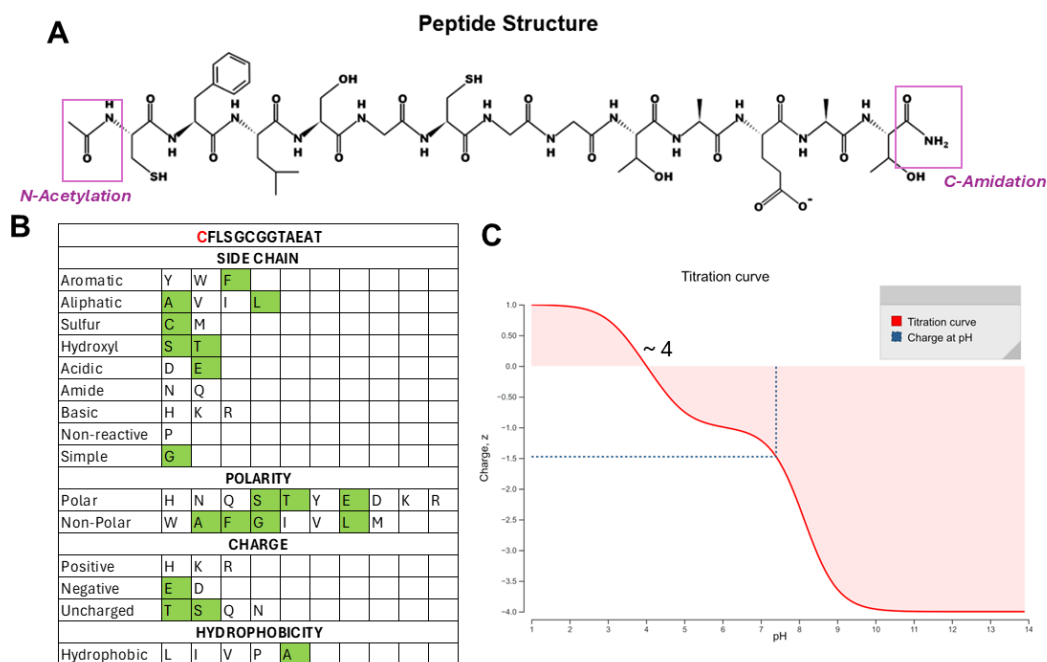


Figure 5.10: **A.** Chemical structure of P1, including N-termini acetylation and C-amidation. **B.** Physicochemical parameters of P1. **C.** Titration curve and isoelectric point of P3.

Peptide hydrophobicity

Hydrophobicity, which measures the affinity of peptides for non-polar environments, can be expressed in terms of the hydrophobic index or as the percentage of hydrophobic amino acids, such as Leucine (L), Isoleucine (I), Valine (V), Proline (P), and Alanine (A).¹⁸⁶ Hydrophobic amino acids confer antioxidant properties to peptides, which can be leveraged during interaction with lipid membranes and hydrophobic regions of proteins to generate free radicals. However, excessive hydrophobicity can reduce solubility, limiting their application in aqueous environments.¹⁸⁶ The addition of Cysteine (C) and Glycine (G) did not significantly influence the hydrophobic index of P1, P2, and P3, which changed from 19.81, 9.08, and 13.96 kcal/mol to 20.94, 10.21, and 13.94, respectively (Table 5.6). P1 has the greater hydrophobic index, which may decrease solubility during peptide-nanoparticle conjugation experiments in water environments. However,

the advantage of a higher hydrophobic index is that P1 may be more resistant to enzymatic degradation than P2 and P3.¹⁸⁶

Charge and isoelectric point

Molecular interactions, bioactivity, solubility, and stability properties of peptides are influenced by the charge and the isoelectric point (pI).¹⁸⁶ Charged peptides are more soluble in aqueous environments and also interact with oppositely charged molecules such as proteins, lipids, or ions.¹⁸⁶ The net charge of peptides depends on the ionization state of their amino acids at different pH ranges.¹⁸⁶ For example, at pH $\sim$ 7.4, amino acids Lysine (K), Arginine (R), and Histidine (H) contribute a positive charge to the peptides, as occurs with P2, which possesses a net charge of +1. Positively charged peptides may act as anticancer peptides because of their ability to recognize and disrupt the negatively charged phospholipid membrane of cancer cells.¹⁸⁶ In contrast, amino acids Glutamic acid (E) and Aspartic acid (D) contribute a negative charge to the peptides, as occurs with peptides P1 and P2, which possess a net charge of -1.

The isoelectric point (pI) is the pH at which a peptide has a net neutral (zero) charge and is determined by the balance between positively and negatively charged amino acids.¹⁸³ At this pH (pH = pI), peptides are least soluble and may precipitate; therefore, for in vivo applications, a pI far from 7 is recommended. As shown in Fig. 5.8, 5.9, and 5.10, P1, P2, and P3 have pI values of 4.26, 9.02, and 4.0, respectively, which guarantee that they will remain charged at neutral pH and even at tumor acidic pH of 6. Moreover, it is important to consider the values of pI, since at pH < pI, P1, P2, and P3 will be positively charged, thus acidic environments are recommended for suitable solubilization. In contrast, for pH > pI, they will be negatively charged; thus, alkaline environments are recommended.

Optical properties of P1, P2 and P3

Using UV-VIS spectrophotometry, the optical properties of P1 and P2 were measured. Generally, the absorbance of a peptide solution from 275 nm to 290 nm is attributed to the functional groups

of the chromophores Tryptophan (W), Tyrosine (Y), and cystine*. ^{187,188} Absorbance in this region enables the calculation of peptide extinction coefficients (ϵ), which then enables the calculation of the concentration of peptides via the Beer-Lambert Law (Equation 6.1). Table 5.7 shows the theoretical and experimental absorbance of P1 and P2 at a concentration of 1 mg/mL. Theoretical absorbances were calculated from free web servers ProtParam, PepDraw, and ProtPi and then compared to experimental absorbances of the synthesized peptides (Fig. 5.11A). Theoretical absorbances for P1 and P2 were 3.358 and 4.879, respectively, and the experimental absorbances were 3.385 and 5. Theoretical extinction coefficients for P1 and P2 were calculated from the equation 6.1. ^{187,188}

$$\epsilon(P) = (nW \times \epsilon_W) + (nY \times \epsilon_Y) + (nC \times \epsilon_C) \quad (5.3)$$

Where nW , nY , and nC are the total number of amino acids in peptide sequences and ϵ_W , ϵ_Y , and ϵ_C are their extinction coefficients: $5500 \text{ M}^{-1}\text{cm}^{-1}$, $1490 \text{ M}^{-1}\text{cm}^{-1}$, and $125 \text{ M}^{-1}\text{cm}^{-1}$, respectively.

After calculation and due to similarities in theoretical and experimental absorbances, we considered $5500 \text{ M}^{-1}\text{cm}^{-1}$, $8480 \text{ M}^{-1}\text{cm}^{-1}$, and $250 \text{ M}^{-1}\text{cm}^{-1}$ as the extinction coefficients of P1, P2, and P3, respectively. Moreover, theoretical and experimental absorbances, as well as the wavelengths at which the maximum of absorbances confirm the adequate chemical synthesis of peptides P1 and P2.

The chemical structure of peptides P1, P2, and P3 was investigated by FTIR measurements (Fig. 5.11B). The characteristic bands corresponding to amino acid side chains appear in the region from 1000 cm^{-1} to 1750 cm^{-1} . Table 5.8 details all the amino acids side chain infrared bands for peptides P1, P2, and P3. ¹⁸⁹ The bands located in the region from 1000 cm^{-1} to 1750 cm^{-1} superimpose with the two most commonly examined regions of the spectrum called amide I (1600 cm^{-1} to 1700 cm^{-1}) and amide II (1480 cm^{-1} to 1580 cm^{-1}) bands. ¹⁹⁰ Amide I band originates primary from C=O stretching vibrations with minor contributions attributed to C-N stretching. Amide II band originates from the bending motions of the N-H groups and stretching

*When two amino acids cysteine (C) form a dithiol bond between their thiol (-SH) functional groups, cystine derivative is formed

of C-N bonds.¹⁹⁰ The amide I and II bands are sensitive to secondary structure since the C=O and N-H bonds of peptides are directly involved in the backbone conformation and hydrogen bond patterns of peptides.¹⁹⁰

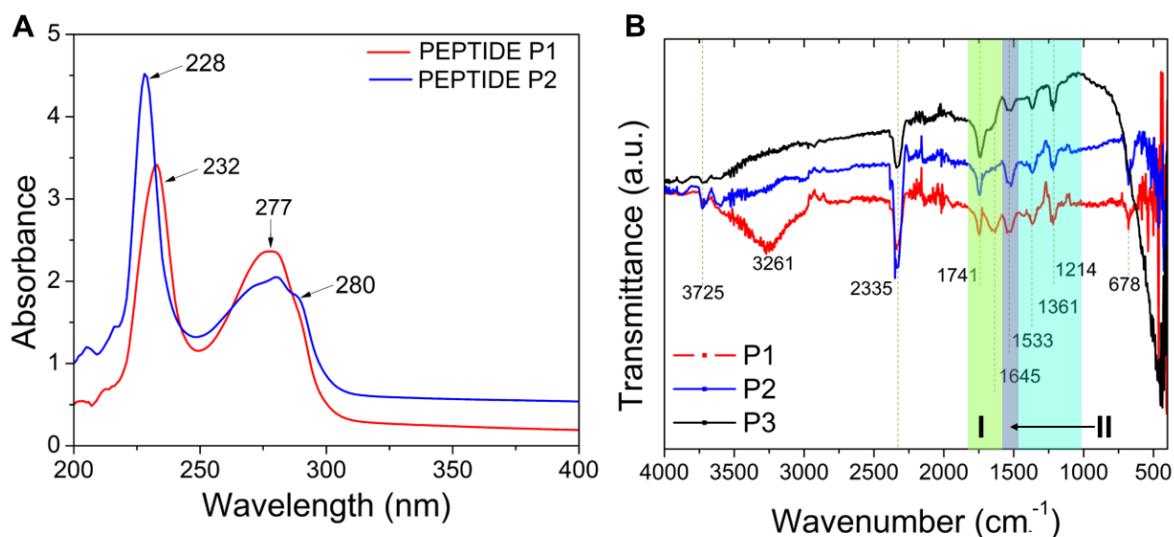


Figure 5.11: A. UV-VIS spectrum for peptides P1 and P2. B. FTIR spectrum of peptides P1, P2, and P3.

Table 5.7: Physicochemical parameters of original and modified P1, P2, and P3.

Peptide sequence	Theoretical Absorbance	Experimental Absorbance	Extinction Coefficient
	1 mg/mL (0.1%)	1 mg/mL (0.1%)	$^{-1}\text{cm}^{-1}$
FKQDAWEAVDIR	3.722	-	5500
TYPYNLQVRHWT	5.375	-	8480
FLSGCGGTAEAT	-	-	-
CFKQDAWEAVDIRG	3.358	3.385	5500
CTYPYNLQVRHWTG	4.879	5.000	8480
CFLSGCGGTAEAT	-	-	250

Table 5.8: FTIR bands of peptides P1, P2, and P3

Amino Acid	Bond	Wavenumber (cm ⁻¹)	Mode of vibration
Cysteine	SH	2551	Stretching
Aspartic Acid	C=O	1716-1788	Stretching
	COO ⁻¹	1574 - 1579	Antisymmetric stretching
	COH	1264 - 1450	In plane bending
Glutamic Acid	C=O	1712-1788	Stretching
	COO ⁻¹	1556 - 1560	Antisymmetric stretching
		1404	Symmetric stretching
	C-O	1120 - 1253	stretching
Asparagine	C=O	1677-1678	Stretching
	HN ₂	1612 - 1622	In plane bending
Arginine	CN ₃ H ₅ ⁺	1672-1673	Antisymmetric Stretching
		1633-1636	Symmetric Stretching
Glutamine	C=O	1668-1687	Stretching
	NH ₂	1586-1610	In plane bending
	CN	1404	Stretching
Histidine	C=C	1630	Stretching
		1575, 1594	Stretching
	CH ₃	1439	In plane bending
	CH	1217	In plane bending
	CN	1229	Stretching
	NH	1199	In plane bending
Lysine	NH ₃ ⁺	1626 - 1629	In plane bending
1526 - 1527			
Tyrosine	CC	1516 - 1518	Stretching
		1599 - 1602	
	C-O, CC	1235 - 1270	Stretching
Tryptophan	COH	1169 - 1260	In plane bending
	CH	1614 - 1621	
	CN	1509	Stretching
	CH		In plane bending
	NH		
	CC	1496	Stretching

5.3.4 Peptide-MBAuNPs Conjugation

Peptides, used as therapeutics, demonstrate some advantages when compared to small molecules, proteins, and antibodies.¹⁸⁴ They exhibit higher affinity and specificity, greater tissue penetration, decreased immunogenicity, and lower production costs.¹⁸⁴ However, peptides are susceptible to proteolytic degradation, which reduces their in vivo stability and half-life, and their potential use in therapeutics.¹⁸⁴ This is attributed to the susceptibility of the amide bonds that link the amino acids to hydrolysis by proteolytic enzymes in vivo, as well as the lack of secondary and tertiary structure.¹⁸⁴ To solve this problem, nanoparticles have been demonstrated to act as protective nanocarriers to avoid peptides from protease-mediated degradation.¹⁹¹ Conjugation with polymers such as polyethylene glycol (PEG) is another strategy to improve the stability of peptides in vivo. By conjugating PEG to the termini of the peptides, glomerular filtration and renal clearance can be prevented. PEG also protects the peptides from enzymatic degradation by forming a protective shell and reducing their immunogenicity through steric masking of potential antigenic sites in the peptides to prevent recognition by the immune system.¹⁸⁴

In this study, we propose the conjugation of peptides to PEGylated MBAuNPs to leverage the advantages of PEG polymer and MBAuNPs as protective structures. For this purpose, we employed the Michael's addition reaction to form a bond between PEG-MAL and peptides P1, P2, and P3. However, to carry this reaction under optimal synthetic conditions, we propose a design of experiment to establish adequate pH conditions to avoid the hydrolysis of the maleimide functional group from PEG-MAL and the formation of the thioether linkage with cysteine.

Hydrolysis of PEG-MAL Polymer

To achieve peptide-MBAuNPs conjugation, we considered the use of PEG-MAL, which has been used as a linking intermediate to conjugate therapeutic proteins or peptides to nanoparticles through Michael's addition reaction of thiol to maleimide double bond to form a stable thioether linkage.¹⁷⁰ The maleimide reactive group possesses fast reaction kinetics and high specificity at physiological pH, room temperature, and aqueous buffers.¹⁷⁵ However, under certain conditions of pH, the maleimide group is susceptible to hydrolysis, resulting in ring opening and forming a

maleic acid amide derivative that is not reactive towards thiol groups.¹⁹²

The hydrolytic conditions of PEG-MAL were explored at different pH ranges (5.1, 6.4, 7.4, and 8.4) over time via UV-Vis before proceeding with the PEGylation of MBAuNPs. As shown in figure 5.12, PEG-MAL shows an intense peak at 220 nm, which is attributed to the PEG chain, and a less intense peak at 300 nm, which is associated with the maleimide group.^{175,193} At pH 5.1 and 6.4, no significant changes were evidenced over time. In contrast, at pH 7.4 and 8.4, the band at 220 nm acquired a shoulder-like aspect, and its intensity decreased. The intensity of the peak at 300 nm decreased at pH 8.4, suggesting the conversion of the cyclic amide to maleic acid, which lacks absorbance at this wavelength.¹⁷⁵ These results confirm the susceptibility of PEG-MAL to alkaline pH and suggest mild acidic and neutral media as the optimal conditions to preserve maleimide stability.⁹⁴ For further conjugation of PEGylated MBAuNPs with peptides P1, P2, and P3 via maleimide-thiol reaction, a pH of ~7 will be considered.¹⁹²

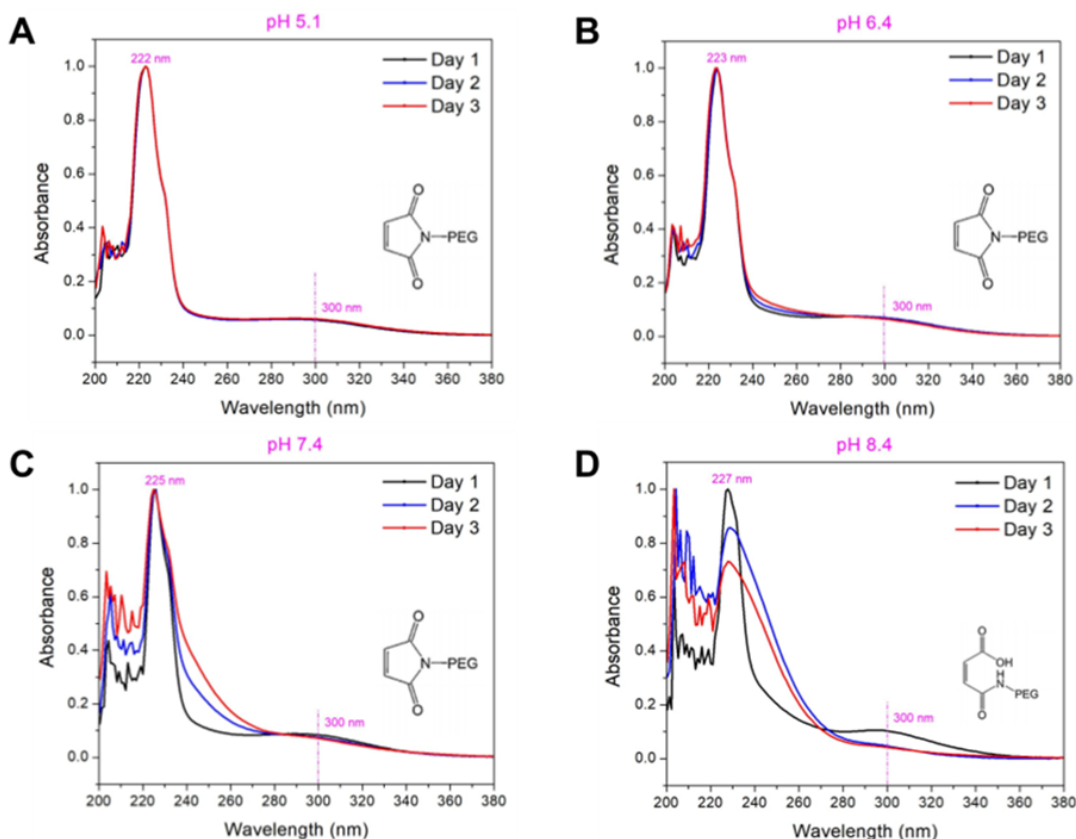


Figure 5.12: Evaluation of the hydrolysis of PEG-MAL at different pH ranges. **A.** At pH=5.1 and **B.** pH=6.4, no significant changes were evidenced with time. In contrast, at **C.** pH=7.4 and **D.** pH=8.4, the band at 220 nm acquires a shoulder-like aspect and the intensity decreases. Also, the maximum at 300 nm significantly decreases at pH 8.4, suggesting the conversion of the cyclic amide to maleic acid, which lacks absorbance at this wavelength.

Conjugation of PEG-MAL and L-Cysteine

Our initial investigations focused on the evaluation of the specific reactivity of PEG-MAL with L-cysteine amino acid to establish suitable experimental conditions before conjugation experiments using peptide P1, P2, and P3. For this purpose, reactions between PEG-MAL and L-Cysteine were conducted in 50 mM HEPES buffers (pH=6.4 and 7.4) using two molar ratios between the ligand (L-Cysteine) and the reactive group maleimide: 1:6 and 1:10. Conjugation was studied

using UV-Vis and FTIR characterization.

Figure 5.13 shows the UV-VIS spectra for Maleimide:L-Cysteine conjugation. Absorbances indicate that the intensity of the characteristic band of maleimide group decreases at pH 6.4 (Fig. 5.13A, C) and 7.4 (Fig. 5.13B, D), which suggest the formation of the thioether bond between the maleimide group of PEG-MAL and the -SH group of cysteine. Also, the maleimide: cysteine molar ratio does not influence greatly the coupling. However, a molar excess of maleimide is recommended to ensure maximum conjugation efficiency.¹⁷⁵

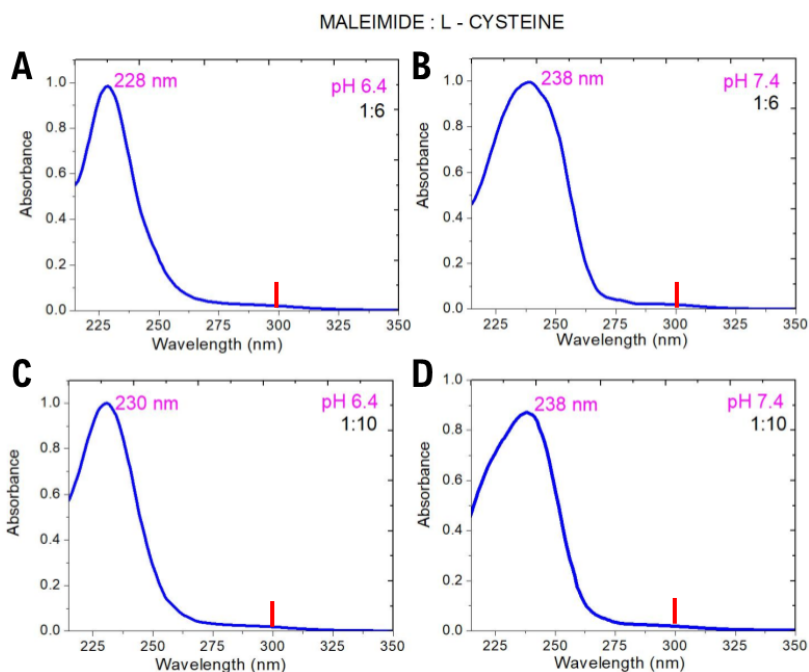


Figure 5.13: UV-VIS spectra for Maleimide:L-Cysteine conjugation. Results indicate that the intensity of the characteristic band of maleimide group (300 nm) decreases at pH 6.4 (A, C) and 7.4 (B, D), which suggest the formation of the thioether bond between the maleimide group of PEG-MAL and the -SH group of cysteine. Also, the maleimide: cysteine molar ratio does not influence greatly the coupling.

We evaluated the vibrational modes to confirm the coupling between PEG-MAL and L-Cysteine using FTIR. Fig. 5.14 shows the spectra for L-Cysteine, PEG-MAL, and PEG-MAL/L-Cysteine at pH 6.4 and 7.4. Spectrum for L-Cysteine shows the bands at 1063, 1404, 1510,

2550, 2960, and 3400-3000 cm^{-1} which correspond to S-H in-plane bend, COO- stretching, NH₂ bending, S-H stretching, C-H vibration in the alkyl chain, and O-H and N-H stretching, respectively.^{194,195} The spectrum for PEG-MAL shows the band at 600-900, 1100, 1380, 1660, 1600-1800, and 2850-300 cm^{-1} which corresponds to N-H wagging, C-O-C stretching, C-H bending, C=O stretching and N-H bending, the maleimide ring, and CH₂ stretching, respectively.^{196,197} A notable change in the intensity of these bands after the coupling reaction was evidenced, especially in the region from 1700-1000 cm^{-1} , which suggest the modification of the S-H functional group in L-Cysteine and the maleimide ring in PEG-MAL due to the formation of a thioether bond. In addition, no effect on the coupling results was observed when using pH 6.4 or pH 7.4, suggesting that the reaction can be carried out at both pHs.

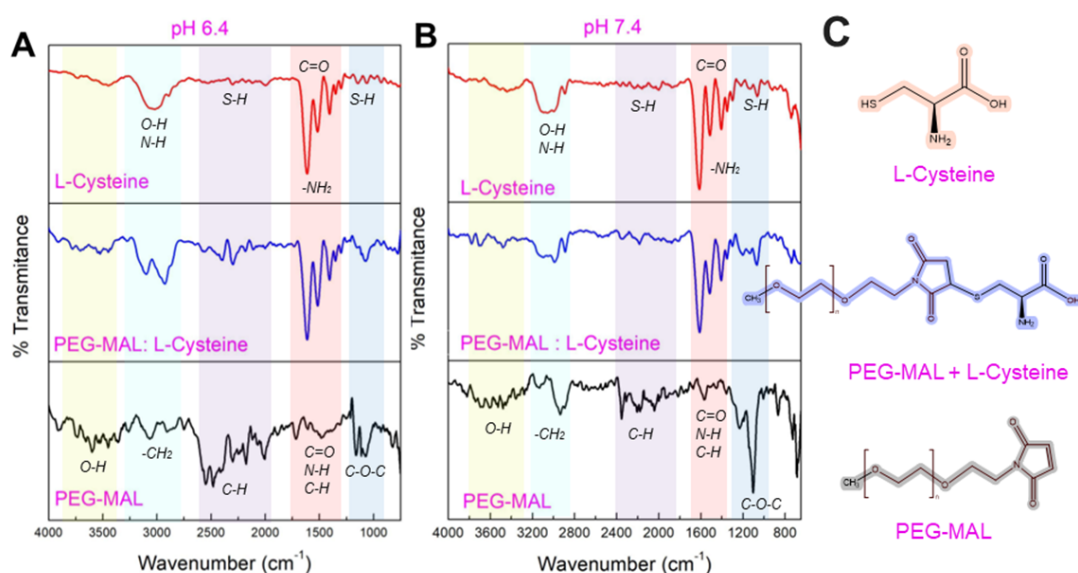


Figure 5.14: FTIR results show the conjugation between L-Cysteine and PEG-MAL at different pH conditions: 6.4 (A) and 7.4 (B). L-Cysteine, thioether bond formed between PEG-MAL and L-Cysteine, and PEG-MAL (C).

5.3.5 Conjugation of the MBAuNPs with Peptides P1, P2, and P3

The experimental conditions used to achieve PEG-MAL: L-Cysteine conjugation were applied to functionalize the peptides P1, P2, and P3 to PEGylated MBAuNPs. This conjugation ensures

that the MBAuNPs carrying P1, P2, and P3 specifically recognize and interact with receptors overexpressed in HeLa and MDA cells to increase MBAuNPs uptake.¹⁹¹ In this regard, receptor-mediated targeting requires the covalent conjugation between P1, P2, and P3 and the maleimide group from PEGylated MBAuNPs.¹⁷⁵

The Michael's addition reaction leverages the high reactivity of maleimide under mild conditions such as room temperature, aqueous buffers, and neutral pH.¹⁷⁵ Michael-type additions cross-linking avoids the use of cytotoxic free radicals and UV light, but instead requires a nucleophilic buffer like HEPES.¹⁹² Maleimide groups have high selectivity towards thiol groups to form a succinimidyl thioether bond that is relatively stable under most conditions.^{175,192} Therefore, we leveraged the addition of a cysteine amino acid to the N-terminal of peptides P1, P2, and P3 to form a thioether bond with PEG-MAL as shown in Fig.5.15).

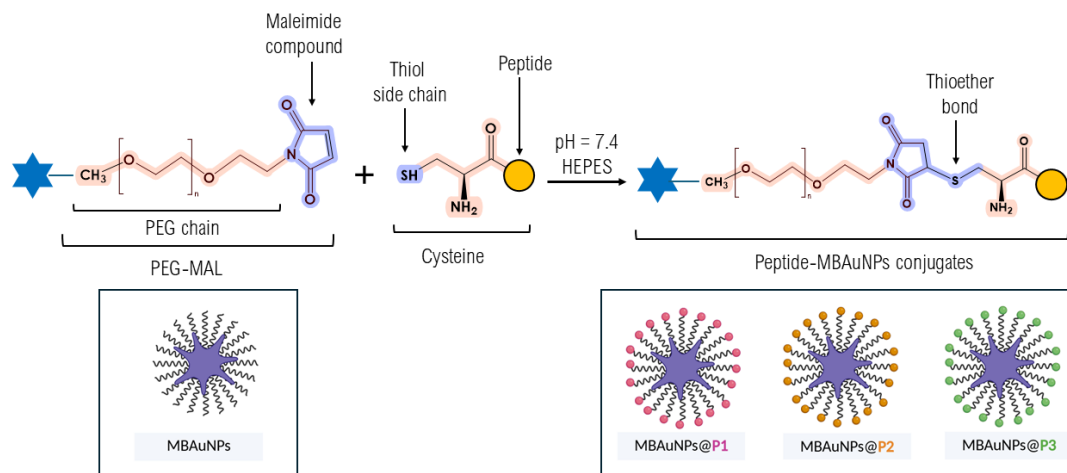


Figure 5.15: Michael's addition reaction between PEGylated MBAuNPs and peptides P1, P2, and P3.

Figure 5.16 shows the UV-VIS spectra of pure MBAuNPs (pH=7.4), PEGylated MBAuNPs, and peptide-MBAuNPs conjugates. PEGylated MBAuNPs (Fig. 5.16-B) exhibit an intense maximum of absorbance around 210 nm, which is attributed to the PEG-chain. As mentioned in previous sections, the signal at 300 nm, which is attributed to the maleimide group from PEG-MAL, enables the conjugation with cysteine from peptides P1, P2, and P3. After conjugation of PEGylated MBAuNPs with peptides P1 (MBAuNPs@P1, Fig. 5.16-C) and P2 (MBAuNPs@P2,

Fig. 5.16-D), a maximum of absorbance appears around 280 nm, confirming the presence of P1 and P2 on the surface of PEGylated MBAuNPs. Since the absorbance at 280 nm is attributed mainly to the presence of amino acids tryptophan and tyrosine in the peptides, both of which are absent in P3, the system MBAuNPs@P3 shows a broad absorbance from 200 nm to 400 nm without an evident maximum around 280 nm (Fig. 5.16-D). To confirm the chemical conjugation of P1, P2, and P3 with the PEGylated MBAuNPs, FTIR measurements were carried out.

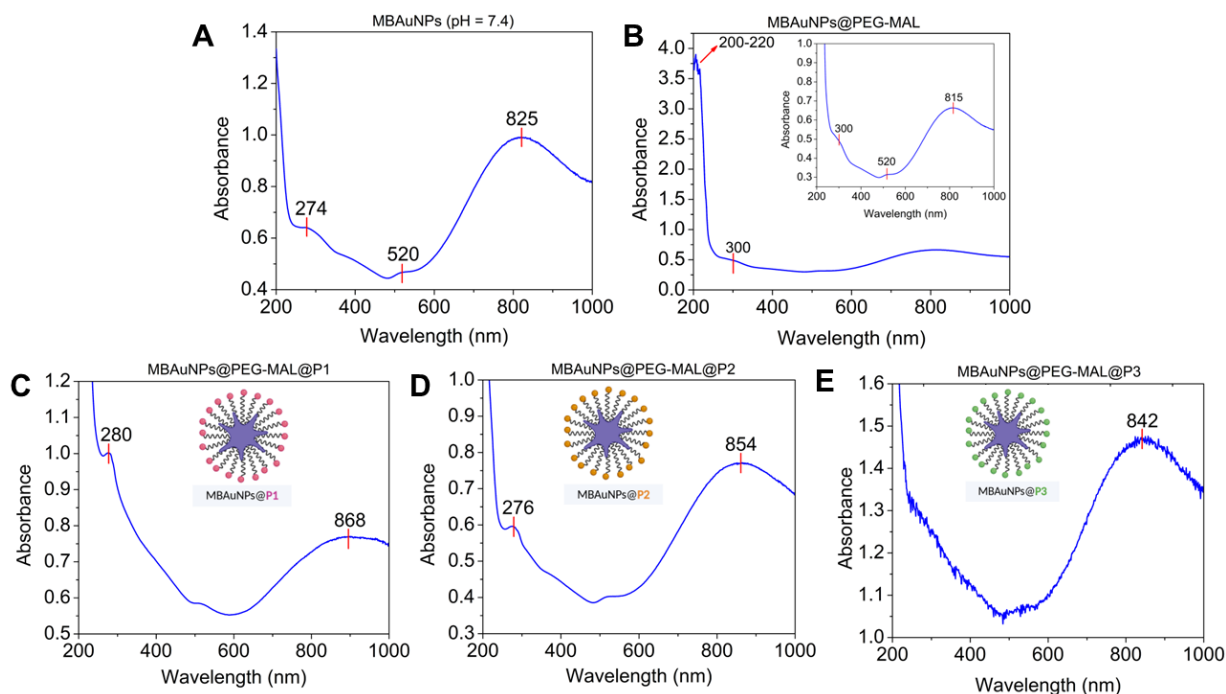


Figure 5.16: UV-VIS spectra of MBAuNPs (A), MBAuNPs@PEG-MAL (B), MBAuNPs@P1 (C), MBAuNPs@P2 (D), and MBAuNPs@P3 (E).

Pure MBAuNPs (Fig. 5.17-a) were synthesized using ascorbic acid as a reduction agent, so a broad band in the range from $3800\text{--}3200\text{ cm}^{-1}$ arise due to the presence O-H groups on the surface of the MBAuNPs. The bands at 1529 cm^{-1} and 1066 cm^{-1} , corresponding to C=O, O-H, and C-H bonds, arise due to the presence of ascorbic acid. On the other hand, the bands of pure PEG-MAL (Fig. 5.17-b) appear located in the region from $3000\text{--}2850\text{ cm}^{-1}$, 1100 cm^{-1} , and around 900 cm^{-1} correspond to the C-H vibrations, whereas the bands at 1500 cm^{-1} and 1150 cm^{-1} correspond to

the C=C and C-H-C of the maleimide ring, respectively. For PEGylated MBAuNPs (Fig. 5.17-c), a combination of the band from pure MBAuNPs and PEG-MAL is evidenced. However, two new bands appear at 1407 cm^{-1} and 1185 cm^{-1} , suggesting decomposition of the double bond of the maleimide ring to form a single bond C-C, and a succinimide C-N-C bond. Interestingly, both bands increase in intensity after peptide conjugation (Fig. 5.17-d, e, f). Moreover, peptide conjugation is confirmed due to the presence of the S-H vibration at 700 cm^{-1} attributed to the thioether bond.

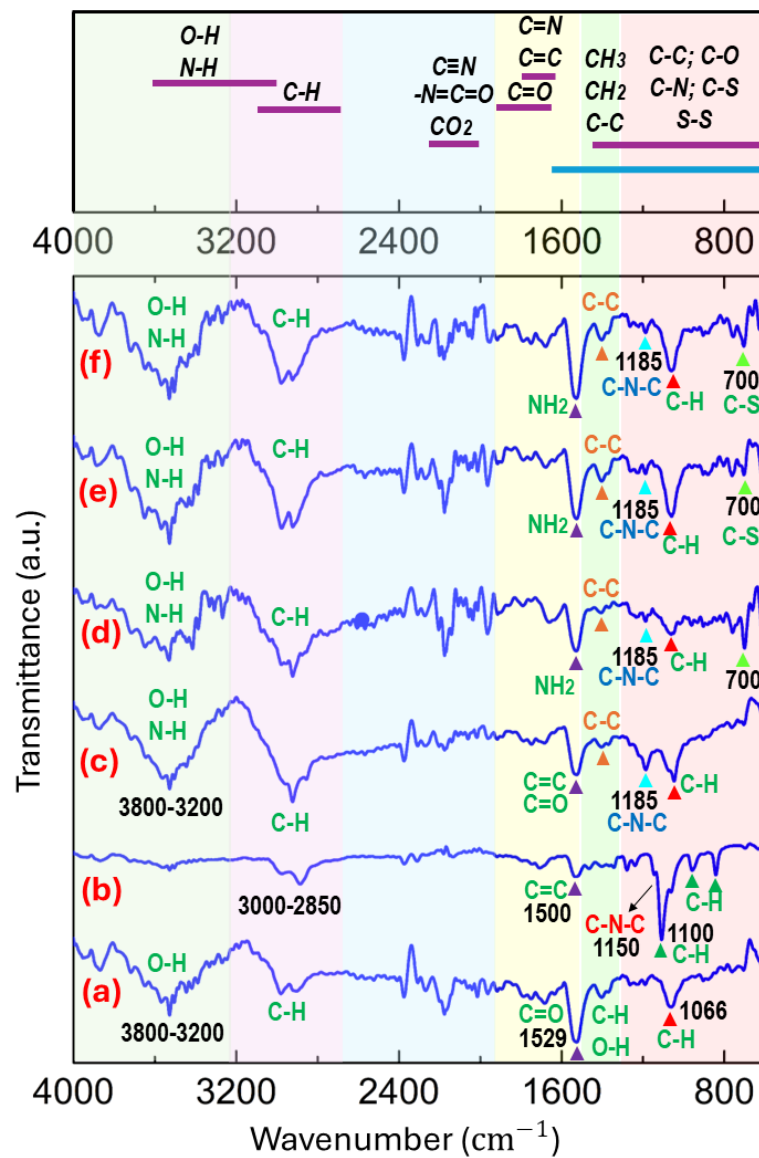


Figure 5.17: FTIR spectra of MBAuNPs (a), PEG-MAL (b), MBAuNPs@PEG-MAL (c), MBAuNPs@P1 (d), MBAuNPs@P2 (e), and MBAuNPs@P3 (f)

5.4 Conclusions

This chapter addressed the following question: How can we conjugate peptides to nanoparticles to obtain nanopharmaceuticals for the active transport of APIs? To venture in this question, we hypothesized that peptide-nanoparticle conjugates can be obtained via the Michael's Addition Reaction. To confirm the hypothesis, we focused on three tasks: 1) the synthesis of MBAuNPs with optimal physicochemical properties, 2) the synthesis of peptides and the characterization of their physicochemical properties, and 3) the establishment of a Design of Experiment (DoE) to conjugate the peptides to MBAuNPs.

We carried out an in-depth study on the physicochemical properties of MBAuNPs using diverse characterization techniques, such as SEM, TEM, XRD, ICP-OES, and UV-VIS. SEM and TEM allow the calculation of the size, area and volume of the MBAuNPs. The values for each parameter were 78.49 ± 2.12 nm, 1.59×10^4 nm², and 1.09×10^5 nm³, respectively. Via XRD characterization, the composition of MBAuNPs was confirmed to be entirely gold, which allow us to approximate their crystallite size (10.70 nm) and the unite cell volume (0.0679 nm³). By complementing XRD results with ICP-OES analysis of Au atomic concentration in MBAuNPs (2.41×10^{-4} mol/L) de concentration of MBAuNs in suspension was calculated to be 2.08×10^{13} MBAuNPs/L. Using LSPR obtained after UV-VIS characterization and the concentration of MBAuNPs/L, we estimated the extinction coefficient for MBAuNPs (4.80×10^{10} M⁻¹cm⁻¹). Finally, the molar concentration of MBAuNPs was calculated using the extinction coefficient and the maximum absorbance ob MBAuNPs (33.35 pM). The molar concentration and the extinction coefficient of MBAuNPs will be further used to carry internallization kinetics experiments in HeLa cells. The synthesis and characterization of the chemical properties of peptides P1, P2, and P3 was presented in this chapter. UV-VIS and FTIR characterization of P1, P2, and P3 confirmed their optimal synthesis via SPPS. We also proposed a DoE for proper peptide-MBAuNPs conjugation. The DoE included the establishment of the optima experimental conditions of pH to avoid the hydrolysis of PEG-MAL and achieve the conjugation of PEG-MAL to L-Cysteine. The DoE demonstrated to be effective to achieve the optimal conjugation of peptides P1, P2, and P3 to PEGylated MBAuNPs via the Michael's addition reaction to obtain the nanoplatforms MBAuNPs@P1, MBAuNPs@P2, and MBAuNPs@P3.

Chapter 6

Core-Shell Nanoplatforms for Hyperthermia

6.1 Introduction

The synthesis and characterization of multifunctional nanostructures is of importance in nanotechnology research due to their potential and practical applications in nanomedicine. CSNSs with magnetic and plasmonic properties have potential future in a wide range of applications, such as cell separation, enzyme immobilization, protein and nucleic acid purification, targeted drug delivery, environmental remediation separation, catalysis, and magnetic resonance imaging.¹⁵⁴

Magnetite nanoparticles (MNPs) have important properties that make them attractive candidates for use in the human body.¹⁹⁸ Some remarkable properties include biocompatibility and low toxicity, superparamagnetic behavior at room temperature, and a large saturation magnetization which implies a response under the application of an external magnetic field.¹⁹⁸ However, bare MNPs have strong magnetic dipole-dipole interaction which causes the formation of big clusters and contributes to hydrophobicity, making necessary the application of coatings. Silica coated MNPs (CSNS) have been demonstrated to effectively enhance biocompatibility, prevent corrosion and agglomeration of MNPs, and improve their stability in aqueous solution.³¹ On the other hand,

gold nanoparticles (GNPs) also have made significant progress among inorganic nanoparticles used in medical applications such as transport, targeting, hyperthermia, and drug release control. They have raised interest because of their optical and electrical properties, and low toxicity.³

Herein, we present the synthesis and characterization of hierarchical nanostructures composed of a core of magnetite (Fe_3O_4) nanoparticles (MNPs) enclosed in a SiO_2 layer to constitute a CSNS and decorated with gold nanoparticles with spherical (AuNPs) and multi-branched (MBAuNPs) morphology. Results suggested that the systems CSNS, CSNS@Au, and CSNS@MBAu comprise magnetothermal properties, suitable for a wide range of applications.

6.2 Methodology

6.2.1 Reagents

Gold (III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) 99.99% trace metals basis, trisodium citrate dihydrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$) $\geq 99\%$ FC, silver nitrate (AgNO_3) ACS reagent $\geq 99.0\%$, L-ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) ACS reagent $\geq 99.0\%$, sodium hydroxide (NaOH) ACS reagent $\geq 97\%$, hydrochloric acid (HCl), sodium nitrate (NaNO_3) ReagentPlus $\geq 99\%$, iron (II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) ACS reagent $\geq 99\%$, tetraethylorthosilicate (TEOS) reagent grade 98%, 3-aminopropyltriethoxysilane (APTES) 99%, and ammonium hydroxide (NH_4OH) solution 28% NH_3 in H_2O $\geq 99.99\%$ trace metals basis were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol 96% was purchased from REPROQUIFIN (Estado de México, México). All samples were prepared using Milli Q water (18 M Ω).

6.2.2 Synthesis of Core-Shell Nanostructures (CSNS)

Synthesis of AuNPs and MBAuNPs

Citrate-stabilized AuNPs were synthesized using the inverse Turkevich method with certain modifications.^{149,199} Briefly, 1.5 mL of an aqueous solution of sodium citrate (0.34 mmol) was injected into 10 mL of an aqueous solution of HAuCl_4 (0.01 mmol) at boiling temperature under vigorous magnetic stirring. The mixture was allowed to react for 10 minutes until the color of the solution turned red-wine and cooled at room temperature. Sample was stored at 4 °C for further analysis.

MBAuNPs were prepared using a seed-mediated growth method according to the protocol of Yuan et al.¹⁴⁹ Briefly, 40 μL of an aqueous solution of HCl (10 mL, 0.01 mol) and 400 μL of the as-synthesized AuNPs were added to 40 mL of an aqueous solution of HAuCl_4 (0.01 mmol) under vigorous magnetic stirring. Immediately, and simultaneously, 400 μL and 200 μL of aqueous solutions of AgNO_3 (1 mL, 0.003 mmol) and $\text{C}_6\text{H}_8\text{O}_6$ (10 mL, 0.001 mol) were injected into the mixture, respectively. Immediately, a color change from light red to blue-gray was noted. The mixture was removed from the plate and stored at 4 °C.

Synthesis of magnetite nanoparticles (MNPs)

The one-step synthesis of magnetite nanoparticles (MNPs) was based on the protocol of Hui et al. to obtain hydrophilic nanoparticles on the gram scale.²⁰⁰ An aqueous solution of $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ (19 mL, 1 mmol) was mixed with NaOH (4 mmol), and NaNO_3 (0.2 mol) under magnetic stirring. The temperature of the mixture was raised to 100 °C until a completely transparent solution was formed. An aqueous solution of $\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$ (1 mL, 2 mmol) was quickly added and the mixture was maintained under magnetic stirring at 100 °C for 1 h. After letting the MNPs cool at room temperature, they were separated from the solvent by a magnet and the supernatant was discarded. The MNPs were rinsed three times with distilled water, dried in an oven at 120 °C for 20 hours, and stored at room temperature.

Synthesis of Fe₃O₄@SiO₂ nanoparticles (CSNS)

The coating process was based on the protocol of Ji et al. with certain modifications.²⁹ In a typical procedure, 10 mg of MNPs were dispersed in 3 mL of distilled water using an ultrasonic bath for 15 minutes, at room temperature. Then, ethanol (40 mL) was added and the sample was ultrasonicated for an additional 10 minutes. 7 mL of NH₄OH were added and the ultrasonication conditions were maintained for another 30 minutes. Subsequently, 0.5 mL of TEOS were added to the mixture and the sample was ultrasonicated for 2h keeping the temperature of the bath below 40 °C. The mixture was allowed to sit for 18 h at room temperature. The as-synthesized CSNS were separated from the solvent by centrifugation at 6500 rpm for 10 minutes, the supernatant was discarded, and the volume was made up to 10 mL with ethanol to rinse them by ultrasonication for 10 minutes. This process was performed three times. Afterward, the CSNS were dried in an oven at 60 °C for 5 hours and stored at room temperature.

Silanization of the CSNS (CSNS@APTES)

10 mg of CSNS were redispersed in 10 mL of ethanol using an ultrasonic bath for 15 minutes at room temperature. Thereafter, APTES (30 µL) was added to the mixture and the sample was sonicated for 1 h, keeping the temperature of the bath between below 40 °C. To discard the excess APTES, the CSNS were rinsed three times with ethanol by ultrasonication and centrifugation processes for 10 minutes, dried in an oven at 80 °C for 1 hour and stored at room temperature.

Decoration of CSNS with AuNPs and MBAuNPs

Before proceeding with the deposition of AuNPs and MBAuNPs onto the surface of the CSNS, they were prepared by resuspending 10 mg of the CSNS@APTES in 10 mL of ethanol for 15 minutes in an ultrasonic water bath. Afterward, 2 mL of the ethanolic CSNS@APTES solution were injected in 4 mL of as-synthesized MBAuNPs under ultrasonication. On the other hand, 2 mL of CSNS@APTES were centrifuged and redispersed in 2 mL of distilled water prior to injection into the 4 mL of as-synthesized AuNPs suspension. Both samples were sonicated for 1

hour.

6.2.3 Characterization of the CSNS

UV-VIS Spectroscopy

UV-Vis spectra of the MNPs, CSNS, AuNPs, MBAuNPs, CSNS@Au, and CSNS@MBAu were recorded on a Cary 60 UV-Vis spectrophotometer with a 4 mL-quartz cuvette of 10-mm optical path length to analyze their absorbance. Samples were prepared as follows: 10 mg of MNPs powder and 10 mg of CSNS powder were resuspended in 10 mL of distilled water via ultrasonication for 15 minutes and their UV-Vis spectra were recorded. AuNPs, MBAuNPs, CSNS@Au, and CSNS@MBAu solutions were characterized as synthesized.

FTIR Spectroscopy

To confirm the presence of amine ($-NH_2$) groups onto the surface of CSNS after amination, pure and functionalized nanostructures were analyzed by FT-IR. Measurements were performed in an IRTracer-100 Fourier Transform Infrared Spectrometer (Shimadzu) equipped with an attenuated total reflection (ATR) diamond accessory to register transmittance over the wavenumber (ν) interval of $4000 < \nu < 400 \text{ cm}^{-1}$. A total of 50 scans were averaged to produce the spectrum and the experiments were performed in triplicate to ensure reproducibility. The interferometer was scanned at 4 resolution, and spectra were obtained by performing a Fourier transform of the interferogram after apodization with a Happ-Genzel. Transmittance spectra were recorded using CSNS and CSNS@APTES as dried powders.

Scanning Electron Microscopy (SEM)

The morphological characteristics of the MNPs, CSNS, AuNPs, MBAuNPs, CSNS@Au, and CSNS@MBAu were analyzed with a Dual Beam Scanning Electron Microscope FEI-Helios

Nanolab 600 from which micrographs were obtained at acceleration voltages of 8 kV and 10 kV, current of 86 pA, and 4 mm working distance, with a secondary electrons (SE) detector and a backscattered electrons (BSE) detector. Prior to the characterization, samples were washed and prepared as follows: 200 μ L of each sample was deposited in 2 mL microcentrifuge tubes and the volume was made up to 1 mL with ethanol (MNPs, CSNS, MBAuNPs, CSNS@Au, and CSNS@MBAu) or distilled water (AuNPs). The tubes were immersed in an ultrasonic bath for 5 min and centrifuged for 10 min at 13500 rpm. The supernatant was removed by a careful aspiration to conserve the pellet, the volume was made up to 1 mL with ethanol, and the process of ultrasonication/centrifugation was repeated two more times. After this, a drop of each sample was deposited on aluminum sample holders using capillary tubes (CAT. NO. 2502, Kimble-Chase, USA). Samples were allowed to dry overnight. ImageJ software (imagej.net) was used to calculate the size distribution of more than fifty nanostructures randomly selected from SEM micrographs.

Transmission Electron Microscopy (TEM) & Energy Dispersive X-Ray Spectroscopy (EDS)

Structural and morphological characterization of the samples was carried out with a Transmission Electron Microscope HR-TEM FEI Tecnai F30 from which micrographs were obtained using the high-angle annular dark field (HAADF) imaging mode (commonly referred to as Z-contrast mode) at an acceleration voltage of 300 kV and point resolution of 3 Å. Elemental (EDS) analysis was performed using a spectrometer with an ultrathin-window Si-Li detector. Previously washed samples were dropped onto a carbon-film-supported copper grid using capillary tubes and allowed to air-dry overnight.

X-Ray Diffraction (XRD)

XRD analysis was performed in a Bruker D8 Advance Equipment with Cu K α radiation (1.5418 Å) generated at 40 kV and 30 mA. The signal was measured in a 2θ range of 10 to 80 degrees, with steps of 0.01 degrees. To prepare the samples, MNPs and CSNS powders were deposited on glass sample holders. To characterize CSNS@Au and CSNS@MBAu, sample preparation was

modified since the signal of the core (MNPs) was not detected even in long-term measurements (15 min) due to the high-intensity peaks of GNPs. In this regard, 10 mg of CSNS@APTES were deposited in 10 mL of AuNPs and MBAuNPs, respectively, and the samples were immersed in an ultrasonic bath at room temperature for 1 hour. Samples were centrifuged, supernatant was discarded and pellets were dried at 80 °C for 1 h. CSNS@Au and CSNS@MBAu powders were deposited on glass sample holders and analyzed.

Physical Property Measurement System (PPMS)

The magnetic properties of the samples were characterized with a Physical Property Measurement System, type "cryogen-free", DynaCool model from Quantum Design Inc., at 300 K, magnetic field range of 2 Tesla, and magnetic field (H) between -20 and 20 kOe. For magnetization measurements, both uncoated MNPs and CSNS were used as dry powders. CSNS@GNPs were dispersed in ethanol by ultrasonication for 10 minutes, dropped on a PPMS sample holder, and dried (drop by drop) at 100 °C.

6.2.4 Calorimetric Measurements)

The heating capacity of the nanostructures (CSNS, CSNS@Au, and CSNS@MBAu) in alternating magnetic fields was measured with the magnetic-calorimeter system (MX Pats. 65,340, Mx/a/2018/002848, Guadalajara, Jalisco, Mexico) working at a frequency of 330 kHz, and magnetic field of $0 < H < 366$ Oe.²⁰¹ The samples CSNS, CSNS@Au, and CSNS@MBAu were prepared in triplicate with a concentration of 10 mg/mL in distilled water and deposited into 2 mL-Eppendorf tubes. Another sample containing 1 mL of distilled water was used as a negative control. The curves of increment of temperature in time (T vs t) were measured by irradiating each sample at five different fixed magnetic fields ($H = 15, 20, 25, 30,$ and 35 mT) for three minutes. The initial slope was calculated for each curve as temperature response is non-linear in time for non-adiabatic systems due to thermal loss, and average SAR values were estimated.²⁰²

6.3 Results and Discussion

6.3.1 Morphological and dimensional analysis

MNPs were synthesized by thermal decomposition of the inorganic salt FeSO_4 at 100 °C in the presence of sodium hydroxide, alkali sodium nitrate, and the biocompatible electrostatic stabilizer trisodium citrate.²⁰⁰ TEM micrographs in Figs. 6.1A and 6.1B show large agglomerates of magnetic nanoparticles with an anisotropic shape instead of a well-formed spherical shape. The formation of agglomerates may be attributed to the strong dipole-dipole interaction between nanoparticles.³¹ The size distribution histogram in 6.1A-inset showed an average size of around 15 nm converging well with a Gaussian distribution for a particle size of 14.88 ± 0.62 nm ($R^2 = 0.79$).

The synthesis of the CSNS was carried out by a multistep sol-gel procedure in which MNPs were coated with amorphous SiO_2 following the Stober method.²⁰³ Figs. 6.1C and 6.1D show TEM micrographs of the CSNS constituted by a magnetic core of MNPs enclosed in a SiO_2 layer. The CSNS have a well-defined spherical shape and most of them are monodispersed. The size distribution histogram in Fig. 6.1C-inset shows an average diameter in the range of 150 nm to 180 nm. The Gaussian distribution calculation was found to be 164.71 ± 1.39 nm ($R^2 = 0.89$) for CSNS.

TEM micrographs in Figs. 6.1F and 6.1H confirmed the decoration of the CSNS with the AuNPs shown in 6.1E, which have a diameter 12.45 ± 0.02 nm ($R^2 = 0.99$) and the MBAuNPs shown in 6.1G of length 49.98 ± 1.13 nm ($R^2 = 0.77$) to constitute the systems CSNS@Au and CSNS@MBAu, respectively. In addition, the Gaussian distribution of size for CSNS@Au was calculated to be 190.74 ± 1.11 nm ($R^2 = 0.83$).

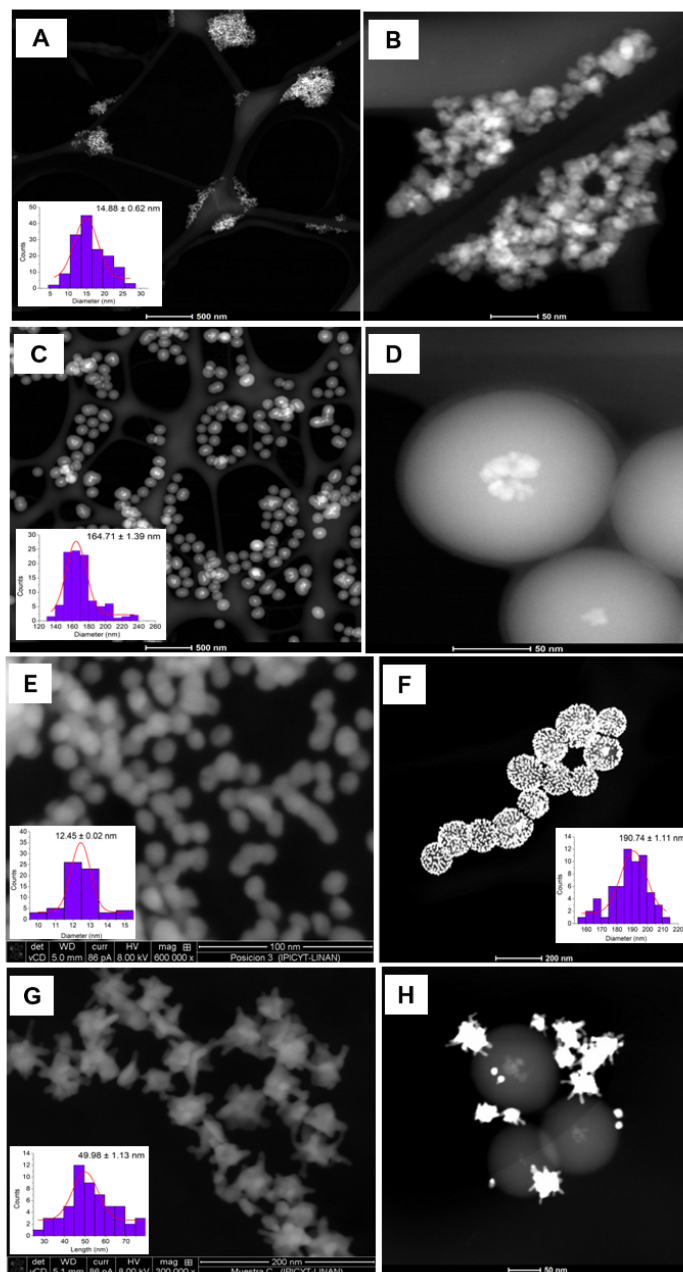


Figure 6.1: Representative TEM and SEM micrographs, and size distributions of MNPs (A, B), CSNS (C, D), spherical AuNPs (E), CSNS@Au (F), multibranched MBAuNPs (G), and CSNS@MBAu (H)

6.3.2 Optical properties

Regarding the optical properties of MNPs, a broad absorbance in the range of 350 nm to 600 nm was noted (Fig. 17-A). Pure AuNPs and MBAuNPs exhibit optical properties derived from the collective oscillations of free electrons, known as the surface plasmon resonance effect (LSPR), which causes a maximum absorbance in the visible region of the electromagnetic spectrum.^{15,156,204,205} This plasmon can be shifted to the near-infrared region as a function of particle size, shape, stabilizing ligands, and solvent dielectric.^{160,204} In this aspect, the maximum absorbance of AuNPs and MBAuNPs presented in Fig. 17-B. is located around 517 nm and 735 nm, respectively. These results are consistent with those reported in the literature.¹⁵ After decoration with gold NPs, their plasmonic properties were transferred to the CSNS as shown in Fig. 17-C, which shows the maximum absorbance of the CSNS@Au and CSNS@MBAu systems located around 555 nm and 745 nm, respectively.

To facilitate the deposition of the AuNPs and MBAuNPs onto CSNS, their surface was functionalized with free amine ($-NH_2$) groups from APTES, and this modification was confirmed by FTIR spectroscopy, a suitable technique that offers information regarding the chemical information at the liquid-solid interface or solid surface of nanoparticles.²⁰⁶ Fig. 6.2D and table 6.1 indicate the bands of CSNS measured as dried powders after functionalization with APTES ($((OC_2H_5)_3Si(CH_2)_3NH_2)$), an organotrialkoxysilane molecule constituted by three ethoxy side chains and one $-NH_2$ group (Fig. 6.2D-inset).¹¹² The silica surface was silanized via covalent bonding between the $-OH$ groups on its surface and the $-NH_2$ group from APTES. In the spectra of pure APTES, the broadband in the region of $3700 > \nu > 3200\text{ cm}^{-1}$ is attributed to the stretching mode of the Si-OH groups. The bands associated with the antisymmetric and symmetric stretching modes of $-NH_2$ are located at 3370 cm^{-1} and 3295 cm^{-1} , respectively. The band placed at 2975 cm^{-1} , and the related bands at 2932 cm^{-1} and 2883 cm^{-1} are associated with the stretching modes of CH_2 and CH_3 , respectively. The deformation mode of the Si- CH_2 bond, the antisymmetric stretching of the Si-O-C, and the symmetric stretching of the C-O bond are placed at 1410 cm^{-1} , 1104 cm^{-1} , and 1089 cm^{-1} , respectively.²⁰⁷ On the other hand, two important regions are distinguished in the FTIR spectra of the silanized CSNS; the bands located in the region from $1450 > \nu > 700\text{ cm}^{-1}$ that originates from the bulk of the silica shell and comprise information about the surface of the silica layer that is in contact with the liquid medium, and the

region from $1020 > \nu > 920 \text{ cm}^{-1}$ that provide information about chemical modification of the surface of silica.²⁰⁶

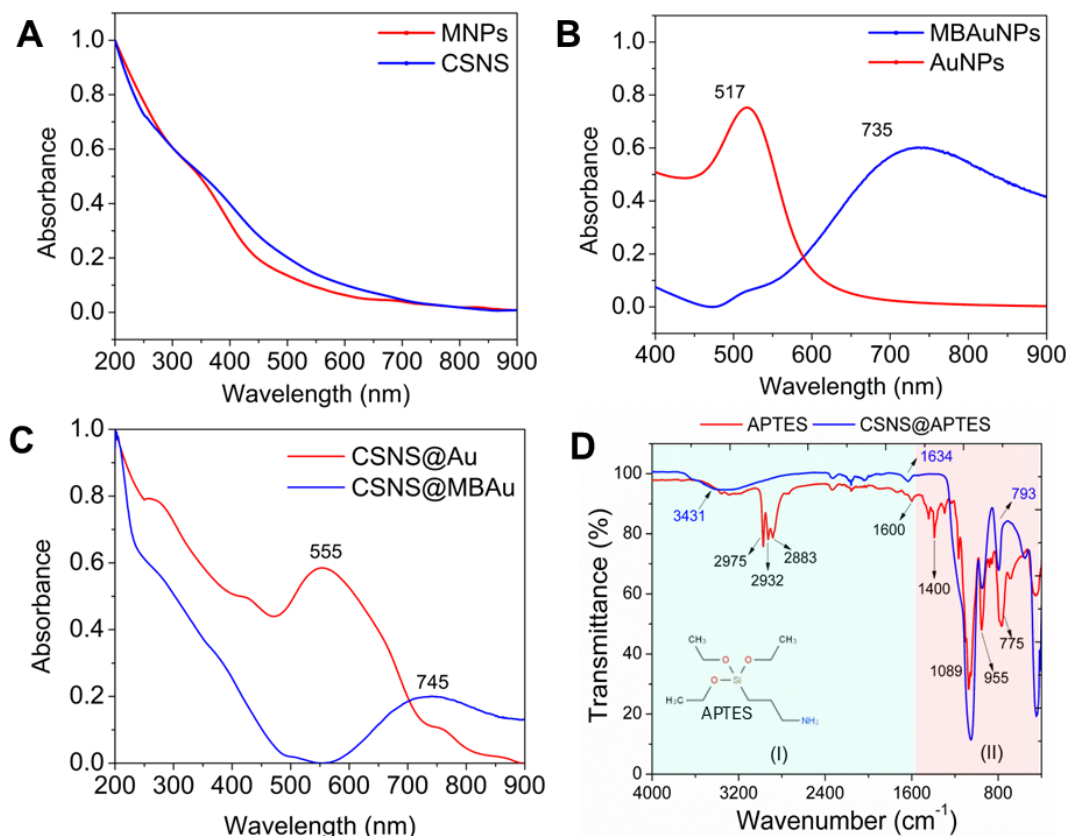


Figure 6.2: Optical properties measured using UV-Vis spectroscopy for: **A.** gold nanoparticles with spherical (AuNPs) and anisotropic (MBAuNPs) morphology; **B.** magnetite nanoparticles (MNPs) and core-shell nanostructures (CSNS); and **C.** gold-decorated CSNS. **D.** FTIR spectrum of CSNS after functionalization with APTES.

Table 6.1: FTIR bands of the CSNS@APTES.

Bond	Wavenumber (cm⁻¹)	Mode of vibration
Pure APTES solution		
Si-OH	3700 - 3200	Stretching
N-H	3370	Asymmetric stretching
N-H	3295	Symmetric stretching
CH ₃	2975	Stretching
CH ₂	2932	Stretching
CH ₂	2883	Stretching
NH ₂	1600	Bending
Si-CH ₂	1400	Deformation
Si-O-C	1104	Asymmetric
C-O	1089	Symmetric
Bulk of the silica shell in the CSNS (1450 > ν > 400 cm⁻¹)		
O-Si-O	1200	Symmetric stretching
Si-O-Si	1200	Symmetric bending
O-Si-O	1120	Asymmetric stretching
O-Si-O	1120	Asymmetric bending
Si-O-Si	1100	Asymmetric stretching
Si-O-Si	801	Symmetric stretching
Si-O-Si	466, 793	Bending from scissoring
Surface of the silica shell in contact with liquid (1450 > ν > 700 cm⁻¹)		
Si-O	1040 (shoulder-like)	Asymmetric stretching
≡Si-OH	961.5	Stretching
O-H	1634	Bending
Surface modification of the silica shell due to silanization (1450 > ν > 700 cm⁻¹)		
NH ₂	3431	Asymmetric stretching
NH ₂	1634	Bending

6.3.3 Crystalline Properties

The MNPs diffraction pattern in Fig. 6.3 shows prominent peaks at 18.30° , 30.03° , 35.65° , 43.25° , 53.65° , 57.12° , 62.70° , 71.32° , and 74.32° which correspond to planes (111), (220), (311), (400), (422), (511), (440), (620) and (533) of cubic spinel structure of Fe_3O_4 (Space Group Fd-3m No. 227), respectively. These results are consistent with the peaks reported in the crystallographic card PDF No. 01-080-6402 for magnetite mineral (Fe_3O_4) and results reported in literature.^{31,154,200,202} Using the Debye-Scherrer equation, the crystallite size of MNPs was calculated from Full Width at Half Maximum (FWHM) of the most intense peak located at 35.65° corresponding to plane (311). The size obtained through this calculation was 13.85 nm which is consistent with the average size of 14.88 ± 0.62 nm reported by TEM analysis, suggesting that the MNPs are single-crystal particles (single-domain structures) and, consequently, they will exhibit superparamagnetic properties.¹⁵⁶

The diffraction pattern for CSNS ($\text{Fe}_3\text{O}_4@\text{SiO}_2$) shows peaks at 30.09° , 35.61° , 43.19° , 57.24° , 62.88° , and 71.32° , which correspond to planes (220), (311), (400), (511), (440) and (620) of MNPs. An additional broad peak between $20^\circ < 2\theta < 30^\circ$ appeared due to the presence of amorphous SiO_2 . The crystallite size of MNPs located at the core of the nanoplatform was calculated using the intense peak (311), located at 35.61° . The size obtained was 15.13 nm suggesting that crystalline properties of MNPs were not modified by silica coating. Advantages of silica coating include the prevention of MNPs agglomeration and further oxidation effects of the magnetic core.³¹

The diffraction patterns for CSNS@Au and CSNS@MBAu showed four new peaks at 38.0° , 44.18° , 64.49° , and 77.67° assigned to planes (111), (200), (220), (311), and (222) lattice planes of gold with spherical shape (AuNPs) and multi-branched shape (MBAuNPs), both formed by a face-centered cubic (Space Group Fm-3m No. 225) crystalline structure according with the data reported in the crystallographic card PDF No. 00-004-0784 and literature.^{31,154} The crystallite sizes calculated for AuNPs ($2\theta = 38.00^\circ$, plane 111) and MBAuNPs ($2\theta = 38.04^\circ$, plane 111) were 10.99 nm and 15.27 nm, respectively.¹⁵⁵ These results revealed that spherical AuNPs may be single-crystal nanoparticles, unlike MBAuNPs, whose reported size in TEM is three times larger, indicating sample polycrystallinity.^{155,156}

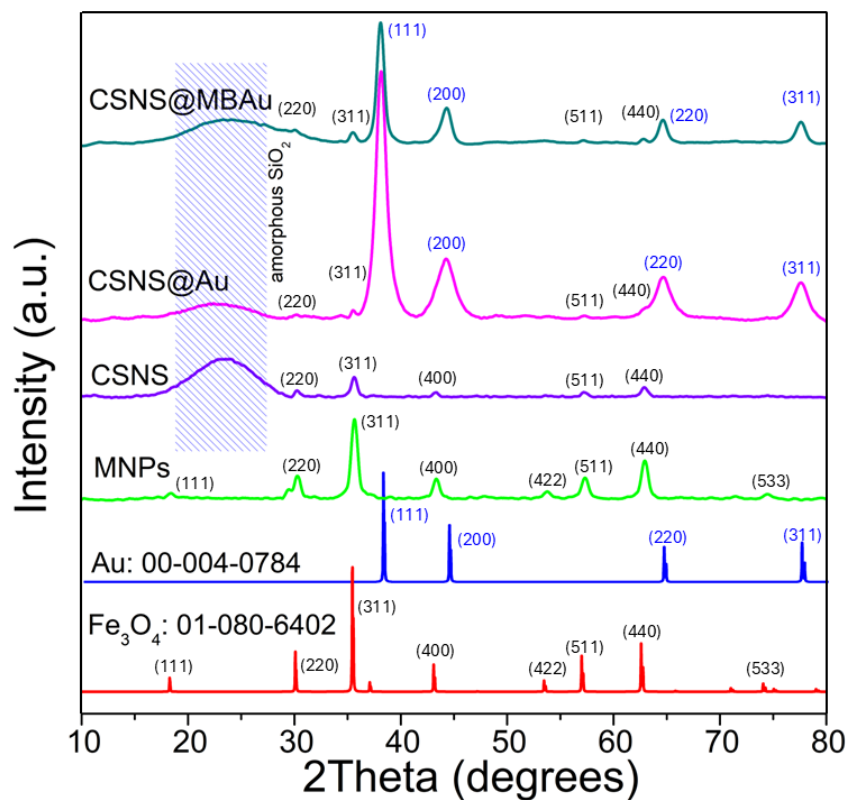


Figure 6.3: Optical properties measured using UV-Vis spectroscopy for: **A.** gold nanoparticles with spherical (AuNPs) and anisotropic (MBAuNPs) morphology; **B.** magnetite nanoparticles (MNPs) and core-shell nanostructures (CSNS); and **C.** gold-decorated CSNS. **D.** FTIR spectrum of CSNS after functionalization with APTES.

Table 6.2: XDR parameters and crystallite size of CSNS.

Sample ID	FWHM (°)	2 θ (°)	D (nm)
MNPs (311)	0.65	35.65	13.48
CSNS (311)	0.57	35.61	15.13
CSNS@Au (111)	0.79	38.00	10.99
CSNS@MBAu (111)	0.57	38.04	15.27

6.3.4 Elemental Analysis

Fig. 6.4 confirmed the elemental composition of the nanostructured systems CSNS, CSNS@Au, and CSNS@MBAu via the collection of the EDS spectra during TEM characterization. Spectra shown confirmed the hierarchical structure of the systems. According to the elemental analysis on individual particles, it was confirmed that the MNPs are enclosed in the nucleus of the SiO_2 (Fig. 6.4B_{1,2}). The analysis also confirmed the homogeneous decoration with spherical AuNPs (Fig. 6.4A₁₋₄), and the deposition of a few MBAuNPs onto the surface of the SiO_2 layer (Fig. 6.4C₁₋₃). The presence of Fe (6000-7000 eV), Si (1700 eV), and Au (2250, 9700, 11500, and 13500 eV) was expected from the protocol of synthesis. Silver (Ag) resulted from the protocol of synthesis of MBAuNPs with AgNO_3 to generate the multibranched morphology and Cu (8000-10000 eV) originates from the copper grid used as sample holder during characterization. The relative intensities of the peaks indicate that the CSNS are largely composed of Si and Fe in minor quantities, as well as Au and Ag, for gold decorated CSNS.

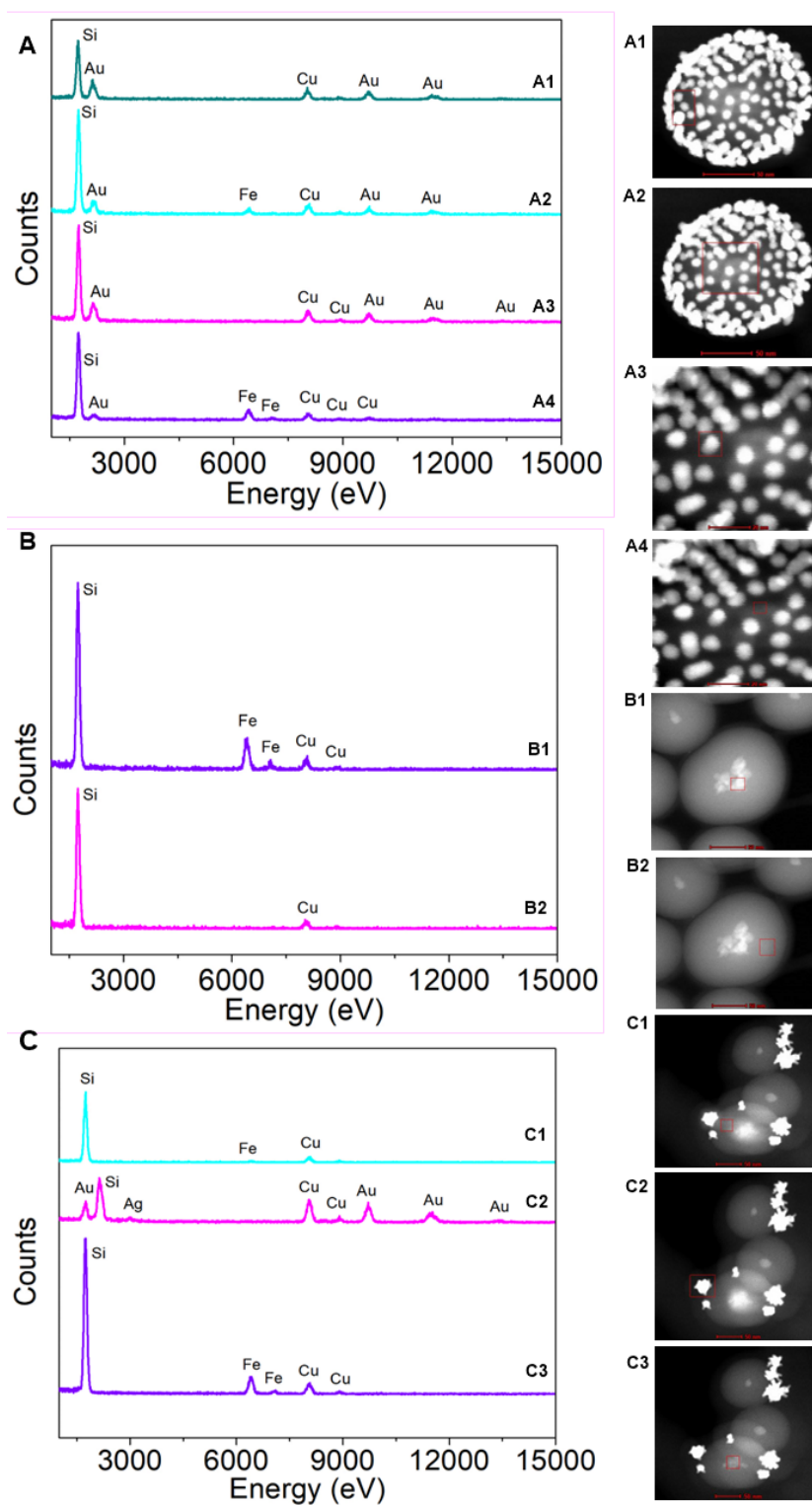


Figure 6.4: Elemental analysis of **A.** CSNS@Au, **B.** CSNS, and **C.** CSNS@MBAu

6.3.5 Superparamagnetic Properties

The superparamagnetic properties of the MNPs, CSNS, CSNS@Au, and CSNS@MBAu were measured using a physical property measurement system (PPMS) at room temperature. Hysteresis curves are shown in Fig. 6.5A-D and the insets are plots of $M(H)$ in the enlarged region between -2 and 2 kOe.

The hysteresis loop for MNPs in Fig. 6.5A does not display magnetic remanence (M_r), it was completely reversible, and the saturation magnetization (M_s) moment per unit mass (emu/g) was about 51.34 emu/g at 20 kOe, as reported in literature.^{29,200} These findings confirmed the superparamagnetic behavior of MNPs expected from XRD and TEM results. PPMS magnetometry also reveals that overlaying MNPs surfaces with a shell of SiO_2 (65 nm thickness) to constitute the CSNS has a significant effect on magnetic behavior, attenuating the M_s of the MNPs to 3.13 emu/g at 20 kOe, which represents about 6.09% of the M_s for MNPs (Fig. 6.5B). The first reason for the decrease of the M_s values is the relative decrease of MNPs content encapsulated in the CSNS which is in accordance with results reported in literature, such as the one described by Ji et al.²⁹ (3.5 emu/g). Another reason is the thickness of the diamagnetic silica shell which attenuates the magnetic properties of the core.²⁰⁸

After the deposition of AuNPs, the M_s of the CSNS decreases to 0.76 emu/g as shown in Fig. 6.5C. In contrast to the system decorated with MBAuNPs, the M_s was 2.30 emu/g as evidenced in Fig. 6.5D. These values represent about 1.48% and 4.47% of the M_s for MNPs, respectively. According to the literature, a gold-silica nanoshell without MNPs inside the core exhibits diamagnetic behavior, then the results suggest that the decrease in the M_s values after coating with SiO_2 and GNPs is most likely the result of a large volume of the two components instead of a modification of the superparamagnetic behavior of MNPs.²⁹

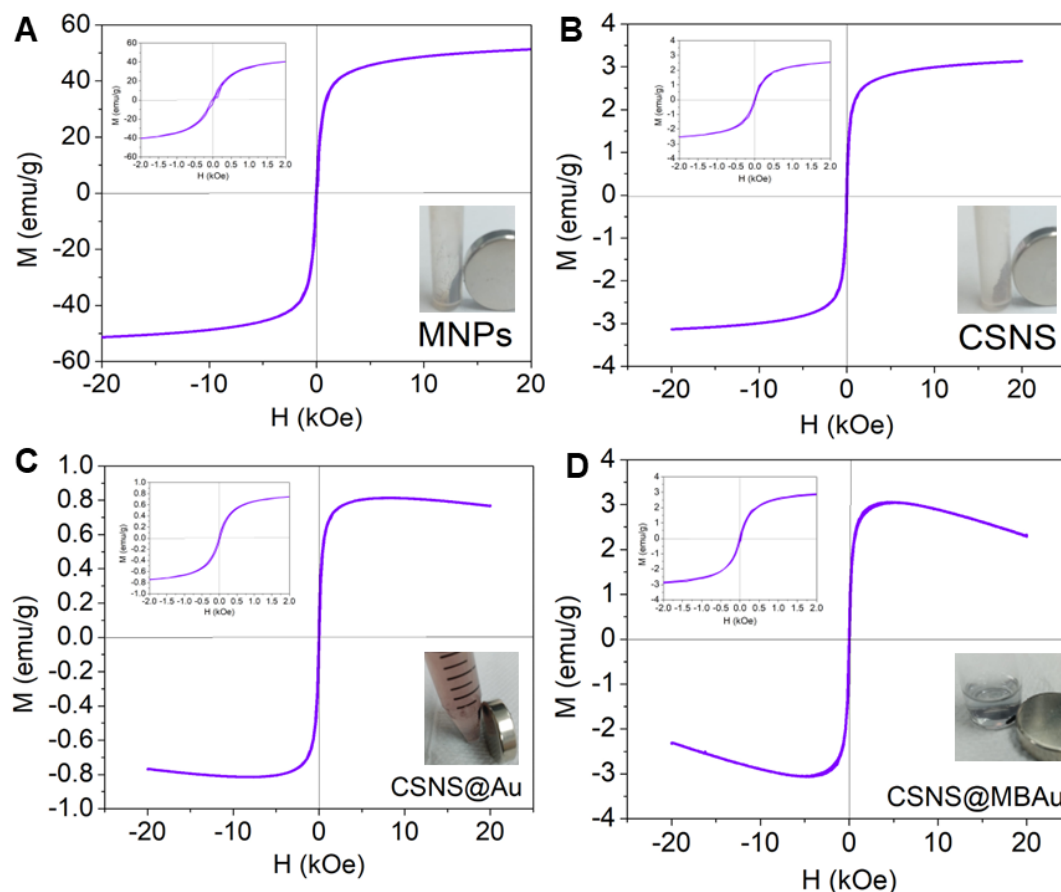


Figure 6.5: Hysteresis loop for MNPs (A), CSNS (B), CSNS@Au (C), and CSNS@MBAu (D), showing a super-paramagnetic behavior and a saturation magnetization (M_s) of 51.34 emu/g, 3.1 emu/g, 0.76 emu/g, and 2.30 emu/g, respectively. Insets are plots of $M(H)$ in the enlarged region between -2 and 2 kOe.

6.3.6 Calorimetric heating performance of CSNS

The heating capacity of the nanostructured systems was evaluated by irradiating each sample at five different fixed amplitudes of magnetic field ($H = 15, 20, 25, 30$, and 35 mT) and a frequency of 330 kHz for three minutes and obtaining the curves of increment of temperature in time (T vs. t) as shown in Fig. 6.6A,C, and E. Magnetic parameters and temperature increment are summarized in table 6.3. At a concentration of 10 mg/mL, all the systems showed a clear

dependence on the intensity of the alternating magnetic field.^{209,210} The CSNS system showed the best heating performance within 160 seconds, reaching 4.78 °C at 35 mT, in contrast with the systems decorated with gold CSNS@Au (3.30 °C) and CSNS@MBAu (2.51 °C). Results are consistent with the superparamagnetic hysteresis curves, particularly, with the significant decrement of the saturation magnetization after the coating process with silica and gold which translates into loss of heating capacity.

Table 6.3: Heating capacity of the nanostructures.

Parameter	CNSN	CSNS@Au	CSNS@MBAu
Saturation Magnetization	3.1	0.76	2.30
H (mT)	Temperature increment at 160 s (°C)		
15	1.37	0.65	0.74
20	1.91	0.91	1.06
25	2.87	1.52	1.71
30	4.19	2.27	1.93
35	4.78	3.30	2.51

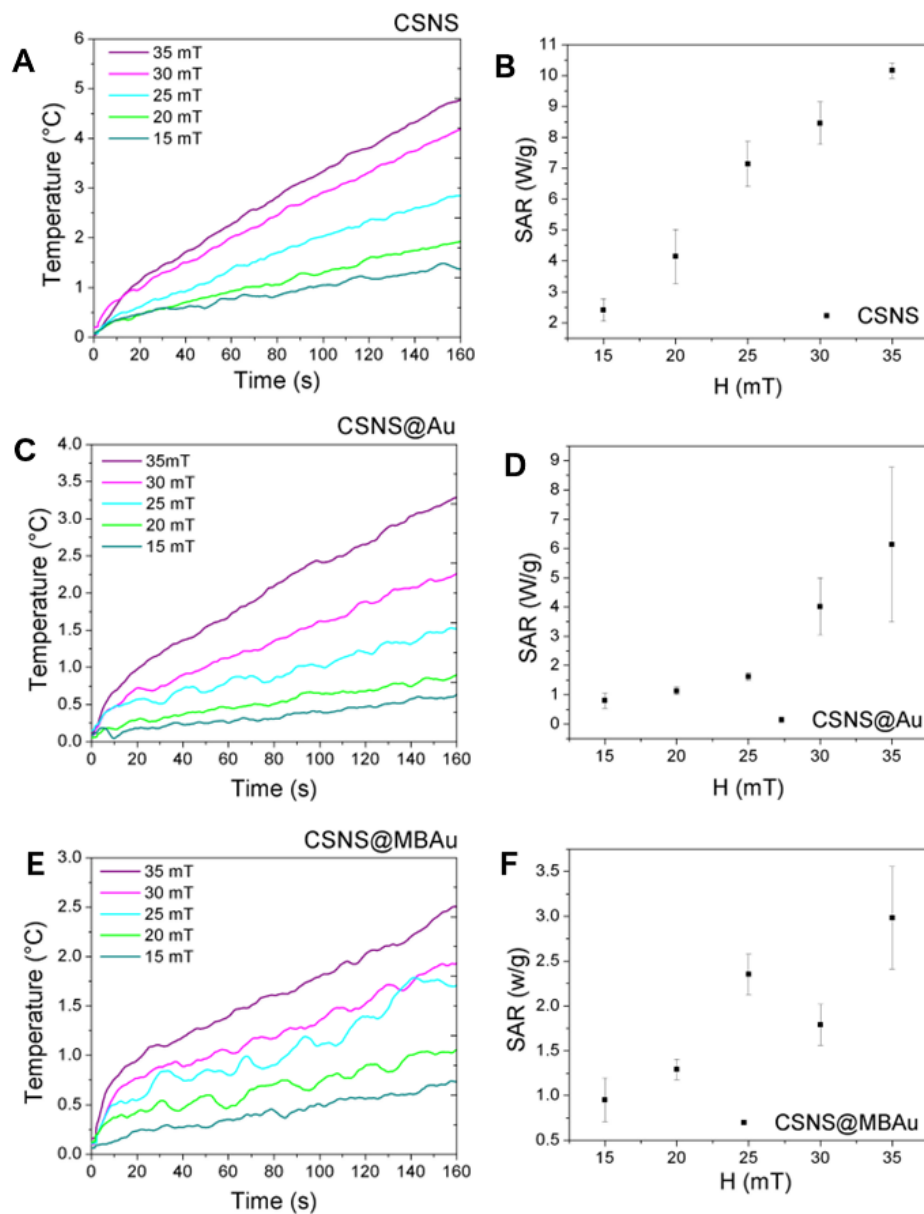


Figure 6.6: Calorimetric measurements of the nanostructured systems CSNS (A), CSNS@Au (C), and CSNS@MBAu (E) irradiating a magnetic field of 330 kHz frequency and amplitudes from 15 to 35 mT. SAR measurements for CSNS (B), CSNS@Au (D), and CSNS@MBAu (F)

Specific Absorption Rate (SAR)

An important parameter that describes the amount of heat produced by superparamagnetic materials is the specific absorption rate (SAR), defined as a function of the specific heat of the aqueous solvent ($C_v=4.18 \text{ J/g } ^\circ\text{C}$), the mass fraction of nanoparticles (m_{NPs}) in the sample, and the initial slope for each T vs t curve (Fig. 6.6A, C, and D).^{201,211,212}

$$SAR = \frac{C_v}{m_{NPs}} \times \frac{dT}{dt} \quad (6.1)$$

SAR is affected by frequency and strength of the magnetic field, and the morphological properties of the material, such as size, stability, shape, and saturation magnetization.^{201,212} Fig. 6.6B, C, and D shows the SAR values for all the nanostructured systems exposed to five different magnetic stimuli for three minutes, using a frequency of 330 kHz. It was noted that the absorption rate for CSNS and CSNS@Au increases with increment of the intensity of the magnetic field. However, this dependence was not observed for the system CSNS@MBAu, which comprises the lowest SAR values (Table 6.4).

Table 6.4: Heating capacity of the nanostructures.

Sample	CNSN	CSNS@Au	CSNS@MBAu
H (mT)	SAR (W/g)		
15	2.41 ± 0.36	0.79 ± 0.25	0.95 ± 0.24
20	4.14 ± 0.87	1.13 ± 0.13	1.29 ± 0.11
25	7.13 ± 0.73	1.62 ± 0.13	2.35 ± 0.22
30	8.45 ± 0.69	4.01 ± 0.97	1.79 ± 0.22
35	10.16 ± 0.24	6.13 ± 2.64	2.98 ± 0.56

6.4 Conclusions

This chapter solved the following question: Active targeting-mediated nanopharmaceuticals can be designed to be stimuli responsive? To address this question, we propose the synthesis and characterization of three multifunctional hierarchical nanostructured systems, designated CSNS, CSNS@Au, and CSNS@MBAu. We successfully synthesized these nanostructured systems by combining the magnetic properties of magnetite nanoparticles (Fe_3O_4 , designated MNPs), the biocompatibility, high loading capacity, and protective functionalities of silica (SiO_2), and the plasmonic and photothermal properties of spherical (AuNPs) and multi-branched gold nanoparticles (MBAuNPs). Using UV-Vis, SEM, TEM-EDS, and XRD, we studied the optical, morphological, chemical, and crystalline properties of the nanostructures. Results confirmed a well-formed hierarchical structure. We characterized the magnetic properties of each nanostructure and confirmed a superparamagnetic behavior. Calorimetric measurements were performed to evaluate the heating capacity of the materials. Our results reported a maximum increase of temperature of 4.78 °C, 3.30 °C, and 2.51 °C for CSNS, CSNS@Au, and CSNS@MBAu, respectively. The heat production of the hierarchical nanostructures may be leveraged during hyperthermal therapy.

Chapter 7

General Conclusions & Outlook

Figure 7.1 summarizes the general conclusions of the thesis.

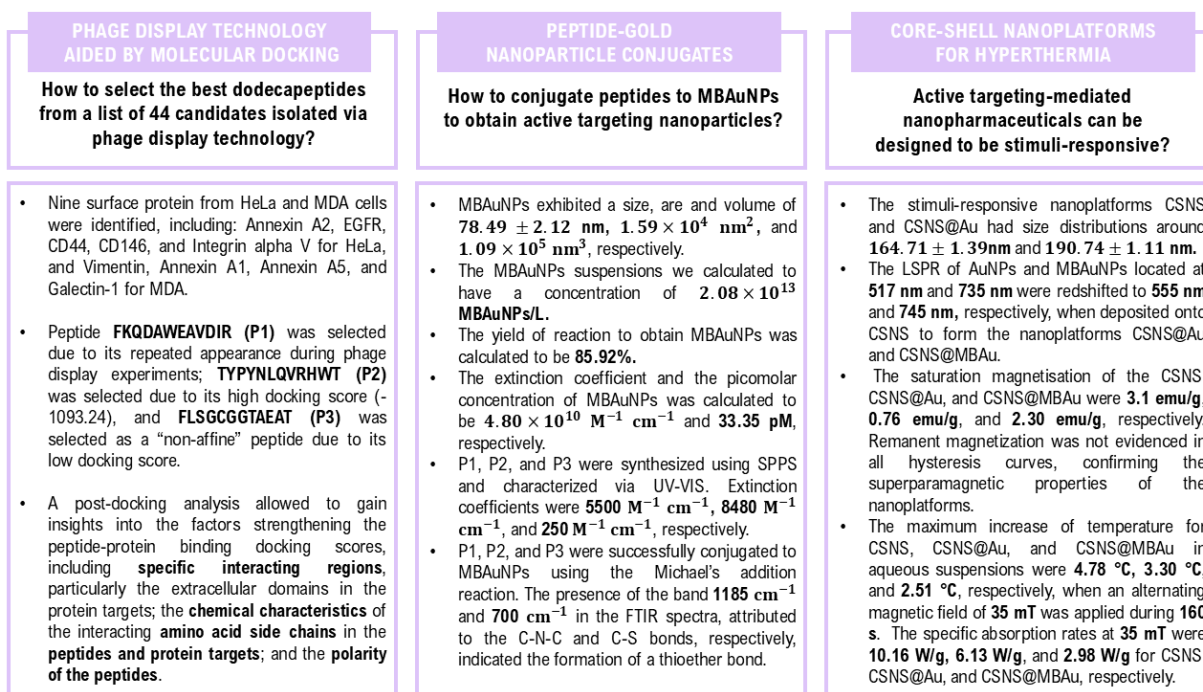


Figure 7.1: General conclusions.

Figure 7.2 summarizes the perspectives of the thesis.

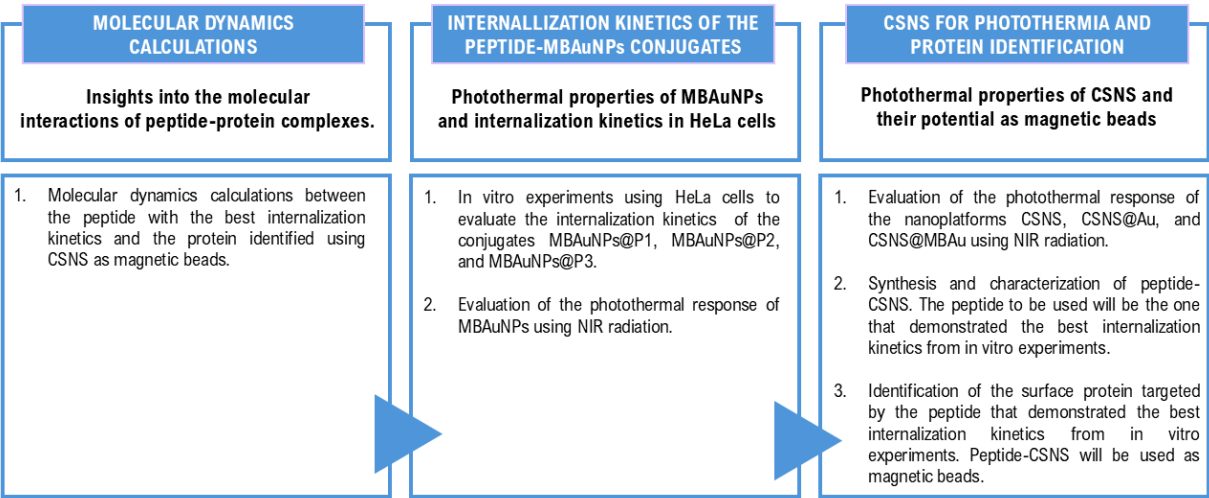


Figure 7.2: Perspectives of this study.

Appendix A

Supporting Information

A.1 Supporting Information Chapter 2

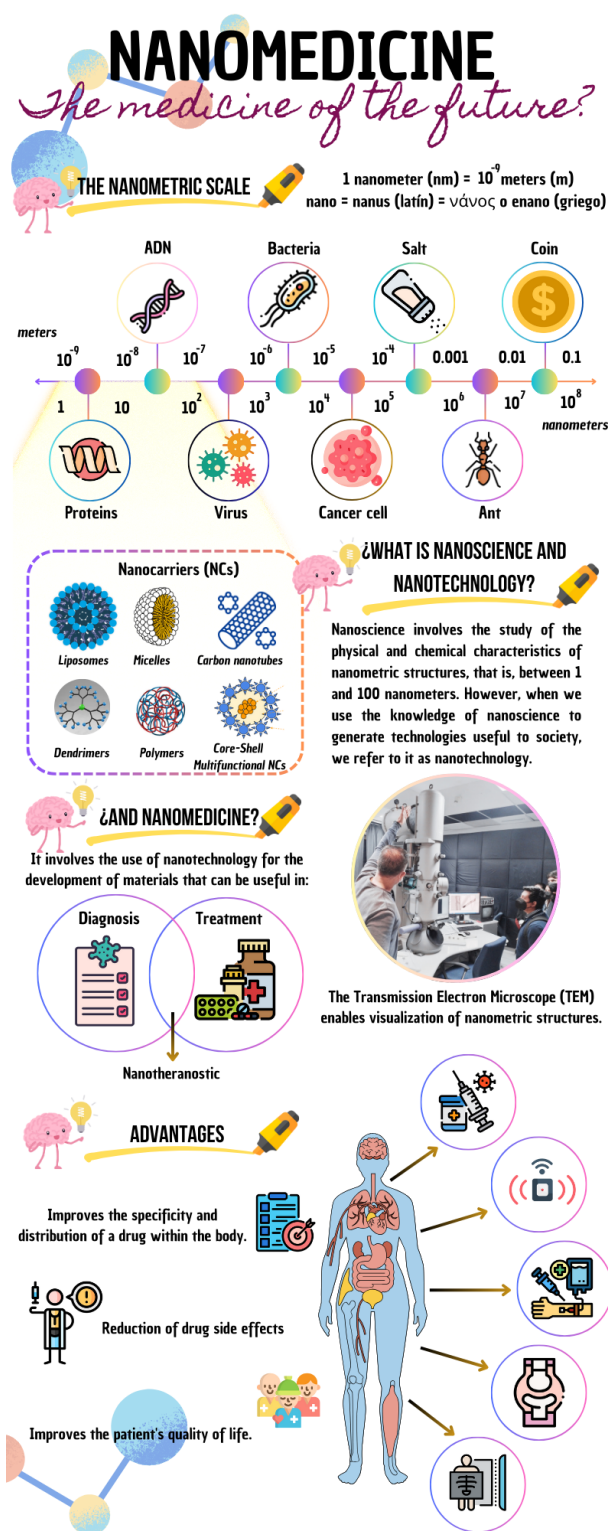


Figure A.1: Nanomedicine and its applications. The infographic summarizes the concepts of nanometric scale, nanoscience and nanotechnology, and applications of nanomedicine.

Table 1. FDA and/or EMA-approved nanoformulations. ¹⁻⁵ N/A: non-applicable; SOF: Some of Europe; PCS: Polyglucose sorbitol carboxymethyl ether

Name	Active substance	Ligand	Size (nm)	Treatment area	Improvement	Reg.	App.
LIPID-BASED NANOPARTICLES							
Abelcet	Amphotericin B	N/A	1-10	Antimycotics	Decreased toxicity	FDA	1995
Diprivan	Propofol	N/A	180.5	Anaesthetics		FDA	1989
Doxil (Caelyx, Evacet, Lipodox)	Doxorubicin	PEG coating	80-100	Kaposi's sarcoma, Ovarian cancer, Multiple myeloma	Increased site-specific Decreased systemic toxicity	FDA	1995
Amphotec	Amphotericin B (AmB)	N/A	100	Antimycotics	Reduced haemolysis Reduced acute toxicity Reduced lipoprotein binding	FDA	1996
DepoCyt	Cytarabine/Ara-C	N/A	300-3000	Lymphomatous	Increased site-specific Decreased systemic toxicity	FDA	1996
DaunoXome	Daunorubicin	N/A	35-65	Kaposi's sarcoma	Improved pharmacokinetics Increase site-specific delivery	FDA	1996
AmBisome	Amphotericin B (AmB)	N/A	100	Antimycotics	Reduced nephrotoxicity	FDA	1997
Curosurf	Proteins SP-B and SP-C	N/A	N/A	Lung activator for stress disorder	Decreased toxicity Increased delivery for smaller volume	FDA	1999
Epaxal (virosome)	Formalin-RG-SB-HAV	N/A	150	Hepatitis A	N/A	EMA	2000
Inflexal V	Haemagglutinin	N/A	150	Influenza A and B	N/A	EMA	2000
Mycet	Doxorubicin	N/A	150-250	Breast cancer	Reduced adverse effects	EMA	2000
Visudyne	Verteporfin	N/A	150-300	Ocular histioplasmosis, Myopia, Decreased vision	Increase site-specific delivery, Photosensitive release	FDA	2000
Definity	Perflutren	N/A	1100-3300	Ultrasound contrast agent		FDA	2001
DepoDur	Morphine sulphate	N/A	1700-2300	Analgescics	Prolonged release	FDA	2004
Mepact	Mifamurtide	N/A	<100	Osteosarcoma	Improved pharmacokinetics	EMA	2009
Exparel	Bupivacaine	N/A	2400-3100	Analgescics	Prolonged release	FDA	2011
Marqibo	Vincristine (VCR)	N/A	100	Acute lymphoblastic leukaemia (ALL)	Passive targeting of VRC Increased site-specific Decreases toxicity Prolonged plasma circulation	FDA	2012
Onivyde	Irinotecan	PEG coating	110	Pancreatic cancer	Increased site-specific Decreases toxicity Increased half-life	FDA	2015

Lipusu	Paclitaxel	N/A	400	Breast, ovarian & non-small-cell lung cancer		FDA	2016
Nocita						FDA	2016
CYP-351/Vyxeos	Cytarabine: Daunorubicin (5:1)	N/A	100	Acute myeloid Leukemia	Delivers two drugs in a synergistic ratio	FDA EMA	2017 2018
Shingrix	Recombinant glycoprotein E	N/A	-	Shingles		FDA	2017
Onpatro	siRNA targeting transthyretin	N/A	-	Transthyretin (TTR)-mediated amyloidosis	Silencing of genes	FDA	2017
Patisiran		N/A	-		siRNA targets transthyretin	EMA	2018
ALN-TTR02		N/A	-		Sustained release of AMK in the lungs with minimal systemic exposure.	EMA	2018
ALIS/Arkayce	Amikacin sulfate	N/A	200-300	Respiratory tract infections		FDA EMA	2018 2020
Comirnaty	mRNA vaccine Tozinameran	N/A	-		N/A	FDA EMA	2020 2022
mRNA-1273	mRNA vaccine Moderna	N/A	-		N/A		
Pfizer-BioNTech	mRNA vaccine	N/A	-		N/A		
Spikevax	mRNA vaccine Elasmomeran	N/A	-	COVID-19 Immunisation		FDA EMA	2020 2021
INORGANIC NANOPARTICLES							
CosmoFer	SPIONs	Dextran	≤10	Iron deficiency in CKD	Increased dose capacity	FDA	1957
INFeD	SPIONs	Dextran	~20	Iron deficiency in CKD		FDA	1992
Dexferum	SPIONs	Dextran	~20, <50	Iron deficiency in CKD		FDA	1996
Feridex/Endorem	Iron	Dextran	<200	MRI contrast agent		FDA	1996
DexIron	Iron	Dextran		Iron deficiency anaemia		FDA	1996
Venofer	SPIONs	Sucrose	<<10	Iron deficiency in CKD		FDA	2000
GastroMARK; Unirem	Iron	Silicone	400	MRI contrast agent	Superparamagnetic character	FDA	2001
Resovist/Ciavist	Iron	Carboxydextran	75	MRI contrast agent		Europe	2001
Zevalin	90Y-ibritumomab tiuxetan	N/A	-	Lymphoma	Therapeutic radiopharmaceuticals	FDA	2002
Feraheme	Ferumoxytol-SPIONs	PSC	≤10; <200	Iron deficiency in CKD		FDA	2009
Rienso	Iron oxide NPs	PSC	-	Iron deficiency in CKD		FDA	2009
Ferinject	SPIONs	Carboxymaltose	≤10	Iron deficiency anaemia		EMA	2013
Ferriject	SPIONs	Sodium ferric gluconate-sucrose	<<10	Iron deficiency in CKD		EMA	2013
Ferumoxtran-10	Ultrasmall SP10	Dextran T10	21	MRI contrast agent		NL	2013

Injectable	Iron	Carboxymaltose	≤10	Iron deficiency anaemia		FDA	2013
Hensify	Hafnium oxide NPs	Phosphate	50	Radiotherapy for Squamous cell carcinoma	Enhances external radiotherapy.	CE MARK	2019
Diafer	Iron	Isomaltoside		Iron deficiency anaemia		SOE	-
Ironate(generic)	SPIONs	Dextran	~20; <50	Iron deficiency anaemia		SOE	-
Monofer	SPIONs	Isomaltoside	<10	Iron deficiency anaemia		SOE	-
Ferisat	Iron dextran colloid	Dextran				SOE	-
POLYMER-BASED NANOPARTICLES							
Adagen	Adenosine deaminase (ADA)	PEGylated		Adenosine deaminase (ADA) immunodeficiency disorder		FDA	1990
Oncaspar	L-asparaginase	PEGylated protein		Leukemia		FDA	1994
Copaxone	Glatiramer acetate	N/A		Multiple sclerosis (MS)		FDA	1996
Renagel	Sevelamer carbonate	Poly(allylamine hydrochloride)		Hyperphosphatemia caused by chronic kidney disease (CKD)		FDA	2000
PegIntron	Alpha interferon (INF)	PEGylated protein		Hepatitis C		EMA	2000
Eligard	Leuprolide acetate	Artigel polymer		Prostate cancer		FDA	2002
Neulasta	Filgrastim	PEGylated protein		Febrile neutropenia		FDA	2002
Pegasys	Recombinant human alfa-2a interferon	PEGylated protein		Hepatitis C, hepatitis B		FDA	2002
Somavert	Analog of human growth hormone	PEGylated protein		Acromegaly	Increased circulation time Less immunogenicity	EMA	2002
Restasis	Cyclosporine			Chronic dry eye	Less plasma clearance	FDA	2003
Macugen	PEGatinib sodium	PEGylated protein		Neovascular age-related macular degeneration	Increased half-life	FDA	2004
Renvela	Sevelamer HCL	Poly(allylamine hydrochloride)		Hyperphosphatemia caused by CKD		EMA	2007
B03XA03/Mircera	Epoetin β (EPO)	PEGylated		Anemia		FDA	2018/2007
Cimzia	IgG Fab' fragment			Rheumatoid arthritis, Crohn's disease, psoriatic arthritis, and ankylosing spondylitis		FDA	2008
Krytexxa	Puricase	PEGylated protein		Chronic gout		FDA	2010
Plegridy	Recombinant IFN-β	PEGylated protein		Multiple sclerosis (RRMS)		FDA	2014
Adynovate	Coagulation factor VIII	PEGylated protein		Hemophilia A		FDA	2015

Rebiny/Refixia	Recombinant DNA-derived coagulation factor IX	PEGylated		Hemophilia B		FDA EMA	2017
FX006/Zilretta	Triamcinolone acetonide	PLGA-hydrogel		Knee osteoarthritis		FDA	2017
Sublocade (Pre)	Buprenorphine	N/A	-	Opioid use disorder	-	FDA	2017
NANOCRYSTALS							
Avinza	Morphine sulfate			Psychostimulant	Mimics bone structure	FDA	2002
Ritalin LA	Methylphenidate	N/A	-	Mental stimulant	Increased bioavailability	FDA	2002
Zanaflex	Tizanidine HCl			Muscle relaxant	Decreased release of poor water-soluble drugs	FDA	2002
Emend	Aprepitant	N/A	-	Anti-anorexic (antiemetic)	Better water solubility Higher absorption in the GI Higher bioavailability Reduced posology		
OsSatura	Hydroxyapatite				Mimics bone structure by cell adhesion and growth	FDA	2003
Vitoss	β -tricalcium phosphate	N/A	100 nm	Bone substitute		FDA	2003
OsSim	Ca hydroxyapatite	N/A	20 nm			FDA	2004
TriCor	Fenofibrate	N/A	-	Antihyperlipidemia	Increased bioavailability	FDA	2004
Megace ES	Megestrol acetate			Antiemetic drug	Increased absorption and bioavailability	FDA	2005
NanOss	Hydroxyapatite			Bone substitute	Allows higher administration at higher doses	FDA	2005
Focalin XR	Dexmethyphenidate HCl			Mental stimulant	Increased drug loading and bioavailability	FDA	2008
Ivemend	Fosaprepitant dimeglumine			Antiemetic drug		FDA	2008
Zypadhera	Olanzapine pamoate			antihyperlipidemia	Increases bioavailability simplifies administration	EMA	2008
EquivaBone	Hydroxyapatite			Bone-grafting material	Mimics bone structure allowing cell adhesion and growth	FDA	2009
Invega	Paliperidone			Schizophrenia	Increased bioavailability and decreased posology	FDA	2009
Invega Sustenna	Paliperidone palmitate			Schizophrenia	Increased bioavailability and decreased posology	FDA	2009
Rapamune	Sirolimus	N/A		Immunosuppressant	Increased bioavailability	FDA	2010
Xepion	Paliperidone palmitate			Schizophrenia		EMA	2011
Ryanodex	Dantrolene sodium			Malignant hyperthermia		FDA	2014
Rapamune	Sirolimus	N/A		Lymphangioleiomyomatosis	Increased bioavailability	FDA	2015

Brixadi/Buvidal (PRE)	Buprenorphine	N/A	-	Opioid use disorder	-	FDA EMA	2021 2018
PROTEIN-BASED NANOPARTICLES							
Abiraxane	Paclitaxel (ABI-007)	Albumin	130	Breast cancer (2005) Lung cancer (2012) Pancreatic cancer (2013)	Higher response rates Longer time to tumor progression Increase site-specific delivery	FDA	2005 2012 2013
Ontak	IL-2 and diphtheria toxin	N/A	-	Cutaneous T-cell lymphoma	Targeted T-cell specificity	FDA	1999
Optison	Perflutren in HSA	N/A	2500-4000	Contrast agent		FDA	1997
MICELLES							
Estrasorb	Estradiol	PEGylated		Menopause	Sustained release	FDA	2003
Genexol-PM	Paclitaxel			CANCER: Breast cancer		FDA	2007
Apealea	Paclitaxel	N/A		CANCER: Ovarian cancer, peritoneal cancer, fallopian tube cancer		EMA	2018
DENDRIMER							
VivaGel BV	Astodimer sodium			Anti-infective for prevention of recurrent bacterial vaginosis (BV)		FDA	2015
MICROBUBBLES							
SonoVue	Sulphur hexafluoride microbubble		2500	Ultrasound imaging		EMA	2001

A.2 Supporting Information Chapter 3

A.2.1 Ultraviolet Visible Spectrophotometry (UV-Vis)

According to the atomic absorption theory, electrons in atoms absorb incident light at a specific wavelength (λ). After absorption, the incident light photons (λ_{in}) having energy $E = E_m - E_n$ will cause an electronic transition of the electrons from the highest occupied molecular orbital (HOMO; E_n = ground state) to the lowest unoccupied molecular orbital (LUMO; E_m = excited state), as seen in figure A.2-A.²¹³ The excited state of the electron is unstable, which causes an immediate return to its ground state by emitting a photon with wavelength λ_{out} and energy $E = E_n - E_m$. Substances are composed of different molecules that absorb at different wavelengths, so different electronic transitions will be produced within the molecules (Fig. A.2-A). The photons that are not absorbed by the substances will be transmitted.^{78,214,215}

UV-VIS spectroscopy is used to measure the absorption of light by a sample, in the ultraviolet (UV; 200-400 nm) and visible (VIS; 400-800 nm) regions of the electromagnetic spectrum.²¹⁴ The principle of the technique relies on the Beer-Lambert Law (Equation A.1), which states that the amount of absorbed light is proportional to the concentration (c) of the absorbing substance and the path length l .²¹⁴ Due to the interactions between the incoming photons having intensity I_o and the absorbent molecules, the transmitted beam power will have an attenuated intensity I .

$$A = -\log T; \quad (A.1)$$

$$T = \frac{I}{I_o}; \quad (A.2)$$

Therefore, substituting A.2 in A.1, the absorbance A can be written as:

$$A = \log_{10}\left(\frac{I_o}{I}\right); \quad (A.3)$$

or

$$A = \epsilon cl; \quad (\text{A.4})$$

Where A is the absorbance, ϵ is the molar absorptivity (or molar extinction coefficient of the absorbent), c is the concentration, and l is the path length in cm.

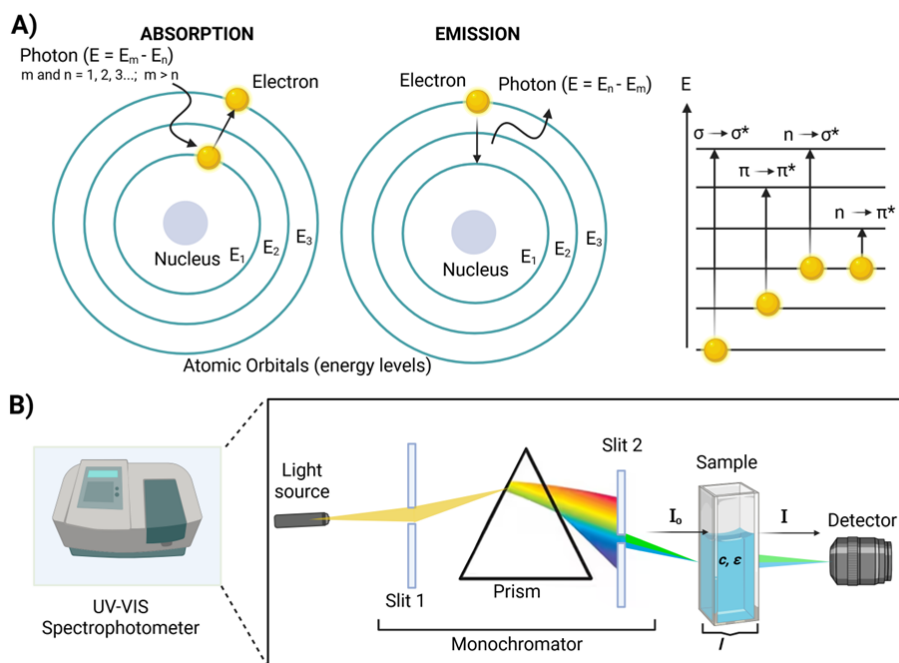


Figure A.2: **A.** Process of absorption of incident light with wavelength λ_{in} and energy $E = E_m - E_n$ carried by the atom. Incident light has enough energy to excite an electron to another electronic state with higher energy (unstable). Due to instability, the electron falls to a state of lower energy by emitting a photon λ_{out} and energy $E = E_m - E_n$. **B** Basic instrumentation of a UV-VIS spectrophotometer.

The basic setup of a UV-VIS spectrophotometer includes a source of UV-Vis light, a monochromator, a cuvette (plastic, crystal, quartz, or silica) containing the sample, and the detector (Fig. A.2-B). During the characterization of a sample, the reference beam's intensity should be measured, so the beam is allowed to travel from the light source to the detector without sample interaction (blank). Then, the process is repeated, allowing the beam to pass through the

sample. The difference between the blank intensity and the sample intensity is plotted as an absorbance/transmittance vs wavelength spectrum in the UV-VIS region.

A.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

Atoms with different electronegativities that are forming a bond have a dipole moment, which is a measure of the separation of positive and negative charges in a molecule.²¹⁶ In contrast to UV-VIS radiation, IR radiation is not energetic enough to excite electrons and cause transitions, but it can cause the chemical bonds in molecules to vibrate in different ways (Fig. A.11).^{213,217} In this regard, molecules absorb those frequencies of IR radiation that match the natural vibration frequency of their bonds so that the amplitude of the vibrational motions of the atoms in the molecule will increase, changing their dipole moment.²¹⁵ If the vibration changes the dipole moment of the molecule, it is considered IR active; otherwise, it is considered IR inactive.²¹⁸ IR active vibrations occur in non-linear molecules with polar bonds, such as CH₄, C₂H₆, NH₂.²¹³ These molecules, constituted by N atoms, will have 3N-6 vibrational motions, also called fundamental vibrations or normal modes.²¹⁸ On the other hand, inactive vibrations occur in symmetric nonpolar molecules due to the presence of a center of symmetry (symmetric vibrations), such as H₂, O₂, among others.^{213,215}

FTIR spectroscopy is a suitable characterization technique to detect IR-active molecular vibrations in organic, inorganic, and polymeric materials.⁷⁸ Based on specific molecular vibrations, it enables the investigation of the chemical structure of materials by irradiating them with IR radiation (10 - 10000 cm⁻¹).²¹⁸ Vibrations that involve bond-length changes (stretching vibrations, ν), and vibrations that change bond angles (δ -in plane bending or π -out of plane bending) are observed in the region from 400 - 4000 cm⁻¹.^{213,216,218} Symmetric (ν_s) and asymmetric (ν_{as}) stretching vibrations, as well as wagging, rocking, twisting, and scissoring bending modes, are more complex vibrations.²¹³ In addition, stretching and bending vibrational modes occur at specific allowed energy levels, and then alterations in the characteristic pattern of absorption bands indicate changes in the material composition and chemical structure.^{78,216}

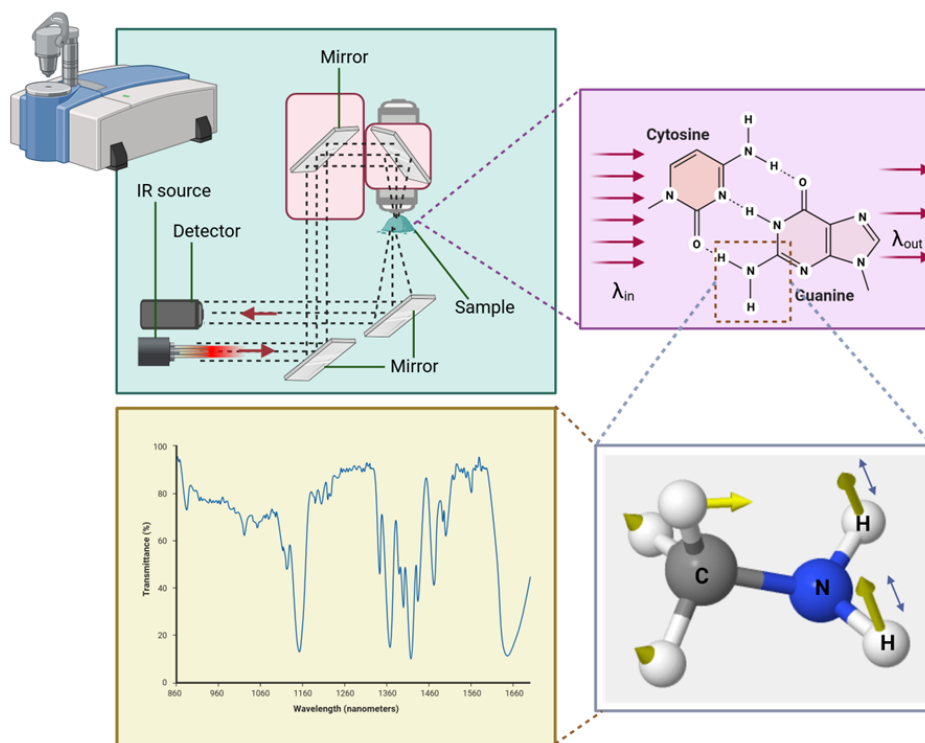


Figure A.3: Representation of the basic setup of a FTIR spectrometer. The incoming photon (λ_{in}) interacts with the molecules in the sample. Absorption of light by the molecules causes a change in their dipole moment (normal modes of vibration). Transmitted light is attenuated after absorption (λ_{out}).

The basic setup of an FTIR spectrophotometer consists of a source of IR light, an interferometer, a sample compartment, a detector, an amplifier, an analog-to-digital converter, and a computer.^{213,215} During the characterization of a sample, the light source generates radiation that passes through the sample, followed by the interferometer and the detector. An interferogram is obtained after amplifying the signal detected and converting it to a digital signal. Then, the interferogram is converted to a FTIR spectrum by applying the fast Fourier transform algorithm.²¹³

The FTIR spectrum is a plot of radiation absorbed by atoms in molecules.²¹³ The plot shows the absorbance or transmittance (%) versus the energy range in wavenumber (cm^{-1}).^{213,216} Similar to UV-VIS spectroscopy, the absorbance is calculated from equation A.4 and a plot of absorbance versus wavenumber is generated. On the other hand, the FTIR y-axis can plot transmittance

instead, which can be calculated from equation A.1.²¹³ As shown in Fig. A.4-A, when the y-axis plot absorbance, the band of each characteristic functional group of the compound is pointed up; otherwise, if the y-axis is plotted as transmittance, the band of each characteristic functional group of the compound is pointed down (Fig. A.4-B).

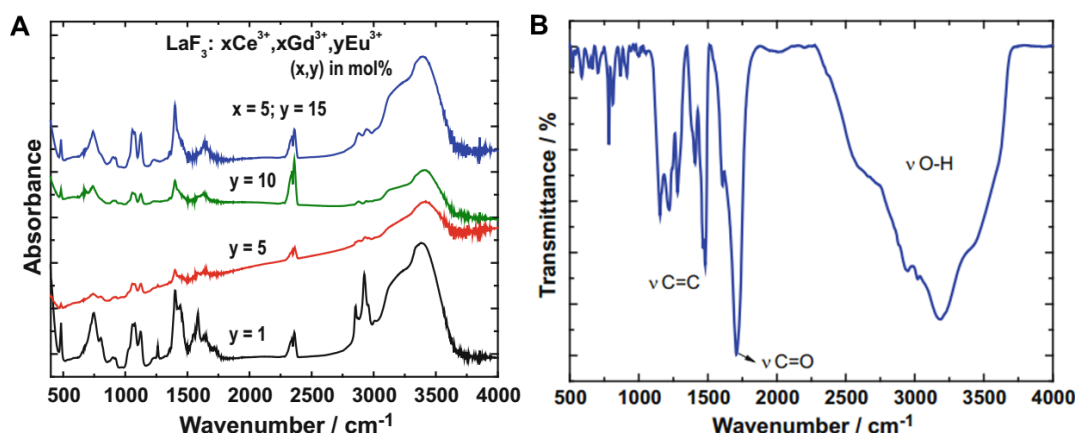


Figure A.4: **A.** The FTIR spectrum plots the absorbance/transmittance versus wavenumber for $\text{LaF}_3:x\text{Ce}^{3+},x\text{Gd}^{3+},y\text{Eu}^{3+}$ ($x = 5$; $y = 1, 5, 10$, and 15 mol%) nanomaterials functionalized with oleic acid. When the y-axis plots absorbance, the band of each characteristic functional group of the compound is pointed up. **B.** FTIR absorption spectrum of p-tetrakis (carboxymethyl)calix-4-arene tetrol macrocycle ligand. When the y-axis is plotted as transmittance, the band of each characteristic functional group of the compound is pointed down. Figure A and B were reprinted from reference 213.²¹³

A.2.3 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

ICP-OES is an analytical technique used for the analysis of multiple elements in different samples.²¹⁹ Fig. A.5 shows the basic setup of an ICP-OES spectrometer, which consists of nebulizers, spray chambers, a sample injector, an ICP torch, a radiofrequency (RF) generator, optics or the spectrometer, emission detectors, signal processing, and software.²¹⁹

For the elemental analysis, the liquid sample* is injected towards the central channel of radiofrequency (RF) induced argon plasma by a peristaltic pump with a nebulizer system, which converts the sample to an aerosol.^{219,220} Once introduced into the plasma, which possesses temperatures from 8000 K to 10000K, the aerosol is rapidly dried, vaporized, atomized, and ionized, and the electrons in the atoms are eventually excited, causing electronic transitions.²¹⁹ Due to the instability of the excited state, electrons return to lower energy levels, releasing photons (λ_{out}), also called ionic or atomic emissions, which are collected by an optics system formed by a concave mirror or lens, and then separated by the values of λ_{out} through a monochromator, to finally be converted into an electrical signal by a photodetector, amplified by integrators and recorded by a computer.²¹⁹ The counts of photons detected correlate with the abundance of the elements in the sample.²¹⁹

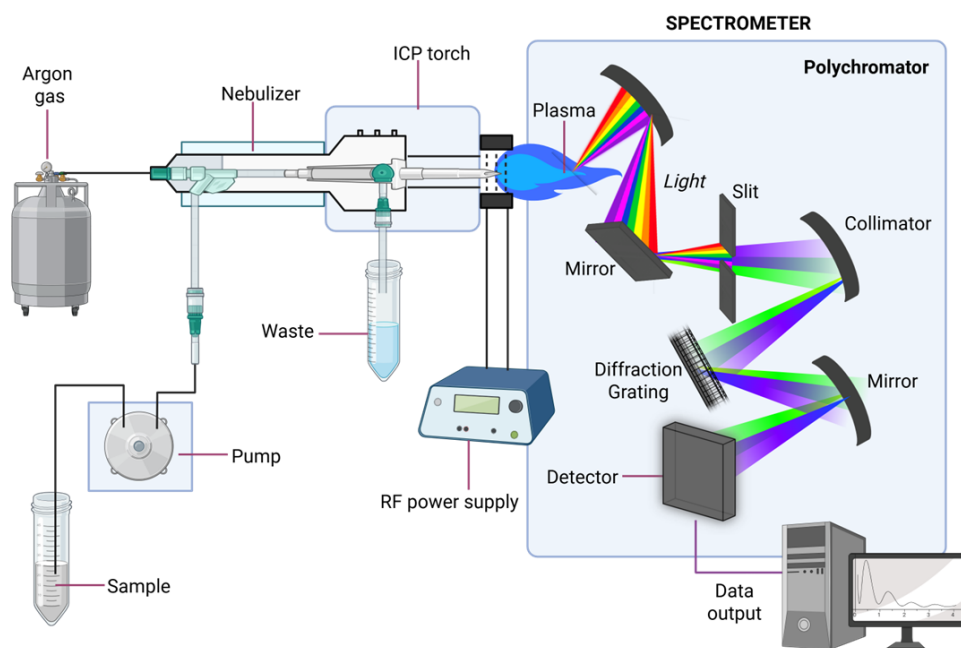


Figure A.5: Representation of the basic setup of an ICP-OES spectrometer.

*Solid samples require acid digestion, while gas samples could be introduced directly into the instrument.

A.2.4 Electron Microscopies

Scanning (SEM) and Transmission (TEM) Electron Microscopies

UV-VIS and FTIR use photons emitted by a light source that interact with matter and are absorbed to cause electronic transitions (UV-VIS) or molecular vibrations (FTIR). In contrast, SEM and TEM use electrons generated from a heated filament and accelerated through a potential ranging from 1 kV to 30 kV in SEM, and 30 kV to 300 kV in TEM, to detect materials with micron and submicron sizes and produce highly magnified images of them.⁷⁸

In SEM, the acceleration voltage enables electrons to interact with the surface of a bulk specimen. Some of the incoming electrons (primary electrons) that interact with the specimen will be reflected (backscattered electrons), while others will supply energy to the electrons in the sample causing their transition to unstable excited states, after which they will return to the ground state by emitting characteristic X-rays, secondary electrons, and Auger electrons (Fig. A.6).^{78,221,222} SEM utilizes both secondary and backscattered electrons to generate detailed, high-resolution images. Secondary electrons highlight the surface structure and morphology of the sample, while backscattered electrons provide insight into its composition.²²¹

The basic setup of a SEM equipment includes an electron gun located at the top of the device that generates electrons via a strong electric field that rips electrons from the atom (field emission) or via heating a filament that emits electrons away (thermoionic emission).⁷⁸ The electron beam generated by the electron gun inside the column is concentrated by scanning coils that guide the electron beam through to the final lens, which redirects the beam horizontally and vertically, enabling raster scanning across a rectangular section of the sample's surface. An image of this area is formed by collecting secondary electrons released from each local region of the specimen.²²¹

On the other hand, in TEM, the high-energy electrons can penetrate distances of several microns (μm) into a solid.²²¹ TEM is capable of providing image mode and diffraction mode information from a sample that is crystalline.^{221,223} Similar to SEM, imaging in TEM involves the generation of electrons from the gun, which are focused on the specimen through electromagnetic lenses.²²⁴ In SEM and TEM, the characteristic X-rays are used by a technique known as EDX, for identifying elemental compositions, plotted in a spectrum of counts of the X-rays versus their

energy.⁷⁸

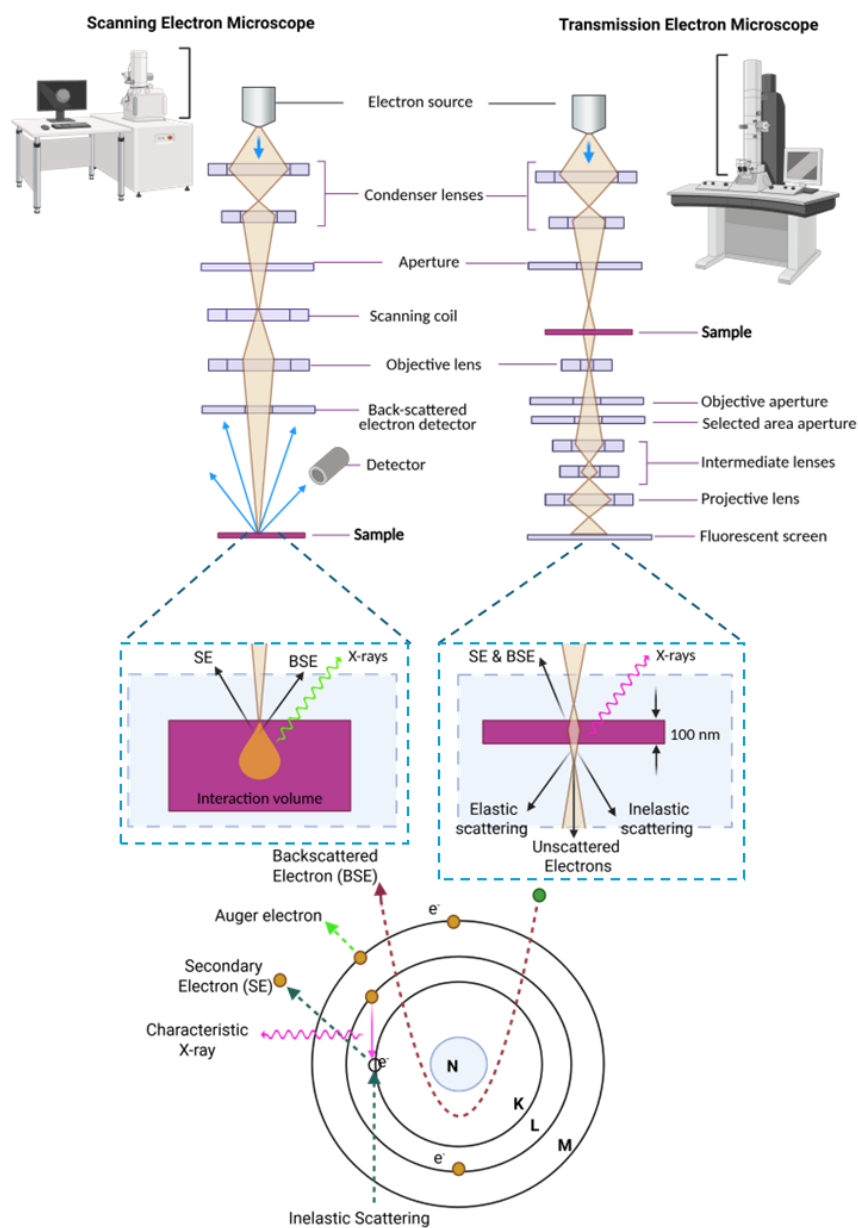


Figure A.6: Scanning and Transmission Electron Microscopies.

A.2.5 X-ray Diffraction (XRD)

XRD is a powerful analytical technique used to investigate the structural properties of crystalline materials, such as phases, impurities, preferred textures, average grain dimensions, defects, crystallinity, strain, and lattice parameters.^{223–225} In a crystal, the orderly arrangement of atoms acts like a three-dimensional diffraction grating for the beam of X-rays whose wavelengths are similar to the spacing between atoms (Fig. A.7-A).²²³ As the beam of X-rays, possessing acceleration voltages between 4 kV and 100 kV, strikes the crystal, they scatter in all directions.²²³ Depending on the direction, the scattered X-rays may cancel each other out (destructive interference) or reinforce each other (constructive interference), producing strong X-ray signals (Fig. A.7-B).^{223,224} The resulting diffraction pattern reflects the reciprocal lattices of the crystal and provides valuable information about the internal structure of the material.²²³ The pattern is governed by Bragg's Law, which relates the wavelength of the X-ray and the angle of incidence to the spacing between atomic planes in the crystal:

$$n\lambda = 2d\sin\theta, \quad (\text{A.5})$$

where n is an integer known as the order of diffraction, λ is the X-ray wavelength, d is the spacing between two consecutive scattering planes and θ is the angle between the atomic planes and the incident (and diffracted) X-ray beam.

Electrons are initially released from a cathode ray tube by heating a filament, and then accelerated toward a sample using an applied voltage.²²⁴ When these electrons possess sufficient energy to dislodge inner-shell electrons from the sample's atoms, characteristic X-ray spectra are produced.²²⁴ The resulting interference pattern of the emitted X-rays aligns with Bragg's law, which defines the relationship between the electromagnetic wave's wavelength, the angle of reflection, and the crystal's lattice spacing (Fig. A.7-C).²²⁴

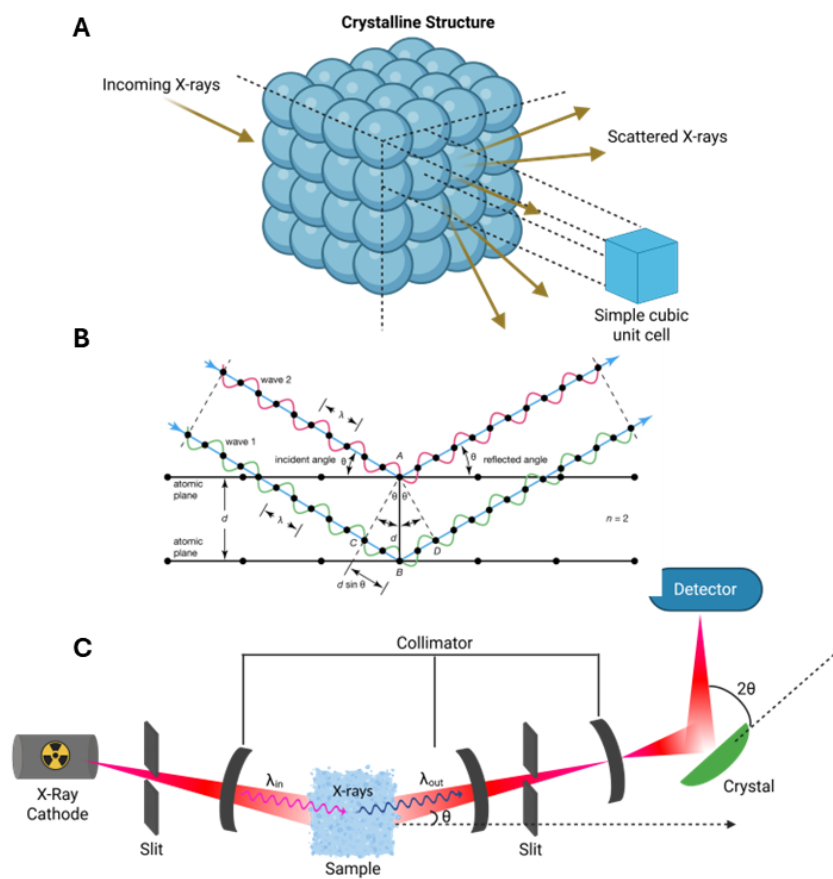


Figure A.7: X-Ray Diffraction. **A.** Periodic arrangement of atoms in a crystalline material. **B.** Bragg's Law. **C.** Basic setup of a X-Ray diffractometer.

A.3 Supporting Information Chapter 4

Table A.1: Potential molecular targets on the MDA cell surface

Protein name	Protein IDs	Genes
Vimentin	P08670	VIME-HUMAN
Annexin A5	P08758	ANXA5-HUMAN
14-3-3 protein zeta/delta	P63104	
Galectin-1	P09382	LEG1-HUMAN
Annexin A1	P04083	ANXA1-HUMAN
14-3-3 protein epsilon	P62258	
Filamin B	Q60FE7	Q60FE7-HUMAN
Plastin 3	P13797	PLST-HUMAN
Lamin A/C	Q5TCJ3	Q5TCJ3-HUMAN
Moesin		
Cathepsin D	P07339	CATD-HUMAN

Table A.2: Potential protein targets on the HeLa cell surface

Protein name	Protein IDs	Genes
Annexin A2	P07355	ANXA2
Epidermal growth factor receptor	P00533	EGFR
CD44	P16070	CD44
CD146	P43121	MCAM
Integrin alpha V	P06756	ITGAV
Fructose-bisphosphate aldolase A	P04075	ALDOA
Transitional Endoplasmic Reticulum ATPase	P55072	MVCP
Receptor-type tyrosine-protein phosphatase F	P10586	PTPRF
Moesin	P26038	MSN
Ezrin	P15311	EZR
Brain acid soluble protein 1	P80723	BASP1
Large neutral amino acids transporter small subunit 1	Q01650	SLC7A5
Basigin	P35613	BSG
HLA class I histocompatibility antigen, A-68 alpha chain	P01891	CD44
Guanine nucleotide-binding protein subunit beta-4	Q9HAV0	GNB4
Guanine nucleotide-binding protein G(i) subunit alpha-2	P04899	GNAI2
Guanine nucleotide-binding protein G(s) subunit alpha	P63092-2	GNAS
Guanine nucleotide-binding protein G(s) subunit alpha	Q5JWF2-2	GNAS
Cofilin-1	P23528	CFL1
Plexin - B2	O15031	PLXNB2
Unconventional Myosin-1e	Q12965	MYO1E
Galectin-3	P17931	LGALS3
Fascin	Q16658	FSCN1
Actin-2	P60709	ACTB
Myosin-9	P35579	MYH9
Ras-related protein Rap-1b	P61224	RAP1B
Integrin alpha 1	P56199	ITGA1
Integrin alpha 2	P17301	ITGA2
Integrin alpha 3	P26006	ITGA3
Integrin alpha 5	P08648	ITGA5
Integrin alpha 6	P23229	ITGA6
Integrin beta 5	P18084	ITGB5
Integrin beta 8	P26012	ITGB8

Table A.3: Molecular docking scores of the dodecapeptides to their potential HeLa molecular targets obtained from conformational model 1.

Target (PDB ID)	ANXA2 (4HRG)	EGFR (3NJP)	CD44 (1UUH)	MCAM (6LYN)	(ITGAV) (1K5G)	Total Score
H1	-184.29	-202.15	-155.05	-204.11	-170.72	-916.32
H2	-161.3	-163.18	-156.76	-159.82	-151.98	-793.04
H3	-191.24	-167.96	-141.46	-197.13	-171.86	-869.65
H4	-162.54	-155.19	-164.46	-174.48	-150.14	-806.81
H5	-152.45	-167.39	-142.31	-178.85	-142.28	-783.28
H6	-220.59	-187.79	-156.25	-221.1	-193.48	-979.21
H7	-145.05	-146.75	-139.8	-172.69	-139.3	-743.59
H8	-178.02	-182.39	-140.67	-203.34	-164.5	-868.92
H9	-156.91	-177.17	-151.47	-167.51	-137.67	-790.73
H10	-190.94	-172.64	-140.44	-208.3	-135.49	-847.81
H11	-154.34	-170.64	-151.27	-154.33	-130.77	-761.35
H12	-178.32	-165.7	-139.5	-173.25	-202.4	-859.17
H13	-163.28	-155.57	-134.02	-172.15	-168.7	-793.72
H14	-157.43	-171.32	-163.26	-172.77	-147.86	-812.64
H15	-165.28	-198.5	-156.47	-180.79	-173.76	-874.8
H16	-180.09	-166.55	-151.23	-181.47	-197	-876.34
H17	-190.96	-198.48	-137.34	-169.13	-176.1	-872.01
H18	-123.29	-147.43	-116.36	-156.84	-134.25	-678.17
H19	-226.15	-252.89	-176.66	-243.03	-194.51	-1093.24
H20	-160.1	-159.41	-134.02	-192.65	-154	-800.18
H21	-163.19	-184.4	-152.08	-177.89	-165.37	-842.93

Table A.4: Molecular docking scores of the dodecapeptides to their potential MDA molecular targets obtained from conformational model 1

Target (PDB ID)	VIME (1GK4)	ANXA5 (1HAK)	ANXA1 (1HM6)	LEG1 (6B94)	Total Score
M1	-136.19	-145.94	-166.55	-160.9	-609.58
M2	-147.03	-162.92	-153.35	-180.96	-644.26
M3	-138.23	-140.5	-144.71	-127.82	-551.26
M4	-153.73	-165.95	-162.68	-152.35	-634.71
M5	-127.43	-171.68	-164.8	-143.59	-607.5
M6	-189.79	-180.04	-191.42	-170.96	-732.21
M7	-159.96	-154.26	-170.81	-159.96	-644.99
M8	-134.78	-164.47	-141.46	-165.89	-606.6
M9	-151.88	-176.96	-165.42	-154.04	-648.3
M10	-140.05	-163.23	-171.29	-154.16	-628.73
M11	-143.68	-163.99	-154.2	-147.74	-609.61
M12	-143.94	-172.15	-159.59	-159.44	-635.12
M13	-133.26	-152.64	-157.66	-137.9	-581.46
M14	-154.86	-162.9	-178.74	-149.33	-645.83
M15	-126.57	-147.18	-152.33	-134.94	-561.02
M16	-156.73	-171.29	-240.14	-177.34	-745.5
M17	-141.83	-167.54	-185.57	-169.19	-664.13
M18	-145.39	-161.26	-170.58	-145.74	-622.97
M19	-169.75	-203.6	-188.72	-177.57	-739.64
M20	-128.57	-173.35	-156.87	-145.58	-604.37
M21	-143.02	-154.83	-172.59	-165.97	-636.41
M22	-157.94	-181.14	-170.36	-190.16	-699.6
M23	-140.63	-199.3	-181.01	-142.3	-663.24

A.4 Supporting Information Chapter 5

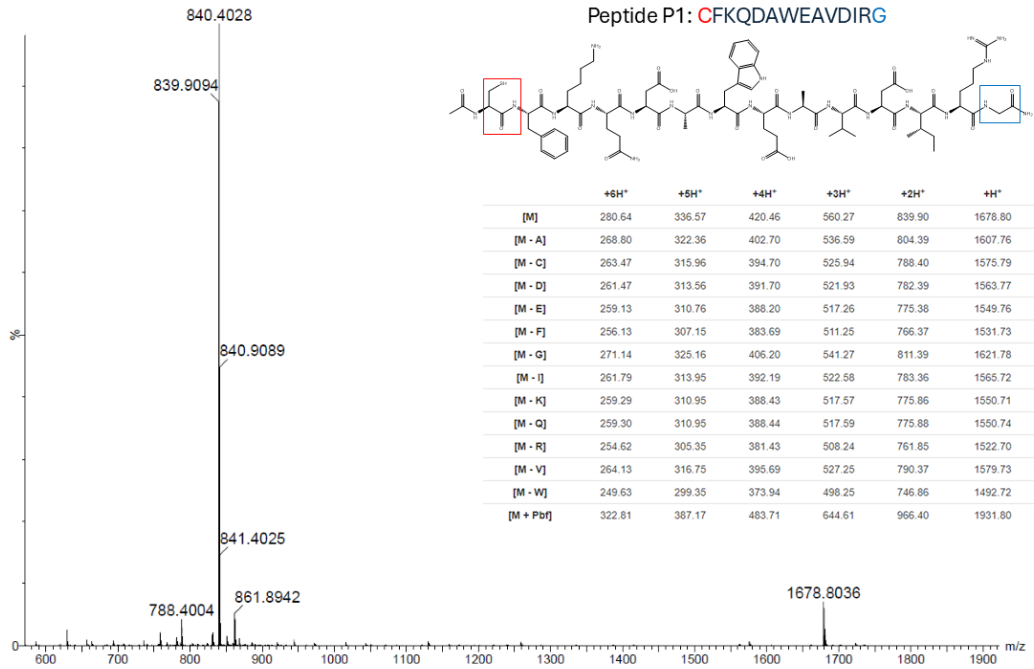


Figure A.8: Mass spectrum of peptide P1

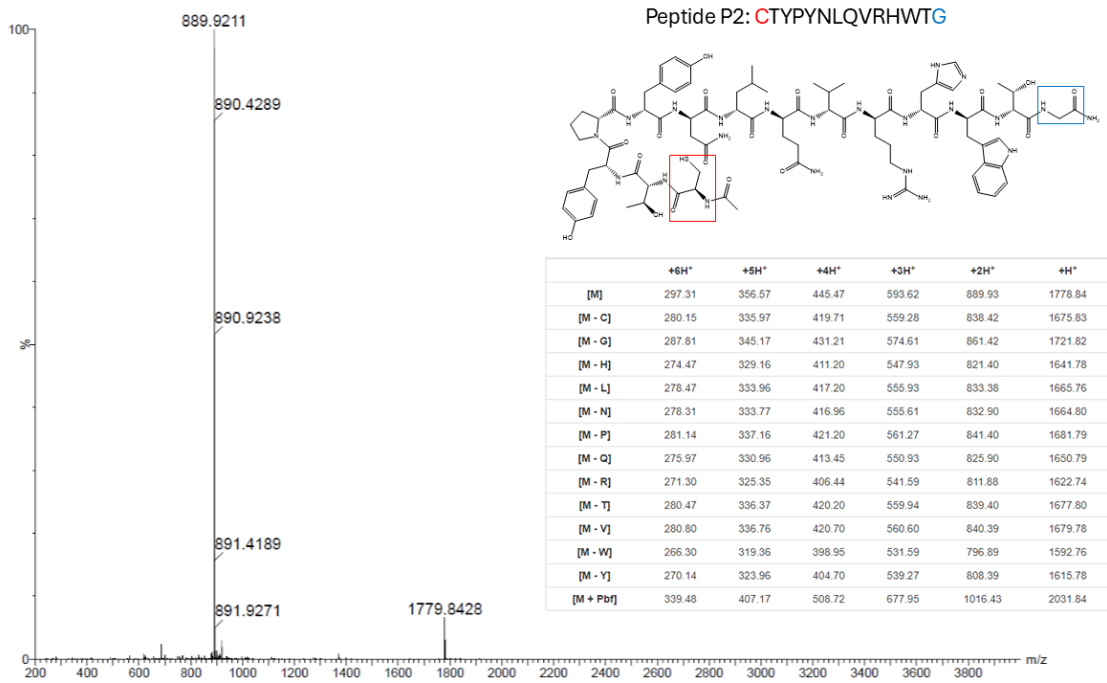


Figure A.9: Mass spectrum of peptide P2

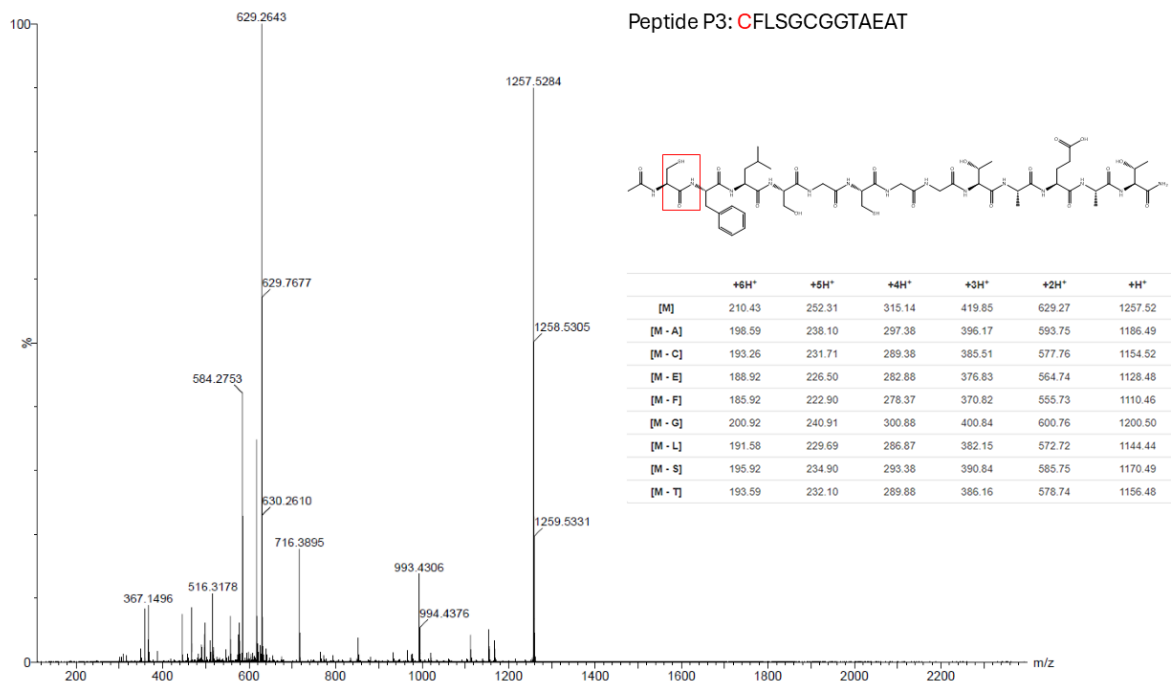


Figure A.10: Mass spectrum of peptide P3

A.4.1 Calculation of the area and volume of the MBAuNPs

To calculate the area and volume of a single MBAuNP, its shape was modelled as a star constituted by a spherical core of radius a , from which N branches originate. These branches of length h_i can be represented as bases of cones with rounded tips attached to the surface of the spherical core. The spherical cap where each branch intersected with the core was subtracted to calculate the volume. Thus, the area (A) and volume (V) can be calculated as follows:

$$A = 4\pi a^2 - 2\pi a \sum_{i=1}^N \left(a - \sqrt{a^2 - R_i^2} \right) + \sum_{i=1}^N \left[\pi(r_i + R_i) \sqrt{(R_i + r_i)^2 + h_i^2} + 2\pi r_i^2 \right] \quad (\text{A.6})$$

$$V = \frac{4}{3}\pi a^3 + \sum_{i=1}^N \left[\frac{h_i}{3}\pi(R_i^2 + R_i^2 r_i + r_i^2) + \frac{2}{3}\pi r_i^3 \frac{\pi}{6} \left(a - \sqrt{a^2 - R_i^2} \right) \left[3R_i^2 + \left(a - \sqrt{a^2 - R_i^2} \right)^2 \right] \right] \quad (\text{A.7})$$

Where,

a = Core average radius of MBAuNPs = 16.08 ± 3.01 nm

r_i = Average hemispherical tip radius of a branch = 3.31 ± 0.54 nm

R_i = Average radius at the base of the branch = 6.55 ± 0.94 nm

h_i = Average branch length = 24.85 ± 5.24 nm

The values assigned to these variables originate from the statistics of the SEM micrographs of the MBAuNPs. The number of branches considered was 8, 12, 16, 17, 18, and 20.

Table A.5: Area and volume of a single MBAuNP with 8, 12, 16, 17, 18, and 20 branches.

Number of branches	Area (nm ²)	Volume (nm ³)
8	8.88×10^3	5.80×10^4
12	1.17×10^4	7.83×10^4
16	1.45×10^4	9.86×10^4
17	1.52×10^4	1.04×10^5
18	1.59×10^4	1.09×10^5
20	1.73×10^4	1.19×10^5

A.4.2 Crystallographic parameters of the AuNPs and MBAuNPs

The crystalline parameters for AuNPs and MBAuNPs were calculated using the Scherrer equation and the Full Width at Half Maximum (FWHM) of the most intense peak assigned to the lattice plane 111:

$$D = \frac{K\lambda}{\beta \cos \theta} = \frac{0.9\lambda}{\beta_{(111)} \cos \theta} \quad (\text{A.8})$$

Where:

D = Average crystallite size,

β = Full Width at Half Maximum (FWHM),

θ = Bragg angle of the most intense peak,

λ = X-ray wavelength = 0.154 nm

Table A.6: Characteristic peaks of AuNPs and MBAuNPs compared with the data reported in the crystallographic card PDF No. 00-004-0784.

PDF No. 00-004-0784	AuNPs	MBAuNPs	Lattice Planes
2θ (degrees)			hkl
38.18	37.94	37.95	111
44.39	44.12	43.77	200
64.57	64.54	64.54	220
77.54	77.48	77.48	311

A.4.3 Concentration of Au ions in the AuNPs and MBAuNPs suspensions

Different volumes (V) of AuNP (0.300 and 1.000 mL) and MBAuNP suspensions (0.150, 0.300, 0.600, 1.000, and 1.500 mL) were centrifuged for 1 hour at 13000 rpm, and the supernatant was discarded to eliminate unreduced Au ions. The samples were digested with 1 mL of HCl:HNO₃, and the volume was made up to 10 mL with distilled water. These data were used to calculate the dilution factors and the final concentration of Au ions in the AuNPs and MBAuNPs suspensions.

Table A.7: The concentration of Au ions in the AuNPs and MBAuNPs suspensions. The density of the solvent (distilled water) was assumed to be 1 g/mL, and standards were used to calibrate the ICP-OES equipment. The final concentration was calculated by multiplying the ICP-OES concentration by the dilution factor. Samples written in blue were not centrifuged to discard unreduced Au ions; they were analysed as synthesized. Also, they were measured in another instrument.

Sample	Volume (V)	Dilution factor	ICP-OES	Final Concentration	
	mL	(10 mL/V)	mg/L	mol/L	
AuNPs	0.300	33.33	6.35	211.64	1.07×10^{-3}
	1.000	10	16.76	167.60	8.51×10^{-4}
	1.000	10	13.94	139.40	7.08×10^{-4}
	1.500	6.666	28.41	189.40	9.59×10^{-4}
Average				177.01	8.97×10^{-4}
MBAuNPs	0.150	66.66	0.74	49.33	2.50×10^{-4}
	0.300	33.33	1.42	47.33	2.40×10^{-4}
	0.600	16.66	2.79	46.48	2.36×10^{-4}
	1.000	10	3.932	39.32	1.99×10^{-4}
	1.000	10	4.174	41.74	2.12×10^{-4}
	1.000	10	3.228	32.28	1.64×10^{-4}
	1.500	6.666	7.62	50.79	2.57×10^{-4}
Average				43.89	2.23×10^{-4}

A.4.4 Concentration of AuNPs and MBAuNPs per litre of solution

Concentration of AuNPs per litre of solution

The concentration of AuNPs in the solution was calculated by estimating the average number of gold atoms per NP through the following formula:

$$N_{at} = \frac{\pi \times \rho \times NA \times D^3}{6 \times M} \quad (\text{A.9})$$

$$N_{AuNPs} = \frac{A_t}{N_{at}} \quad (A.10)$$

Where,

N_{at} = Average number of Au atoms per nanoparticle,

NA = Avogadro's number = 6.022×10^{23} ,

ρ = FCC density of the auric seeds = 1.93×10^{-20} ,

D = Diameter of the nanoparticles = 12.45 ± 0.02 nm,

M = Gold atomic weight = 197 g/mol,

At = Total number of atoms in the sample (atoms/L), obtained from ICP-OES = 177.01 mg/L

Then,

$$N_{at} = \frac{\left(6.022 \times 10^{23} \frac{atoms}{mol}\right) (\pi) \left(1.93 \times 10^{-20} \frac{g}{nm^3}\right) (12.45 nm)^3}{6 \times \left(197 \frac{g}{mol}\right)} = 59612.66 atoms \quad (A.11)$$

$$A_t = \frac{\left(177.01 \frac{mg}{L}\right) \left(0.001 \frac{g}{mg}\right)}{197 \frac{g}{mol}} \left(6.022 \times 10^{23} \frac{atoms}{mol}\right) = 5.41 \times 10^{20} \frac{atoms}{L} \quad (A.12)$$

$$N_{AuNPs} = \frac{5.41 \times 10^{20} \frac{atoms}{L}}{59612.66 atoms} = 9.08 \times 10^{15} \frac{AuNPs}{L} \quad (A.13)$$

Concentration of MBAuNPs per litre of solution

MBAuNPs are formed by a face-centered cubic (fcc; Space Group Fm-3m No. 225) crystalline structure according to the experimental results obtained by X-ray characterization and data reported in the crystallographic card PDF No. 00-004-0784. Then, the unit cell edge length for gold (Au) was assumed to be 0.4078 nm. The unit cell is a perfect cube, so its volume is 0.0678 nm^3 . Therefore, in a single MBAuNP of volume $1.09 \times 10^5 \text{ nm}^3$ (18 branches), there are 1.61×10^6 unit cells. In the fcc unit cell of Au there are 4 atoms. Thus, in 1.61×10^6 unit cells there

are 6.44×10^6 Au atoms, which is equivalent to a density of 59.08 Au atoms per for the overall MBAuNP.

Using the concentration of Au atoms obtained by ICP-OES analysis of the as-synthesized MBAuNPs (43.90 mg/L), the number of MBAuNPs per litter of solution can be calculated as follows:

$$N_{MBAuNPs} = \frac{0.0439 \frac{g}{L}}{\frac{197g}{1mol}} \times \left(\frac{6.022 \times 10^{23} atoms}{1mol} \right) = 1.32 \times 10^{20} \frac{atoms}{L} \quad (A.14)$$

$$= 1.32 \times 10^{20} \frac{atoms}{L} \times \frac{1 unitcell}{4 atoms} = 3.35 \times 10^{19} \frac{unitcells}{L} \quad (A.15)$$

$$= 3.35 \times 10^{19} \frac{unitcells}{L} \times \frac{1.09 \times 10^5 nm^3}{1.61 \times 10^6 unitcells} = 2.27 \times 10^{18} \frac{nm^3}{L} \quad (A.16)$$

$$= 2.27 \times 10^{18} \frac{nm^3}{L} \times \frac{1 MBAuNP}{1.09 \times 10^5 nm^3} = 2.08 \times 10^{13} \frac{MBAuNPs}{L} \quad (A.17)$$

Table A.8: Nanoparticles per litre of solution calculated for MBAuNPs with 8, 12, 18, and 20 branches.

Number of branches	MBAuNPs/L
8	3.91×10^{13}
12	2.89×10^{13}
16	2.30×10^{13}
17	2.18×10^{13}
18	2.08×10^{13}
20	1.91×10^{13}

Note: The calculations presented above were validated by applying the same calculations to the spherical AuNPs, as follows:

In a single AuNP of volume $1.01 \times 10^3 \text{ nm}^3$, there are 1.49×10^4 unit cells. In the fcc unit cell of Au there are 4 atoms. Thus, in 1.49×10^4 unit cells there are 4.41×10^4 Au atoms. **Without considering the packing factor, the number of atoms per AuNPs was 5.96×10^4** , confirming the results obtained using the formula reported by Liu et al. described in section A.4.4

Then, using the concentration of Au atoms obtained by ICP-OES analysis of the as-synthesized AuNPs (177.01 mg/L), the number of AuNPs per liter of solution can be calculated as follows:

$$N_{AuNPs} = \frac{0.17701 \frac{\text{g}}{\text{L}}}{\frac{197\text{g}}{1\text{mol}}} \times \left(\frac{6.022 \times 10^{23} \text{atoms}}{1\text{mol}} \right) = 5.41 \times 10^{20} \frac{\text{atoms}}{\text{L}} \quad (\text{A.18})$$

$$= 5.41 \times 10^{20} \frac{\text{atoms}}{\text{L}} \times \frac{1\text{unitcell}}{4\text{atoms}} = 1.35 \times 10^{20} \frac{\text{unitcells}}{\text{L}} \quad (\text{A.19})$$

$$= 1.35 \times 10^{20} \frac{\text{unitcells}}{\text{L}} \times \frac{1.01 \times 10^3 \text{nm}^3}{1.49 \times 10^4 \text{unitcells}} = 9.29 \times 10^{18} \frac{\text{nm}^3}{\text{L}} \quad (\text{A.20})$$

$$= 9.29 \times 10^{18} \frac{\text{nm}^3}{\text{L}} \times \frac{1\text{AuNP}}{1.01 \times 10^3 \text{nm}^3} = 9.19 \times 10^{15} \frac{\text{AuNPs}}{\text{L}} \quad (\text{A.21})$$

Assuming a yield of 100%, the number of AuNPs in the reaction batch is given by the number of Au atoms (6.022×10^{18}) in the reaction volume (10 mL), divided by the number of Au atoms per AuNPs:

$$N_{AuNPs} = \frac{6.022 \times 10^{18} \text{atoms}}{5.96 \times 10^4 \frac{\text{atoms}}{\text{AuNP}}} = 1.01 \times 10^{14} \text{AuNPs(in10mL)} \quad (\text{A.22})$$

Or $N_{AuNPs} = 1.01 \times 10^{16}$ AuNPs/L. By ICP-OES, the yield of the Au reduction was calculated to be 89.85%, then $N_{AuNPs} = 8.59 \times 10^{15}$ AuNPs/L, consistent with calculations in section A.4.4

A.4.5 Yield of the synthetic procedure and molar concentration of AuNPs and MBAuNPs

The yield of the reaction protocol to synthesize AuNPs and MBAuNPs is estimated by considering the number of Au atoms in the solution calculated from the ICP-OES results and the total Au atoms equivalent to the amount of gold salt added to the reaction.

To prepare 10 mL of AuNPs, a 10 mL 1 mM aqueous solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was employed, which contained 6.022×10^{18} ($N_{\text{Au}^{3+}}^{\text{SYNT}}$). The average concentration of Au atoms in the AuNPs estimated by ICP-OES was 177.01 mg/L, so in 10 mL of AuNPs which is equivalent to 5.411×10^{18} Au^{3+} atoms ($N_{\text{Au}^{3+}}^{\text{ICP-OES}}$). Therefore, the yield of the synthetic procedure following and Inverse Turkevich method was:

$$\frac{N_{\text{Au}^{3+}}^{\text{ICP-OES}}}{N_{\text{Au}^{3+}}^{\text{SYNT}}} \times 100 = \frac{5.411 \times 10^{18} \text{Au}^{3+} \text{atoms}}{6.022 \times 10^{18} \text{Au}^{3+} \text{atoms}} \times 100 = 89.85\% \quad (\text{A.23})$$

On the other hand, to prepare 40 mL of MBAuNPs, we employed 2.164×10^{17} Au atoms from 0.4 mL of AuNP seeds and 6.022×10^{18} atoms Au atoms from a 0.4 mL 25 mM solution of gold salt growth solution. Then,

$$N_{\text{Au}^{3+}}^{\text{SYNT}} = 2.164 \times 10^{17} \text{atoms} + 6.022 \times 10^{18} \text{atoms} = 6.238 \times 10^{18} \text{Au}^{3+} \text{atoms} \quad (\text{A.24})$$

The average concentration of Au atoms in the MBAuNPs estimated by ICP-OES was 43.8 mg/L, then in 40 mL of sample there are 6.360×10^{18} Au^{3+} atoms ($N_{\text{Au}^{3+}}^{\text{ICP-OES}}$). Therefore, the yield of the seed mediated synthetic procedure to obtain MBAuNPs was:

$$\frac{N_{\text{Au}^{3+}}^{\text{ICP-OES}}}{N_{\text{Au}^{3+}}^{\text{SYNT}}} \times 100 = \frac{5.411 \times 10^{18} \text{Au}^{3+} \text{atoms}}{6.022 \times 10^{18} \text{Au}^{3+} \text{atoms}} \times 100 = 85.92\% \quad (\text{A.25})$$

Molar concentration of AuNPs and MBAuNPs in solution

The molar concentrations (C_M) of AuNPs and MBAuNPs in solution were calculated as the ratio between the total atomic gold concentration added to the reaction volume and the previously determined number of atoms per AuNPs or MBAuNPs, as determined via ICP-OES.

$$C_M = \frac{\text{moles from reaction}}{\text{number of atoms per NPs}} \times \text{yield of the synthetic protocol} \quad (\text{A.26})$$

The molar concentration of AuNPs in a 10 mL batch was:

$$C_M = \left[\frac{6.022 \times 10^{18} \text{ Au atoms} \times \frac{1 \text{ mol}}{6.022 \times 10^{23} \text{ Au atoms}}}{59612.66 \frac{\text{Au atoms}}{\text{AuNP}} \times 0.01 \text{ L}} \right] \times 0.8985 \quad (\text{A.27})$$

$$C_M = 1.509 \times 10^{-8} \frac{\text{mol}}{\text{L}} = 15.09 \text{ nM} \quad (\text{A.28})$$

The molar concentration of MBAuNPs in a 40 mL batch was:

$$C_M = \left[\frac{6.022 \times 10^{18} \text{ Au atoms} \times \frac{1 \text{ mol}}{6.022 \times 10^{23} \text{ Au atoms}}}{6.44 \times 10^6 \frac{\text{Au atoms}}{\text{AuNP}} \times 0.04 \text{ L}} \right] \times 0.8985 \quad (\text{A.29})$$

$$C_M = 3.335 \times 10^{-11} \frac{\text{mol}}{\text{L}} = 0.033 \text{ nM} = 33.35 \text{ pM} \quad (\text{A.30})$$

A.4.6 Estimation of the extinction coefficients of the AuNPs and MBAuNPs

The extinction coefficients of as-synthesized AuNPs and MBAuNPs were estimated following the procedure reported by Liu et al., with certain modifications. Different volumes (0.600, 0.300, 0.150, 0.100, and 0.05 mL) of as-synthesized AuNPs and MBAuNPs were deposited in

microcentrifuge tubes, and the volume was made up to 2 mL using distilled water. The samples were sonicated for 20 minutes, and the extinction maximum at the LSPR was measured using a UV-Vis spectrophotometer. 18 samples were prepared for 6 relative concentrations measured in triplicate, and standard deviations of the absorbance were calculated (Table S4). The extinction maximum at the LSPR was plotted versus the molar concentration of the suspension. The extinction coefficient was then obtained from the slope of the linear region of the absorbance-concentration curve, as stated in Beer-Lambert's law (A.4).

Table A.9: Parameters used to calculate the extinction coefficients of AuNPs and MBAuNPs

Volume (V)	Relative Concentration	Absorbance
mL	C, (10^{-8}) mol/L	A_{λ}
AuNPs		
2	1.5100	3.5772 ± 0.0000
0.6	0.4520	1.1338 ± 0.0286
0.3	0.2260	0.5726 ± 0.0047
0.15	0.1130	0.3029 ± 0.0027
0.1	0.0754	0.2027 ± 0.0043
0.05	0.0377	0.1044 ± 0.0008
mL	C, (10^{-11}) mol/L	A_{λ}
MBAuNPs		
2	3.3350	1.5758 ± 0.0000
0.6	1.0005	0.3303 ± 0.0051
0.3	0.5003	0.1647 ± 0.0047
0.15	0.2501	0.1049 ± 0.0025
0.1	0.1668	0.0704 ± 0.0007
0.05	0.0833	0.0355 ± 0.0013

A.4.7 Extinction coefficients of spherical AuNPs and star-shaped/multibranched MBAuNPs reported in the literature

Table B.10. Physicochemical properties of AuNPs reported in the literature and used to find the relation between the volume of the AuNPs and extinction coefficients.

Reducing agent	Solvent	LSP	Diameter	Volume	Molar extinction coefficient
		nm	nm	nm ³	M ⁻¹ cm ⁻¹
Decanethiol	Toluene		3.76 ± 0.65	27.819	(3.61 ± 0.08) × 10 ⁶
DMAB ³	-		4	33.493	1.16 × 10 ⁵
Sodium Citrate	Water		4.61 ± 0.65	51.272	(8.56 ± 0.09) × 10 ⁶
DMAB	-		6	1.13 × 10 ²	3.88 × 10 ⁵
			7.19	2.21 × 10 ²	2.88 × 10 ⁷
DMAB	-		8	2.68 × 10 ²	9.22 × 10 ⁵
			8	2.68 × 10 ²	4.12 × 10 ⁷
Sodium Citrate	Water		8.55 ± 0.79	3.20 × 10 ²	(5.14 ± 0.07) × 10 ⁷
Oleylamine ⁴	THF		8.76 ± 1.11	5.23 × 10 ²	(8.79 ± 0.0.) × 10 ⁷
DMAB	-		10	5.23 × 10 ²	1.81 × 10 ⁶
DMAB	-		12	9.0 × 10 ²	3.10 × 10 ⁶
Sodium Citrate	Water	520	12.45 ± 0.02	1.0 × 10 ³	(2.35 ± 0.02) × 10 ⁸
Seed-mediated - PEGylated	Water	524	14	1.4 × 10 ³	2.80 × 10 ⁸
Sodium Citrate	Water		20.60 ± 1.62	4.5 × 10 ³	(8.78 ± 0.06) × 10 ⁸
Commercial	Water		25.67 ± 5.62	8.9 × 10 ³	(2.93 ± 0.02) × 10 ⁹
			31	1.56 × 10 ⁴	3.86 × 10 ⁸
Seed-mediated - PEGylated	Water		34.46 ± 4.34	2.1 × 10 ⁴	(6.06 ± 0.03) × 10 ⁹
Commercial	Water	531	50	6.5 × 10 ⁴	1.80 × 10 ¹⁰
Seed-mediated - PEGylated	Water	535	54 ± 3.0	8.2 × 10 ⁴	1.90 × 10 ¹⁰
Commercial	Water	536	60	1.1 × 10 ⁵	3.40 × 10 ¹⁰
Seed-mediated - PEGylated	Water	549	75 ± 3.0	2.2 × 10 ⁵	4.50 × 10 ¹⁰
Commercial	Water	549	80	2.6 × 10 ⁵	7.90 × 10 ¹⁰
Decanethiol	-	589	100 ± 10.0	5.2 × 10 ⁵	2.10 × 10 ¹¹
Commercial	-	660	150	1.8 × 10 ⁶	1.50 × 10 ¹¹

³ DMAB: Reduction of gold(I) 6-thioguanosine hydrogel with DMAB

⁴ Oleylamine: Reduction of gold salt in the presence of oleylamine

Table B.11. Physicochemical properties of MBAuNPs reported in the literature and used to find the relation between the volume of the MBAuNPs and extinction coefficients.

Reducing agent	Solvent	Size	LSPR	Volume	Molar extinction coefficient
		nm	nm	nm ³	M ⁻¹ cm ⁻¹
HEPES	Water	21 ± 4	543	3.00 × 10 ³	5.70 × 10 ⁸
HEPES	Water	24 ± 5	568	3.50 × 10 ³	5.80 × 10 ⁸
HEPES	Water	26 ± 6	594	2.90 × 10 ³	4.90 × 10 ⁸
HEPES	Water	26 ± 5	616	3.40 × 10 ³	6.60 × 10 ⁸
HEPES	Water	30 ± 9	668	2.90 × 10 ³	6.40 × 10 ⁸
HEPES	Water	31 ± 7	651	4.10 × 10 ³	8.10 × 10 ⁸
HEPES	Water	41 ± 11	711	8.40 × 10 ³	2.01 × 10 ⁹
HEPES	Water	46 ± 12	745	9.00 × 10 ³	2.44 × 10 ⁹
EPPS	Water	-	755	-	2.30 × 10 ⁹
DMF	Water	-	684	9.48 × 10 ³	5.90 × 10 ⁸
HEPES	Water	55 ± 16	773	1.16 × 10 ⁴	2.68 × 10 ⁹
Hydroxylamine	Water	60.1 ± 5.0	633	—	2.12 × 10 ¹⁰
Ascorbic Acid	Water	~70	778	4.14 × 10 ⁴	3.14 × 10 ⁹
DMF	Water	-	774	1.15 × 10 ⁵	3.60 × 10 ¹⁰
DMF	Water	-	829	4.45 × 10 ⁵	9.61 × 10 ¹⁰
Ascorbic Acid	Water	78.49 ± 2.12	845	1.09 × 10 ⁵	(4.8 ± 0.02) × 10 ¹⁰
DC ₁₄ TAB		350 ± 50	915	-	6.90 × 10 ¹¹
DC ₁₄ TAB		350 ± 50	1064	-	7.40 × 10 ¹¹

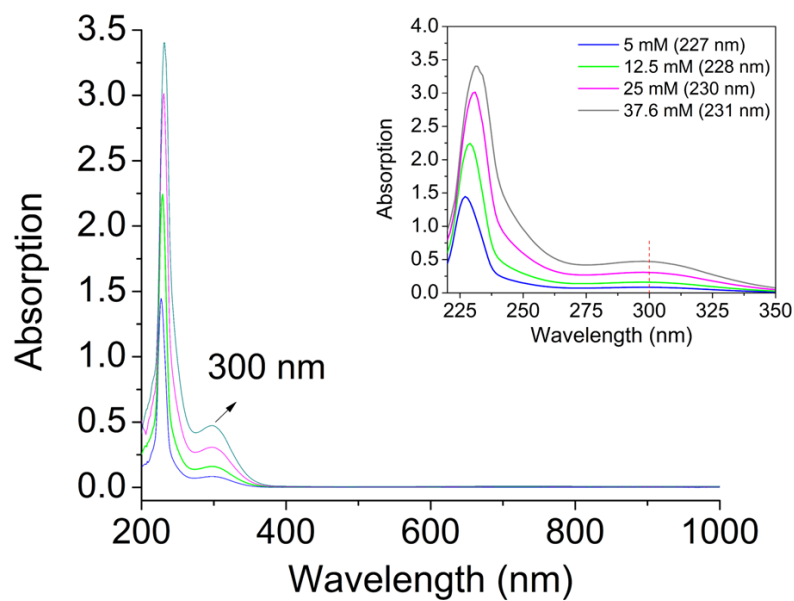


Figure A.11: Optical properties of PEG-MAL. PEG-MAL lacks absorbance in the visible and NIR region. The absorption of the peaks at 231 nm and 300 nm, attributed to PEG chains and maleimide functional groups, respectively, increase with increasing concentration of PEG-MAL (inset).

Appendix B

Appendix

B.1 Science Outreach Article

- [1] Quilumba-Dutan V and Rodríguez-López JL. **Nanomedicina: La Salud de Hoy y Mañana en Buenas Manos**. *Obsidiana Magazine, Ciencia y Cultura por México*. 2024.
<https://obsidianadigital.mx/>

B.2 Young Research Talks

- [1] State Congress of Materials Science and Engineering-CECIM-2025. *"Identification of ligands with high affinity toward cancer cells and their potential use in the design of multifunctional nanostructures."* San Luis Potosí, México
- [2] International Materials Research Congress-IMRC-2024. *"Phage-displayed Peptides Targeting Surface Proteins of HeLa and MDA-MB-231 Cells Assessed by Molecular Docking."* Cancún, México

- [3] Nanoscience Summer School at Yachay 2024. *"Highly specific peptide-nanoparticle conjugates against HeLa and MDA-MB-231 cancer cells"*. Galápagos, Ecuador
- [4] Research Seminar at Nanogroup. *"Multifunctional nanostructured system with potential applications in the treatment of breast and cervical cancer"*. Birmingham, United Kindom
- [5] International Materials Research Congress-IMRC-2023. *"Design of a Multifunctional Nanostructured System with Potential Applications in Breast and Cervical Cancer Treatment"*. Cancún, México
- [6] Nanoscience Summer School at Yachay 2023 *"Design of a Multifunctional Nanostructured System with Potential Applications in Breast and Cervical Cancer Treatment"*. Galápagos, Ecuador

B.3 Poster Sessions and Awards

- [1] International Materials Research Congress-IMRC-2025. *"Peptide-gold nanostar conjugates for site-specific photothermal treatment of breast and cervical cancer."* **Best Student Poster Award (First Place)**. Cancún, México
- [2] State Congress of Materials Science and Engineering-CECIM-2025. *"Peptide-gold nanostar conjugates for site-specific photothermal treatment of breast and cervical cancer."* San Luis Potosí, México
- [3] International Congress of Nanoscience and Nanotechnology-ICNN VI-2025. *"Peptide-gold nanostar conjugates for site-specific photothermal treatment of breast and cervical cancer."* **Best Student Poster Award (Second Place)**. Quito, Ecuador
- [4] First National Symposium on Research and Innovation in Nanosciences-SNIIN-2024. *"Design of a Multifunctional Nanostructured System with Potential Applications in Breast and Cervical Cancer Treatment"*. San Luis Potosí, México

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- [5] International Materials Research Congress-IMRC-2024. *"Design of a Multifunctional Nanostructured System with Potential Applications in Breast and Cervical Cancer Treatment"*. **Best Student Poster Award (Participant)**. Cancún, México

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