Investigation of *in-situ* nanoimprinting of cell surface receptors: potential of a novel technique in biomarker research

Sadia Ahmed

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Brenda S. J. Winkel, Chair

Iuliana M. Lazar

Shihoko Kojima

José Á. Rodríguez-Corrales

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ABSTRACT (ACADEMIC)

Biological markers, also termed biomarkers, are objective and measurable characteristics of a cell or organism that indicate disease, infection, environmental exposure, or the response to therapeutic intervention. Biomarkers are used as primary endpoints in disease research as well as in early drug development. Cell surface receptor proteins have been recognized as significant biomarkers due to the ease of detection relative to intracellular biomolecules. In complex diseases like cancer, these proteins may also display high variance across patients and stages of the disease. Therefore, methods not only for detection of cell surface receptors, but also for characterizing expression patterns are needed to develop these proteins as clinically-relevant biomarkers. Unfortunately, there is still a lack of integrated, robust, and cost-effective techniques for studying receptor expression discriminated by spatial and temporal events during disease. To close this gap, we investigated an *in-situ* imprinting technique for cell surface receptors on silver nanodiscs that could be used to assess expression patterns of a biomarker. We designed a proof-of-principle study using melanocortin receptor 1 (MC1R) as a model. We synthesized a branched (dendrimeric) peptide ligand specific to MC1R and evaluated its binding in WM-266-4, an established melanoma cell line. We also synthesized a europium-labeled ligand and determined MC1R expression levels in the same cell line, which were then used to quantify the number of receptors present on individual cells. This novel nanoimprinting technique could be adapted for more personalized diagnosis as well as for surface receptor phenotyping in basic and applied research.

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ABSTRACT (GENERAL)

Biomarkers are biological characteristics that can be observed or measured during disease conditions, and compared to the healthy state (e.g. grades of fever during infection). Biomarkers have been used in medical history to study disease progression, to develop drugs, or to predict drug efficacy. However, in complex diseases such as in cancer, biomarkers vary tremendously among patients and disease stages. Cell surface receptors, proteins that are located at the cell surface and deliver external signals into the cell, are a significant group of easily-detectable biomarkers. Along with the detection of particular biomarkers related to a disease, extensive characterization of expression patterns is necessary to optimize their application. Therefore, we designed a technique to imprint or capture the expression pattern of these receptors on silver nanoparticles. We incorporated branched molecules that can simultaneously bind to the target receptors and the nanoparticle surface. To develop the technique, we used melanocortin receptor 1 (MC1R), a receptor present at high levels on the surface of melanoma cells, as a test system. We determined optimum binding of this molecule in an established melanoma cell line, WM-266-4. We also synthesized a labeled molecule that was used to estimate the number of MC1R proteins on these cells. These studies indicate that this might be a promising approach for developing sensitive and cost-effective tools to characterize cell surface receptors in studying complex diseases and cell mechanisms.

DEDICATION

I dedicate this thesis to all the great women I have met in my life. From mom to mentors, they taught how to become strong and kind at the same time. They showed how to improve oneself while helping others to grow.

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Graduate school is more of a journey than a destination itself. When I started my journey in United States, far from my family and my country, on the other side of the world, I was excited and scared at the same time. But today, when I am writing this acknowledgment piece, I can say, it was a beautiful and rewarding journey. I still have a long path to walk, but these two years will always show me the way ahead.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Aha	6-aminohexanoic acid
AIDS	Acquired immunodeficiency syndrome
ACTH	Adrenocorticotropic hormone
AGRP	Agouti-related peptide
AgNDs	Silver (Ag) nanodiscs
ASIP	Agouti signaling protein
AuNP	Gold (Au) nanoparticles
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
B_{max}	Maximum binding
BRCA	Breast cancer
CD4	Cluster of differentiation 4
c-myc	Cellular homolog of v-myc, the avian myelocytomatosis viral oncogene
Cryo-EM	Cryogenic electron microscopy
Dap	2,3-diaminopropionic acid
DCM	Dichloromethane
DELFIA	Dissociation enhanced lanthanide fluorescence immunoassay
DIC	*N*,*N'*-diisopropylcarbodiimide
DIEA	*N*,*N'*-diisopropylethylamine
DMEM	Dulbecco's modified essential medium
DMF	*N*,*N*-dimethylformamide
DMSO	Dimethylsulfoxide
DTPA	Diethylenetriaminepentaacetic acid
EC_{50}	50% effective concentration
EDT	Ethanedithiol
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's modified essential medium

EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
ESI	Electrospray ionization
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and drug administration
GPCR	G-protein coupled receptor
GFP	Green fluorescent protein
HCTU	*O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	Hydroxybenzotriazole
HER2	Human epidermal growth factor receptor 2
ICC	Immunocytochemistry
Inp	Isonipecotic acid
K_d	Dissociation constant
LC	Liquid chromatography
LCM	Laser capture microdissection
MALDI	Matrix-assisted laser desorption/ionization
MCR	Melanocortin receptor
MS	Mass spectrometry
MSH	Melanocyte-stimulating hormone
MSH(7)	Ac-Ser-Nle-Glu-His-DPhe-Arg-Trp-NH_2
NDP- α-MSH	Ac-Ser-Tyr-Ser- Nle -Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
PBS	Phosphate buffered saline
POMC	Proopiomelanocortin
PR	Progesterone receptor
PSA	prostate specific antigen
Raf	Rapidly accelerated fibroblastoma, RAF in humans
Ras	Rat sarcoma, RAS in humans
SAR	Structure-activity relationship

SELDI	Surface enhanced laser desorption/ionization
SPPS	Solid phase peptide synthesis
TA	Thioanisole
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIS	Triisopropylsilane
TOF	Time of flight
TRF	Time-resolved fluorescence

1. INTRODUCTION

1.1. Biomarkers

Biological markers, or biomarkers, are objective indications or characteristics of biological or pathological processes in a cell or organism that are indicative of disease, infection, exposure to environmental factors, or response to a therapeutic intervention. Biomarkers are utilized as potential primary or surrogate endpoints in medical research and discovery because of their accuracy and reproducibility.[1,2] The presence or absence, quantity, or molecular pattern of specific biomolecules, e.g. DNA, RNA, proteins, lipids, or sugars, in a specific tissue or body fluid can all serve as biomarkers.[3]

Along with pathological examinations and medical signs and symptoms, biomarkers help in disease diagnosis, classification, prognosis, staging, survival, determination of therapy, and consecutive outcome. Biomarkers provide significant interim evidence in early drug development to predict safety and efficacy, and the detection methods are simple, affordable, and less invasive yet accurate even in small subject groups.[1] Commonly used approaches for the discovery and development of biomarkers include the analysis of gene expression, gene fusion, or gene translocation, and protein presence in body fluids or tissues by mass spectrometry (MS), tissue imaging, and microarrays, among others. A summary of these methods is presented in Figure 1.1.

A classic example of the use of biomarkers as therapeutic targets is breast cancer. Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are the most common biomarkers for this disease. The chemotherapeutic agent trastuzumab (Herceptin®) is used to target HER2 positive tumors, while fulvestrant (Faslodex®) is used to treat tumors that are HER2 negative but ER and PR positive. Patients whose tumors are characterized as negative for all three receptors, such as in the case of triple negative breast cancers, are tested for further biomarkers such as mutations in breast cancer (BRCA) genes in order to develop effective therapies.[4,5] Other widely-used biomarkers include DNA mutations in genes such as rat sarcoma (Ras, RAS in human) and rapidly accelerated fibroblastoma (Raf, RAF in human), which are also

used to select effective therapy,[6,7] RNA biomarkers such as the expression profile of c-myelocytomatosis (c-myc, c-Myc in human) associated with large cell lymphoma,[8] or protein biomarkers such the level of prostate specific antigen (PSA) in serum to screen for prostate cancer.[9] The Food and Drug Administration (FDA) has already approved or is in the process of surveying a number of biomarkers for oncogenic, neurological, systemic, autoimmune, inflammatory, and infectious diseases.[10]

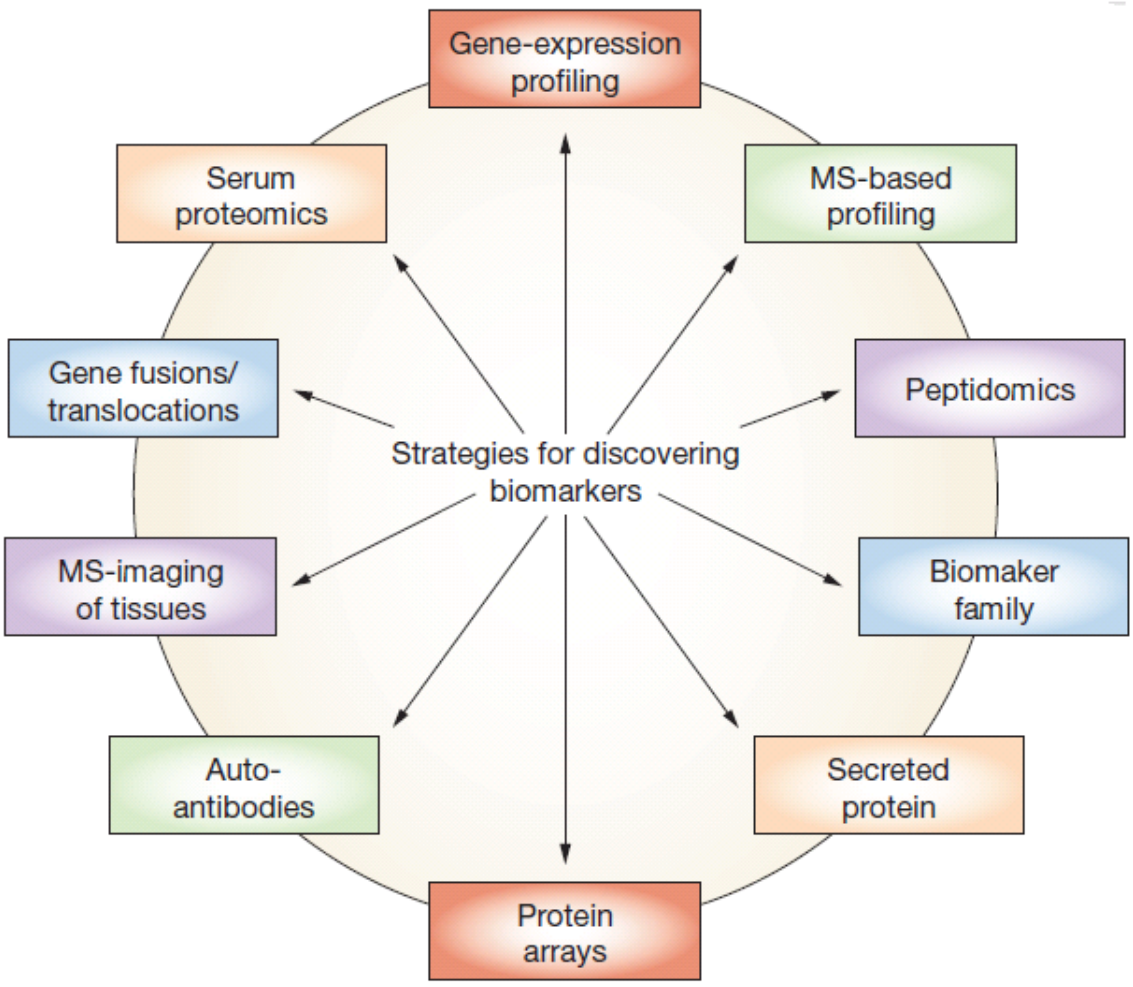

Figure 1.1. Strategies for biomarker discovery.[11] Figure copied from Kulasingam, V.; Diamandis, E. P. Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. *Nature Clinical Practice Oncology* **2008**, *5*, 588, Used by permission of Springer Nature.

1.2. Cell surface receptors as biomarkers

As stated in the central dogma of molecular biology, enunciated by Francis Crick,[12] genetic messages in DNA are transcribed to RNA, followed by translation into protein. Alterations in the optimal state of these biomolecules due to disease or clinical interventions can be assessed by a variety of means, which have given rise to several different types of biomarkers. The screening for such biomarkers has expanded beyond the genome, transcriptome and proteome to include the chemical changes in DNA and histones (epigenome), metabolites (metabolome), sugars (glycome), lipids (lipidome), metals and metalloids (metallome), secreted proteins (secretome),[13] and cell surface proteins (surfaceome).[14]

Although complex diseases, such as cancers, are initiated by genetic alterations due to heredity or the environment, multiple genetic and epigenetic events occur during disease progression. In many cases, these events cause high molecular complexity and variance in different populations and different stages of disease.[3] Changes in the types or amounts of specific proteins, either intracellular or cell surface bound, are indicative of changes in the state of the cell.[15] Among them, cell surface signaling proteins, or cell surface receptors, are easily accessible for detection and analysis, which minimizes sample processing. In addition, these protein have extracellular domains exposed in the cell surface which can be targeted by synthetic ligands or antibodies. Consequently, cell surface receptors are an important group of biomarkers with substantial sensitivity and specificity.[16,17]

Cell surface receptors are glycoproteins and lipoproteins embedded in the cell membrane that receive external physical (e.g., light, temperature, and pressure,) or chemical (e.g., peptides, lipids, hormones, neurotransmitters, and odor molecules) stimuli and transduce the signal across the membrane to the interior of the cell.[18,19] Agonist or antagonist ligands bind to the extracellular domain of the receptors, following dimerization of monomeric receptors or conformational change in the transmembrane domain, which effects an intracellular response by activating or inhibiting specific cellular signaling pathways.[20,21] Three major classes of cell surface receptors are ligand-

gated ion channels controlled by neurotransmitters, enzyme-linked receptors associated with protein kinases, and G-protein coupled receptors (GPCRs).[18] These are depicted in Figure 1.2.

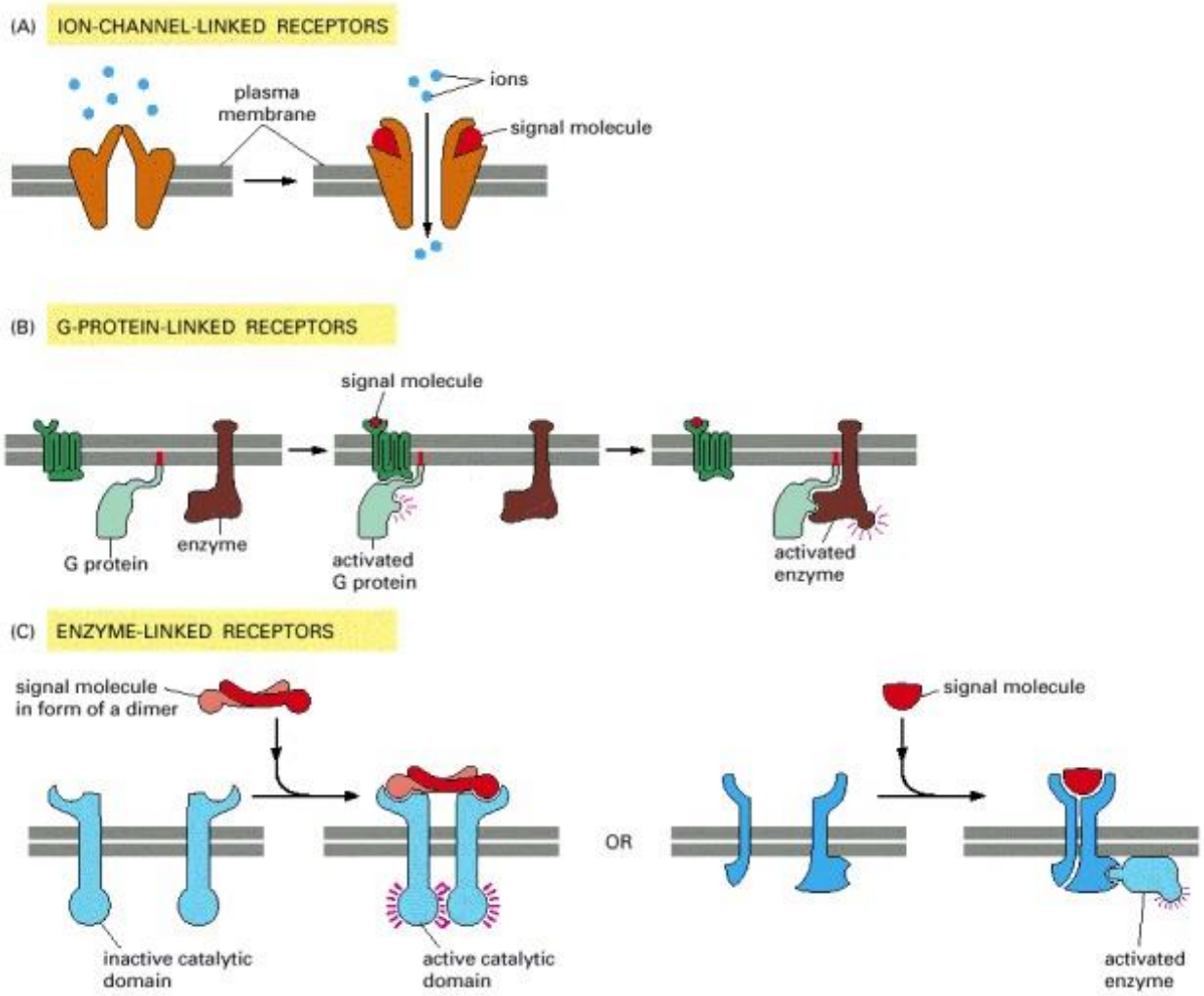

Figure 1.2. Major classes of cell surface receptors and intracellular pathways involved with them.[22] Figure copied from MOLECULAR BIOLOGY OF THE CELL, FOURTH EDITION by Bruce Alberts, et al. Copyright © 2002 by Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter. © 1983, 1989, 1994 by Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D. Watson. Used by permission of W. W. Norton & Company, Inc.

Several surface proteins have been identified as potential biomarkers in cancer and other complex diseases that may offer improved detection and sensitivity. For instance, overexpression of the glycoprotein epithelial cell adhesion molecule (EpCAM) has been observed in cancer-propagating cells in numerous solid tumors,[23] whereas a reduction in the number of T-cells expressing cluster of differentiation 4 (CD4) has been detected in acquired immunodeficiency syndrome (AIDS) patients.[24]

1.3. Research motivation

In the current era of proteomics, cell surface protein receptor identification and characterization have attained significant success, growing from conventional immunoblot analysis to high resolution MS.[25] MS ionizes the chemical species in a sample and allows for identification based on their mass to charge ratio.[26] A variety of ionization and analysis techniques including Electrospray Ionization (ESI), Matrix-assisted Laser Desorption/ionization (MALDI), Surface-enhanced Laser Desorption/ionization (SELDI), ion trap, quadrupole, and Time of Flight (TOF)-MS are available for both small scale research and high-throughput screenings.[27] Although MS is fast, sensitive, reproducible, and adaptable to clinical diagnostics, its use for biomarker applications requires the purification of target tissues or cells from heterogeneous samples prior to analysis. To fraction liquid samples such as body fluids, liquid chromatography (LC) can be coupled to MS, while laser capture microdissection (LCM) can be used to isolate specific cells from a microscopic region of a tissue sample prior to MS analysis.[14,25,28]

Flow cytometry is another technique widely used in protein biomarker studies. Here, cells are labeled with stains, dyes, or fluorescent markers prior to analysis to allow their examination based on fluorescence. Fluorescence activated cell sorting (FACS) coupled to flow cytometry provides the opportunity to sort only the cells of interest from a heterogeneous mixture. Upon containment of a labeled cell suspension in the center of a narrow and rapidly flowing stream, cells are distinguished by their specific light scattering and fluorescent characteristics upon laser excitation. A vibrating mechanism then breaks the stream into individual droplets, with one cell per droplet, and a positive or negative charge is added depending on the previous fluorescent characterization.

Differently charged droplets and cells are then sorted into different containers for further study.[29-31]

Although these techniques are effective in the early stages of cell surface biomarker discovery, different approaches are needed to develop tools and techniques that can be translated to clinical applications. For example, the number and pattern of expression of a particular receptor vary between patients or different stages of disease. Phenomena such as ligand binding, conformational changes, dimerization, internalization, and recycling as well as association with other cellular mechanisms may also be important distinguishing features. To develop efficient tools and techniques for successful "surface phenotyping," these spatial and temporal characteristics must also be assessed.[14,32]

Fluorescently-tagged antibodies designed against specific receptors have allowed the detection of the location, expression, and activity of receptors through a variety of microscopy techniques.[32] Common fluorophores include organic dyes, proteins (e.g., green fluorescent protein or GFP), organic or metal nanoparticles, and quantum dots. Although monoclonal antibody probes can be specific, they possess several caveats. While intact antibodies (~150 kDa) suffer from high background signal and nonspecific accumulation, antibody fragments may be taken up by tissues, which hinders the identification of target proteins, even those that are located exclusively on the cell surface.[33]

Engineered antibody mimetics consisting of only variable chains linked by small peptides (e.g., affibodies, antibody conjugates, and multivalent antibodies) can overcome these limitations. Additionally, thousands of chemical probes have been manufactured based on peptides or small molecules mimicking the receptors' native ligands and provide affinity, specificity, and cost effectiveness. Unlike biologically-produced antibodies, these types of reagents are amenable to structure modification and conjugation to detection molecules, making them ideal tools for studying cell surface receptors. Furthermore, cryogenic electron microscopy (cryo-EM) has recently been gaining popularity for ultra-structural analysis.[34] Despite of all these advances, there

is still a lack of integrated, robust, and cost-effective techniques to obtain "overall snapshots" of spatial and temporal events occurring *in situ*, a prerequisite for cell surface receptor research.

1.4. Research proposal

In order to bridge the research gap discussed above, we propose a novel technique to imprint cell surface receptors. Silver nanoparticles can serve as an immobilization platform for biomolecules, including thiol-bearing peptides that can be covalently linked to the metal surface.[35] The flat surface of silver (Ag) nanodiscs (AgNDs) provides a unique opportunity to capture a 2D-imprint of the cell surface. We have termed this technique "nanocamera" since it provides a snapshot of receptor distribution upon ligand immobilization on the AgNDs.

Figure 1.3 presents a schematic of the proposed approach. Upon saturation of cell surface receptors with a thiol-decorated peptidomimetic dendrimer of a native ligand, cells are washed to remove any unbound ligand. Cells are then incubated with AgNDs in order to immobilize the ligands to the discs. Upon detachment of the AgNDs from the cell surface, the characterization of nanodiscs and bound ligands by electron microscopy using gold (Au) nanoparticles (AuNPs) can provide information on the number and distribution of imprinted receptors, thus allowing for discrimination between different protein expression profiles. Furthermore, this technique can be utilized to study multiple receptors if ligands are synthesized as multivalent scaffolds (i.e., two or more ligands joined by a linker). This knowledge is critical for the development of highly avid and selective cell targeting strategies through the precise phenotyping of the cell surface.

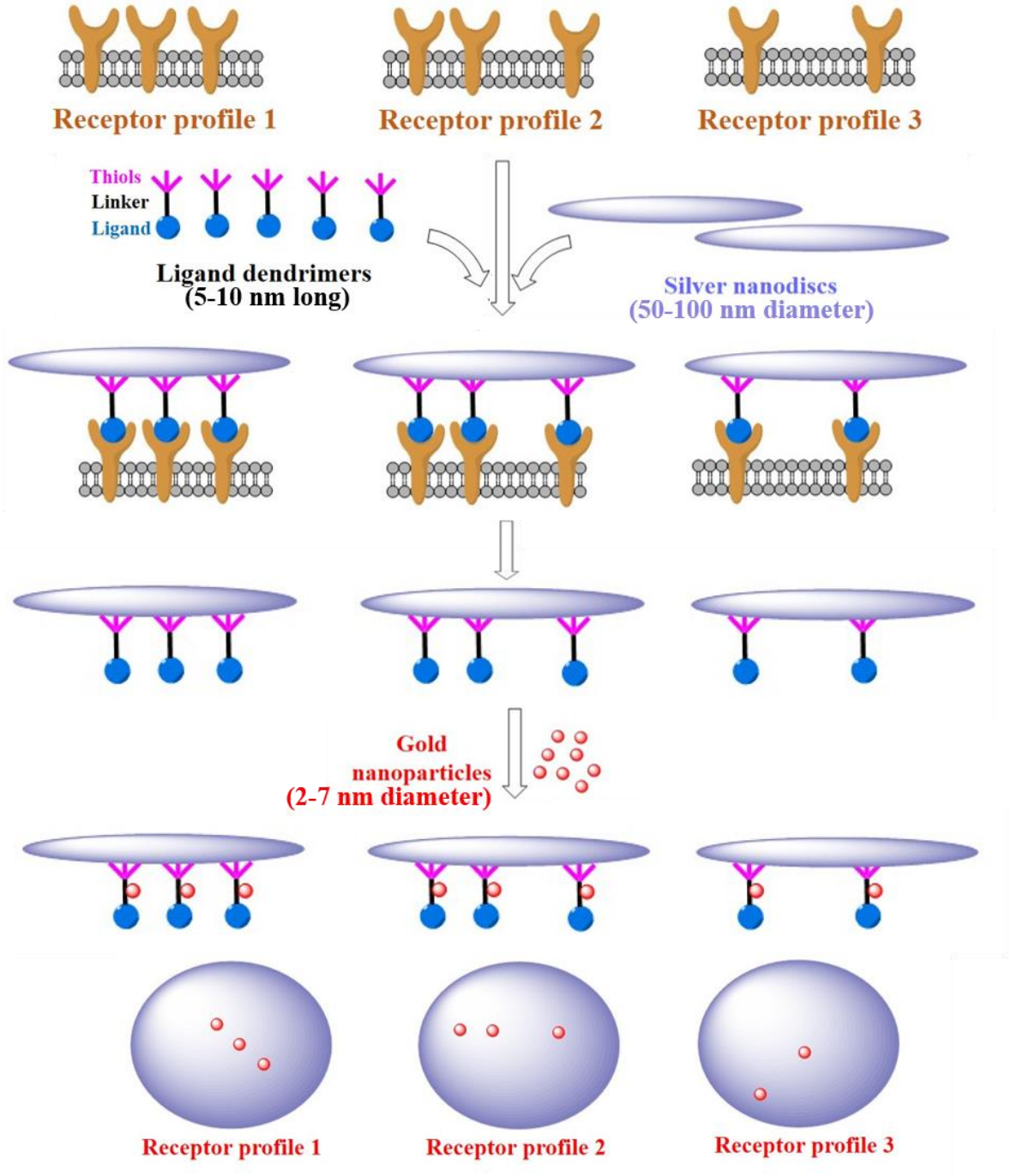

Figure 1.3. Schematic overview of *in-situ* nanoimprinting of cell surface receptors.

1.5. Research strategy

In order to establish the proposed "nanocamera" technology, we first aimed to test and develop a platform for a single protein system. We chose melanocortin receptor 1 (MC1R) as our model system owing to the well-established history of physiological function and ligand development for this protein, which includes multiple peptidomimetic and small-molecule agonists and antagonists of low and high-affinity.[36,37]

Melanocortin receptor 1 (MC1R) is a GPCR generally expressed in melanocytes and associated with pigmentation of hair, eyes, and skin. A relationship between loss-of-function of MC1R variants and risk for melanoma and non-melanoma skin cancers has been proposed.[38] Immunohistochemical staining demonstrates that MC1R expression levels in melanoma are much higher than in non-diseased tissues, suggesting that this receptor could serve as a useful biomarker for melanoma diagnostics and therapeutics.[39]

One of the native agonists for the MCR family is α-Melanocyte-stimulating hormone (α-MSH). This is a tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) secreted from pituitary gland that binds non-selectively to all MCRs with varying affinities.[40] A more potent analog of α-MSH that is also more resistant to proteolysis was developed by substituting the native amino acids at positions 4 (Met) and 7 (Phe) with norleucine (Nle) and D-phenylalanine (D-Phe) respectively; known as [Nle^4, D-Phe^7]-α-MSH or NDP-α-MSH.[41] MSH(7) is a truncated heptapeptide (Ac-Ser-Nle-Glu-His-DPhe-Arg-Trp-NH_2) of NDP-α-MSH that, because of its shorter length compared to the original tridecapeptide, is more amenable for further modifications at the N-terminus to generate bivalent ligands for MC4R.[36]

Our group has designed a convenient route to synthesize a MSH(7) analog that would allow for crosslinking to AgNDs. This molecule, which we named MSH(7)-thio-dendrimer, consists of the MSH(7) ligand with branched thiols spaced by amino acid linkers at the N-terminus shown in figure 1.4. We also designed a series of experimental approaches to test and develop a basic working model for nanoimprinting.

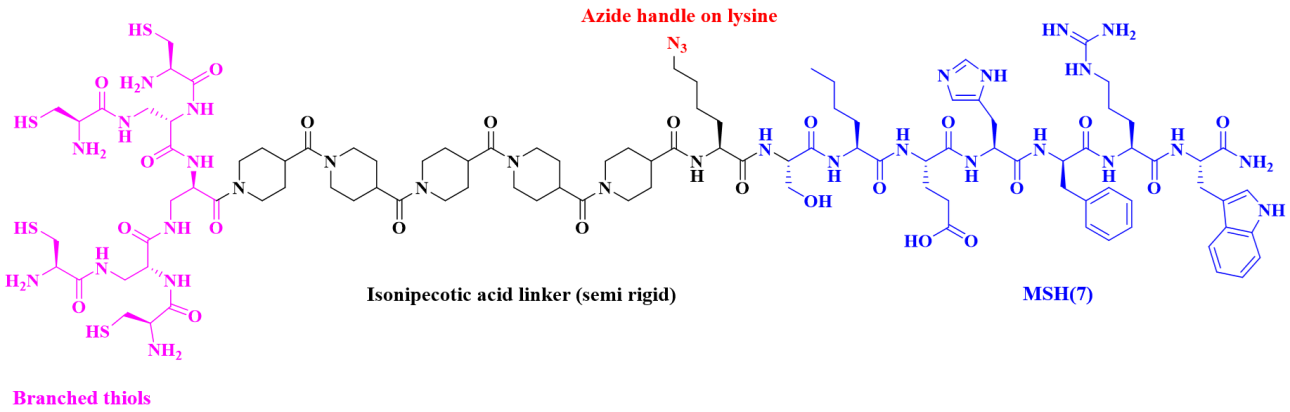

Figure 1.4. Chemical structure of the MSH(7)-thio-dendrimer.

1.5.1. Identification of melanoma cell lines that express MC1R protein on the cell surface

In order to validate our putative target, MC1R, in melanoma cells, an immunocytochemical assay was performed. We examined three melanoma cell lines, WM-266-4, WM-115, and A-375, which were expected to exhibit various levels of MC1R expression, and PC-3, a metastatic prostate cancer cell line, which should have little or no MC1R expression.

1.5.2. Quantification of surface expression levels of MC1R in melanoma cell lines

We determined the level of MC1R surface expression in the selected melanoma cell line. This was necessary to correlate the nanoimprinted ligand density with the receptor density present on the cell lines. Using solid phase peptide synthesis (SPPS), we prepared a highly avid peptide analog of NDP-α-MSH, which is coupled to diethylenetriaminepentaacetic acid (DTPA) and tagged with Eu(III) at the N-terminus (termed Eu-DTPA-NDP-α-MSH) for this purpose.

To determine the level of expression of MC1R (number of receptor copies/cell), we conducted a ligand-based saturation assay.[37] The peptide was incubated with the cells in a 96 well plate format and fluorescence count in maximum binding of the ligand (B_{max}) was determined using a time-

resolved Dissociation Enhanced Lanthanide Fluorescence Immunoassay (DELFIA).[42] In turn, the number of cells per well was determined using a hemocytometer.[37]

1.5.3. Assessment of binding of the MSH(7)-thio-dendrimer

We designed a dendrimeric molecule based on MSH(7) able to bind with MC1R and crosslink to AgNDs. In this system, the N-terminus of MSH(7) was coupled to a dendrimeric region decorated with thiol and amine groups that allow for binding to Ag. A ~25 Å-long semi-rigid linker, consisting of a series of isonipecotic acid (Inp) units, a non-natural amino acid, was incorporated to provide spacing between the ligand and the dendrimer. Finally, a lysine functionalized with an azide group, Lys(N_3), was coupled to the N-terminal of MSH(7) to allow for binding with AuNPs and detection by transmission electron microscopy (TEM).

The peptide was synthesized by solid phase peptide synthesis (SPPS). Binding experiments was performed using DELFIA to determine the dissociation constant (K_d) using europium-labeled Eu-DTPA-NDP-α-MSH as reference.[37]

1.5.4. Crosslinking of MC1R−bound dendrimeric ligands to AgNDs

Incubation of fixed or live cells with the thiol-dendrimer will lead to binding to the expressed MC1R and, after removal of unbound ligand, AgNDs will be added in order to form Ag-S and Ag-N coordinate covalent bonds with the bound ligands. The resulting nanodiscs, which are now "imprinted" with ligands, will be detached from the cell surface using NaCl/acetic acid or urea/tris treatment. Conditions will be optimized to ensure minimal ligand and AgNDs internalization (in the case of live cells), and minimal non-specific binding, along with maximal detachment after immobilization.

1.5.5. Characterization of MC1R dendrimer ligand bound AgNDs by TEM

The MSH(7)-dendrimer bears an azide tag on the linker region that can be used for labeling with gold nanoparticles using click chemistry. Once the dendrimer ligandhas been imprinted into the AgNDs and removed from the cells, we will incubate with cyclooctyne-decorated AuNPs of 2-7 nm (i.e., 7 nm is similar in dimension to a typical GPCR). Since gold provides exquisite contrast for electron microscopy, we will be able to determine ligand distribution through the AgNDs, which will provide fundamental information on receptor distribution (e.g. dimers vs. homogeneous distribution).

A potential caveat is that the thiol-bound ligand can diffuse across the AgNDs. The dendrimer bearing four thiol and amine groups was designed to address this potential issue through the presence of multiple binding points to the AgNP. Nevertheless, we will verify the presence or lack of this crosswalk by probing the same area of the disc at different time points.

1.6. Scope of current study

We have divided the entire investigation scheme into smaller projects to be completed in the available time period. As the beginning of the study, we have completed the synthesis of both europium-labeled peptide, Eu-DTPA-NDP-α-MSH, and dendrimeric heptapeptide, MSH(7)-dendrimer. We have performed chemical characterization of the peptides by high performance liquid chromatography (HPLC) and time of flight-mass spectrometry (TOF-MS) (section 2.3.1). We tested different cell lines for MC1R expression (section 2.3.2) and selected WM-266-4 melanoma cell line for binding studies. Furthermore, we determined the binding affinity of MSH(7)-thio-dendrimer to WM-266-4 (section 2.3.3). All of these results are described in Chapter 2, which circumferences the scope of this thesis. We are currently procuring AgNDs and AuNPs for the next set of experiments. The novelty of this technique and possible future applications are discussed in Chapter 3.

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2. SYNTHESIS AND CHARACTERIZATION OF LANTHANIDE-LABELED AND THIO-DENDRIMERIC ANALOG OF [Nle^4, D-Phe^7]-α-MSH TO TARGET HUMAN MELANOCORTIN RECEPTORS

Sadia Ahmed, José A. Rodríguez-Corrales, Donald H. Clark, Jatinder S. Josan

Attributions

This chapter was written in the form of a manuscript yet to be submitted. The first author, Sadia Ahmed, synthesized and purified peptide **1**, conducted cell experiments, organized data, and wrote the manuscript. Dr. José A. Rodríguez-Corrales, former graduate researcher in the Josan group, synthesized and purified peptide **2**, assisted in planning all experiments, and edited the manuscript. Donald H. Clark, an undergraduate researcher, synthesized and purified peptide **3**. Dr. Jatinder S. Josan, principal investigator, supervised the study, gave feedback, and provided technical and financial support. Individuals who assisted with this project but are not listed among the authors are Dr. Brenda S.J. Winkel, who contributed intellectually during the project and assisted in manuscript editing; Robert J. Grams, former graduate student in the Josan group, who helped during synthesis and purification of peptides **1** and **3**; and Dr. Mehdi Ashraf-Khorasani, research scientist in the Department of Chemistry, who performed the characterization of purified peptides by HPLC and TOF-MS.

2.1. Abstract

Melanocortin receptors (MCRs) comprise a family of G-protein coupled receptors (GPCRs) that are associated with a wide range of physiological functions. The five MCR subtypes, MC1R-MC5R, exhibit distinct expression and distribution patterns across tissues and cell types, including in disease states such as cancer, making these receptors ideal targets for diagnostics and therapeutics. The native peptide ligand for MCRs, α-melanocyte stimulating hormone (α-MSH), has been used to develop a variety of tools for probing MCR expression, localization, and function. These include [Nle^4, D-Phe^7]-α-MSH (NDP-α-MSH), a well-studied analog that demonstrates higher affinity, potency, and stability in biological systems. In this study, we synthesized and characterized a europium-labeled analog of NDP-α-MSH, which we called Eu-DTPA-NDP-α-MSH, in order to determine the level of MC1R expression in the WM-266-4 cell line using dissociation enhanced lanthanide fluorescence immunoassay (DELFIA). Binding analysis showed the dissociation constant, (K_d) of Eu-DTPA-NDP-α-MSH to MC1R as 9.4±5.7 nM. Furthermore, we synthesized and characterized a thio-dendrimeric analog of NDP-α-MSH which offers substantial potential as a multifunctional ligand to target MC1R as well as other melanocortin receptors. Its binding to MC1R in WM-266-4 cells in terms of 50% effective concentration (EC_{50}) relative to Eu-DTPA-NDP-α-MSH was determined to be 0.68 μM. Both of these peptides, especially the novel thio-dendrimeric peptide, can be employed in different techniques and serve as a multifunctional molecule to detect and characterize the expression of melanocortin receptor.

2.2. Introduction

The melanocortin receptor (MCR) family, a member of the class A (rhodopsin-like) family of G-protein coupled receptors (GPCRs), consists of five subtypes with varying tissue expression and functions.[1] Melanocortin receptor 1 (MC1R) is typically expressed in melanocytes and is associated with pigmentation of hair, eyes, and skin.[2] Loss of function in MC1R variants is associated with a risk of developing melanoma and non-melanoma skin cancers.[3] MC2R is expressed in adrenal cortex, skin, and adipose tissue and plays a role in anti-inflammation and the circadian regulation of glucose metabolism.[4,5] MC3R is expressed in the nervous system, placenta,

and gut. In turn, MC4R, being expressed in brain and nervous system, has been associated with eating behavior, metabolism, obesity, eating disorders such as anorexia and cachexia, memory and cognitive disorders such as Alzheimer's disease, sexual behavior, and erectile function in males.[6-8] Finally, MC5R is expressed in brain, muscles, lungs, and kidneys, and is necessary for sebum production, fatty acid oxidation in muscles, erythrocyte differentiation, thermal homeostasis, and inflammation.[9,10] As a result, MCRs are of considerable interest as potential targets for the detection and treatment of a wide variety of diseases.

The ligands for MCRs, melanocortins, are of two types, agonists and antagonists. Agonists are melanocyte stimulating hormone (MSH), (consisting of α-, β-, and γ- subtypes), and adrenocorticotropic hormone (ACTH). These molecules are generated from different cleavage mechanisms of the proopiomelanocortin (POMC) precursor polypeptide and, therefore, share similar sequences.[11] MC2R binds ACTH exclusively, whereas both ACTH and MSHs are recognized by other MCRs (MC1R, MC3R-MC5R), albeit showing higher affinity for MSHs.[11] Among the three MSH subtypes, α-MSH is the major activator, or agonist, for the MCRs. Upon binding to the receptor, MSH gets internalized, transduces the signal by conformational change of the receptor and a G-protein associated signaling cascade is initiated.[2] In contrast, antagonists consist of agouti signaling protein (ASIP) and agouti related protein (AGRP), which turn off this pathway.[12]

α-MSH is a non-selective melanocortin tridecapeptide, with the sequence Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. It displays the highest potency towards MC1R (inhibition constant, K_i, of 0.12 nM), with weaker binding to MC3R , MC4R, and MC5R (K_i of 31, 660, and 5700 nM, respectively), and no detectable binding to MC2R in radiolabeled ligand based assays.[11] A more potent analog of α-MSH was obtained by substitution of the native amino acids located in positions 4 (Met) and 7 (Phe) for norleucine (Nle) and D-phenylalanine (D-Phe) respectively. The resulting peptide, known as [Nle^4, D-Phe^7]-α-MSH or NDP-α-MSH, displays 1000-fold higher potency and enhanced stability to proteolysis.[13] Another analog, MSH(7), is a truncated heptapeptide (Ac-Ser-Nle-Glu-His-DPhe-Arg-Trp-NH_2) of NDP-α-MS that binds with

MC4R and is amenable for further modifications at the N-terminus due to shorter length than the native tridecapeptide.[14]

Structure-activity relationship (SAR) studies showed that all melanocortin agonists bind to the transmembrane region of the MCRs in a β-turn conformation through the His^{6}-(L/D)Phe^{7}-Arg^{8}-Trp^{9} motif. Thus, it serves as "message sequence" for sufficient receptor binding and signaling,[15,16] which has been conserved in a myriad of synthetic ligands with a wide range of affinities. These ligands have been derivatized by modifying side chains,[17] adding bioactive rigid or flexible linkers,[18,19] or conjugating radioisotopes or lanthanides,[20,21] organic fluorophores or fluorescent protein tags,[22] photoactivatable nanoparticles,[23] or quantum dots[24]. These modified ligands retain substantial binding affinity for the MCRs, illustrating the feasibility of constructing homo-multivalent or hetero-multivalent scaffolds,[25,26] polymers,[27] branched dendrimers, micelles,[28] and liposomes[29] based on NDP-α-MSH.

In this study, we synthesized and characterized two novel peptide derivatives of NDP-α-MSH using solid phase peptide synthesis (SPPS), with the aim of targeting MC1R expressed in melanoma cells. A europium-labeled diethyltriaminepentaacetic acid (DTPA)-coupled NDP-α-MSH, hereforth peptide **1** (see Figure 2.1), was developed as an enhanced probe for the detection and quantification of MC1R in biological systems. Although most organic fluorophores absorb and emit energy within 10 ns, lanthanides (e.g., europium, terbium, and samarium) in a singlet excited state can undergo intersystem crossing and emits from a metastable triplet state. Since photon emission is delayed significantly, the background signal is reduced and the signal-to-noise ratio is improved, thus making Time-Resolved Fluorescence (TRF) a highly sensitive technique.[30]

We designed our binding experiments by adapting a previously-reported method involving the use of a DELFIA.[31] This method has been used for quantification of melanocortin 4 (MC4R), delta opioid (δOR), and cholecystokinin (CCK) receptors with europium-tagged ligands.[32-34] Here, we extended the method to determine MC1R expression levels in the melanoma cell line WM266-4, where we validated MC1R expression by immunocytochemistry. We conducted a saturation

binding assay using labeled Eu-DTPA-NDP-α-MSH (peptide **1**) and nonlabeled NDP-α-MSH (peptide **2**, see Figure 2.1) to determine total, nonspecific, and specific binding for peptide **1**.

Furthermore, we designed and synthesized a dendrimeric form of MSH(7), peptide **3** (see Figure 2.1) to be used as a multifunctional ligand for studying MCR expression. In peptide **3**, lysine functionalized with an azide group in the ε-position or Lys(N_3), was coupled to the N-terminal of MSH(7) followed by a semi rigid linker containing five units of isonipecotic acid (Inp), a non-natural heterocyclic amino acid. A generation 2 (G2) dendrimer was assembled on the N-terminus by coupling of two subsequent units of 2,3-diaminopropionic acid (Dap) as branching unit, and cysteine in the four terminal positions, thus completing the synthesis of peptide **3**. We evaluated binding affinity (EC_{50}) of the peptide towards MC1R natively expressed in the melanoma cell line WM-266-4 by a competitive binding experiment, using peptide **1** as the competitor ligand. We determined the half maximal effective concentration or EC_{50} value for peptide **3** as 0.68 μM.

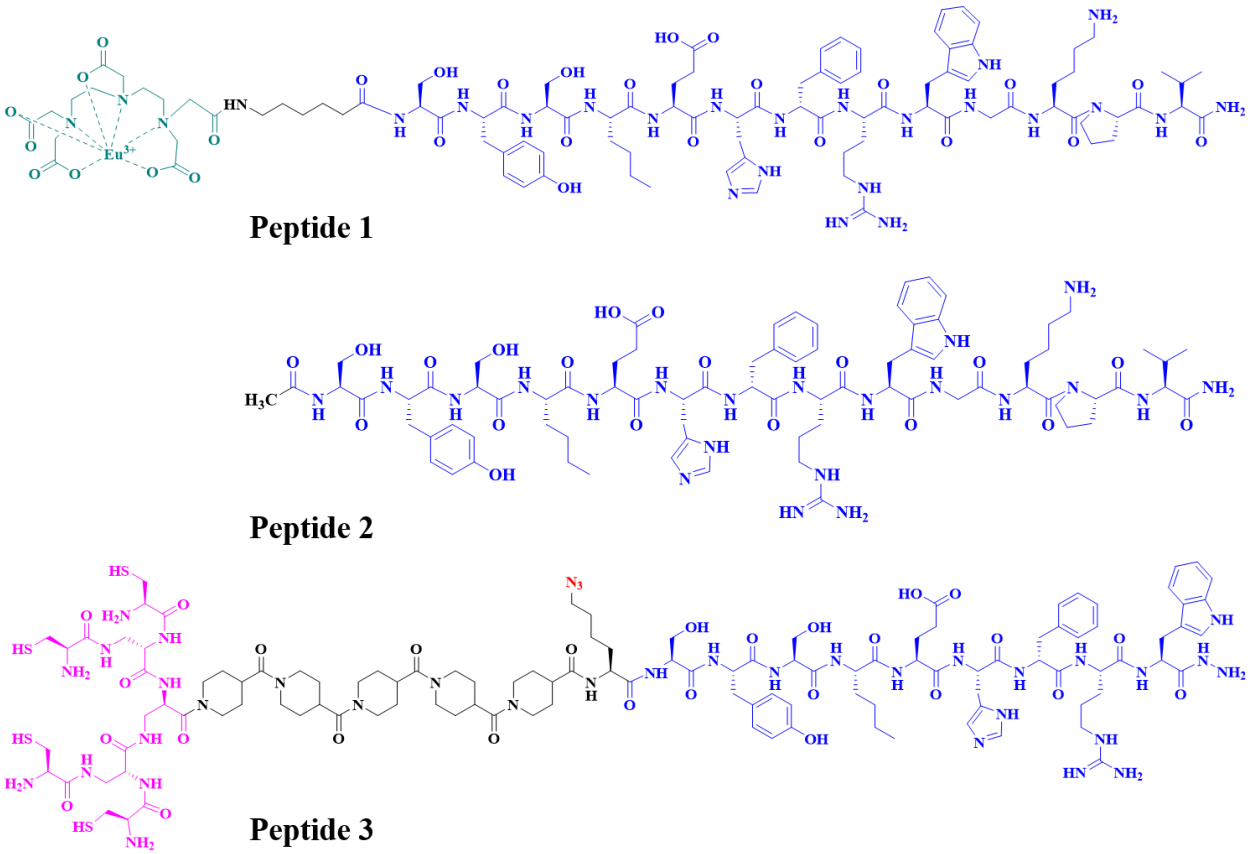


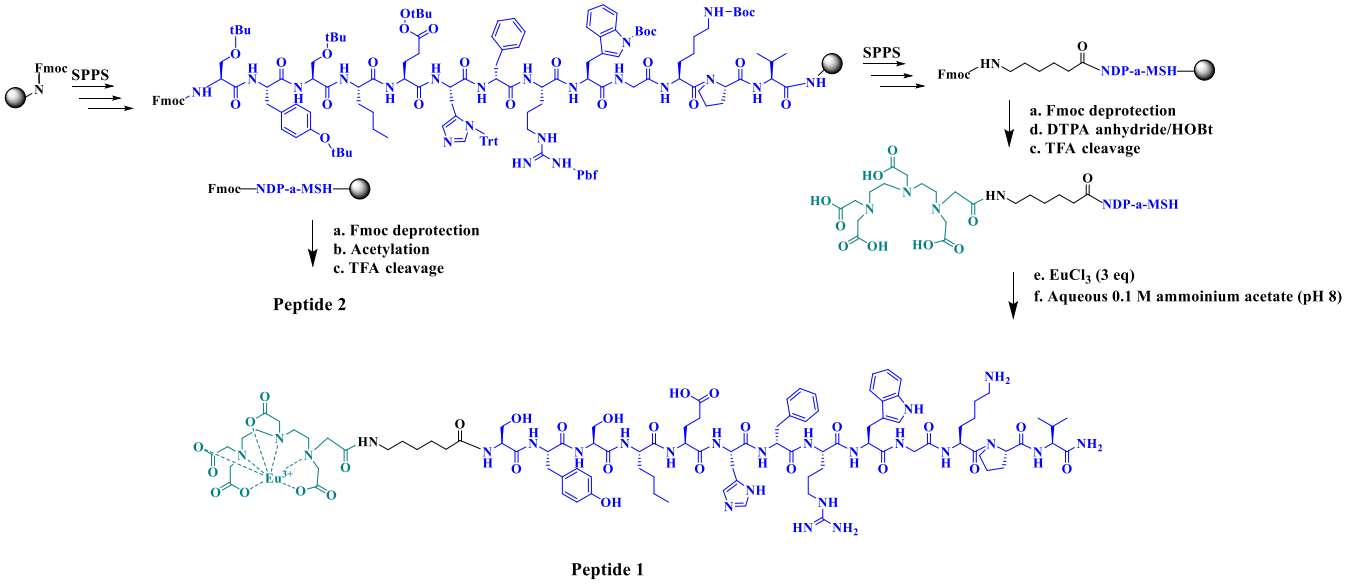
Figure 2.1. Chemical structures of Eu-DTPA-NDP-α-MSH (peptide **1**), Ac-NDP-α-MSH (peptide **2**), and MSH(7)-thio-dendrimer (peptide **3**).

2.3. Results and discussion

2.3.1. Peptide synthesis

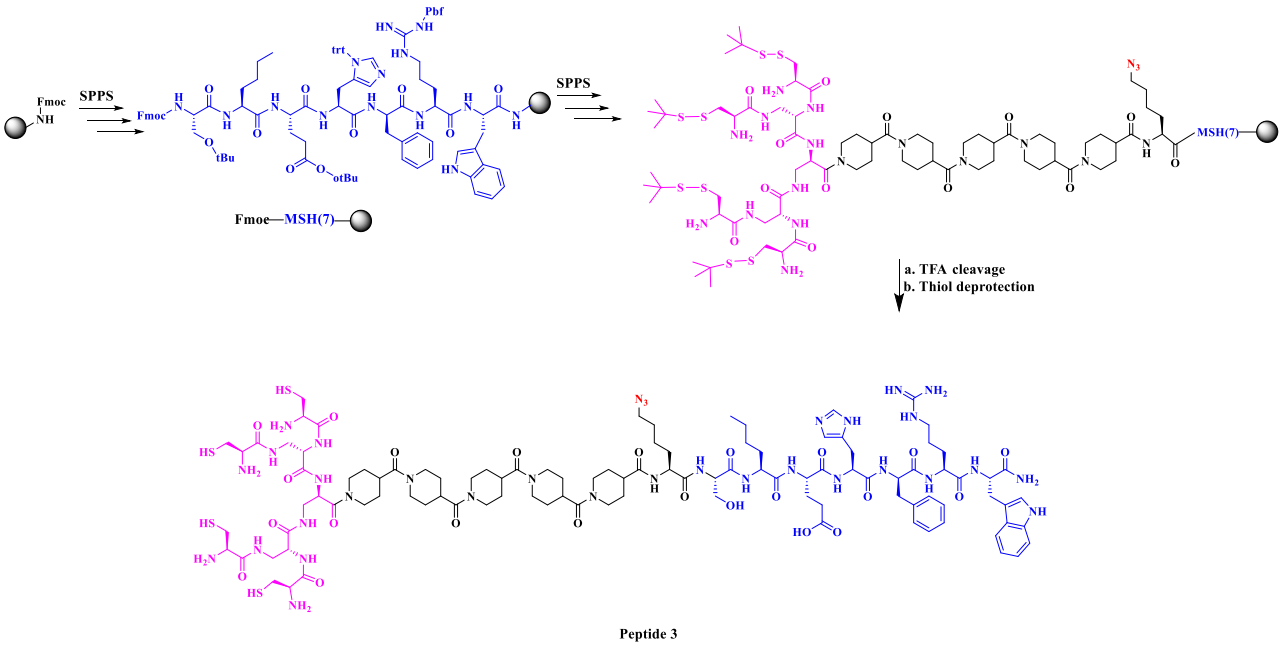
Peptide **1**, **2** and **3** were successfully synthesized using solid phase peptide synthesis (SPPS) following the routes shown in Schemes 1 and 2. Detailed description of the procedures are presented in the Experimental Section (section 2.5.1). In brief, SPPS was performed in Tentagel S RAM (Rink Amide) resin (loading: 0.24 mmol/g) using N-α-Fmoc protecting groups and *N*,*N*′-diisopropylcarbodiimide (DIC)/OxymaPure or *O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU)/ *N*,*N*′-diisopropylethylamine (DIEA) activation. Presence of unreacted amine on the resin was checked by the Kaiser test and capping of unreacted amine was performed by using excess acetic anhydride:pyridine 1:1 for 1 min. The

procedure described above was repeated until all amino acids were coupled. The peptide was cleaved from the resin using Trifluoroacetic acid (TFA) mixture followed by evaporation, precipitation, centrifugation, and washing in ice-cold diethyl ether. Finally, drying of the products yielded white to yellow solid powders that were stored at -20 °C. Peptide purity and identity were checked after coupling every three amino acids and after completing the peptide sequence by HPLC-Ultraviolet (UV)/Visible (Vis)-MS or HPLC-UV/Vis-MS spectrometry.



Scheme 2.1: Synthesis of peptides **1** and **2**. Conditions for different reaction steps are provided in Section 2.5.1. **a.** 20% piperidine in DMF for (2+18) min. **b.** Acetic anhydride:pyridine 1:1, 1 min. **c.** TFA (90%), water (2.5%), triisopropylsilane (2.5%), thioanisole (2.5%), and ethanedithiol (2.5%) for 3 h. **d.** DTPA dianhydride (20 equivalents, final concentration 0.6 M) and HOBt (40 equivalents), dissolved in dry DMSO by heating at 50°C, followed by stirring at room temperature for another 20 min. Mixing resin with dissolved mixture and microwave at 60°C for 30 min (2×). **e,f.** Dissolving purified peptide in 0.1 M aqueous ammonium acetate solution (pH 8.0), adding europium(III) chloride (3 equivalents), stirring at room temperature until complete chelation is confirmed by HPLC-MS.

Peptide **2** was acetylated using acetic anhydride:pyridine before cleavage from the resin. Peptide **1** was synthesized following a modification of a previously-reported protocol,[31] except that coupling was performed under microwave irradiation. We observed that the reaction of activated DTPA mixture for two cycles of 30 min at 60 ^{o}C in the microwave generated optimum coupling. Further cycles promoted formation of DTPA dipeptide as mentioned in the protocol.[31] High concentration and excess amount of DTPA dianhydride (20 eq) and HOBt (40 eq) were used to decrease the extent of side reactions. DTPA-coupled compound was purified by preparative HPLC and analyzed by analytical HPLC-UV/Vis-MS. Europium(III) was chelated using europium chloride ($EuCl_3$) in aqueous ammonium acetate followed by solid phase extraction (SPE) with a C-18 Sep-Pak® cartridge.[35] The protocol followed reported that Eu-DTPA chelation reaction should be fast (~4 h), but can take up to 48 h depending on the peptide sequence, and therefore recommended to confirm chelation by HPLC-MS.[31] In our case, most of the peptide was seen to be chelated in 1 h, while increasing reaction time did not improve product yield or purity. In the case of peptide **3**, the thiols in the cysteine branches were deprotected by reaction with dithiothreitol (DTT) after purification of the peptide (Scheme 2.2).



Scheme 2.2. Synthesis scheme of peptide **3.** Conditions for different reaction steps are provided in Section 2.5.1. **a.** TFA (90%), water (2.5%), triisopropylsilane (2.5%), thioanisole (2.5%), and ethanedithiol (2.5%) for 3 h. **b.** Dissolve peptide in 100 mM aqueous $NaHCO_3$ (1 mg/mL), stir with 10 mM dithiothreitol (DTT) for 3 h.

All peptides were characterized by UV/Vis (Figure 2.2) and time of flight mass spectrometry (TOF-MS) (ESI+) (Figure 2.3). To prevent dechelation of europium from peptide **1** due to change in pH during HPLC analysis, 0.1% TEA/acetic acid buffer (pH=6.0) was added to water to prepare mobile phase A. Mobile phase A was added to acetonitrile (final concentration of solvent A was 10%) and pH was adjusted to 6.0 by adding acetic acid) to prepare mobile phase B.[36] Peptide chemical identity was confirmed by comparison of the calculated charge to mass (m/z) ratio of the protonated forms of the peptide and the measured m/z for each peak in the chromatogram. Purity of the peptides was calculated by using absorption at 280 nm of a sample co-injected with aqueous 0.5 mM H-Trp-OH as standard. All compounds were found to be more than 95% pure. Isotopic distribution of major mass peaks, as shown in Figure 2.4.A-C, for peptides **1**, **2** and **3**, also confirmed that the most abundant isotope of the molecule or the monoisotopic peaks are very close to their calculated mass. LC and MS data of the peptides are summarized in Table 2.1.

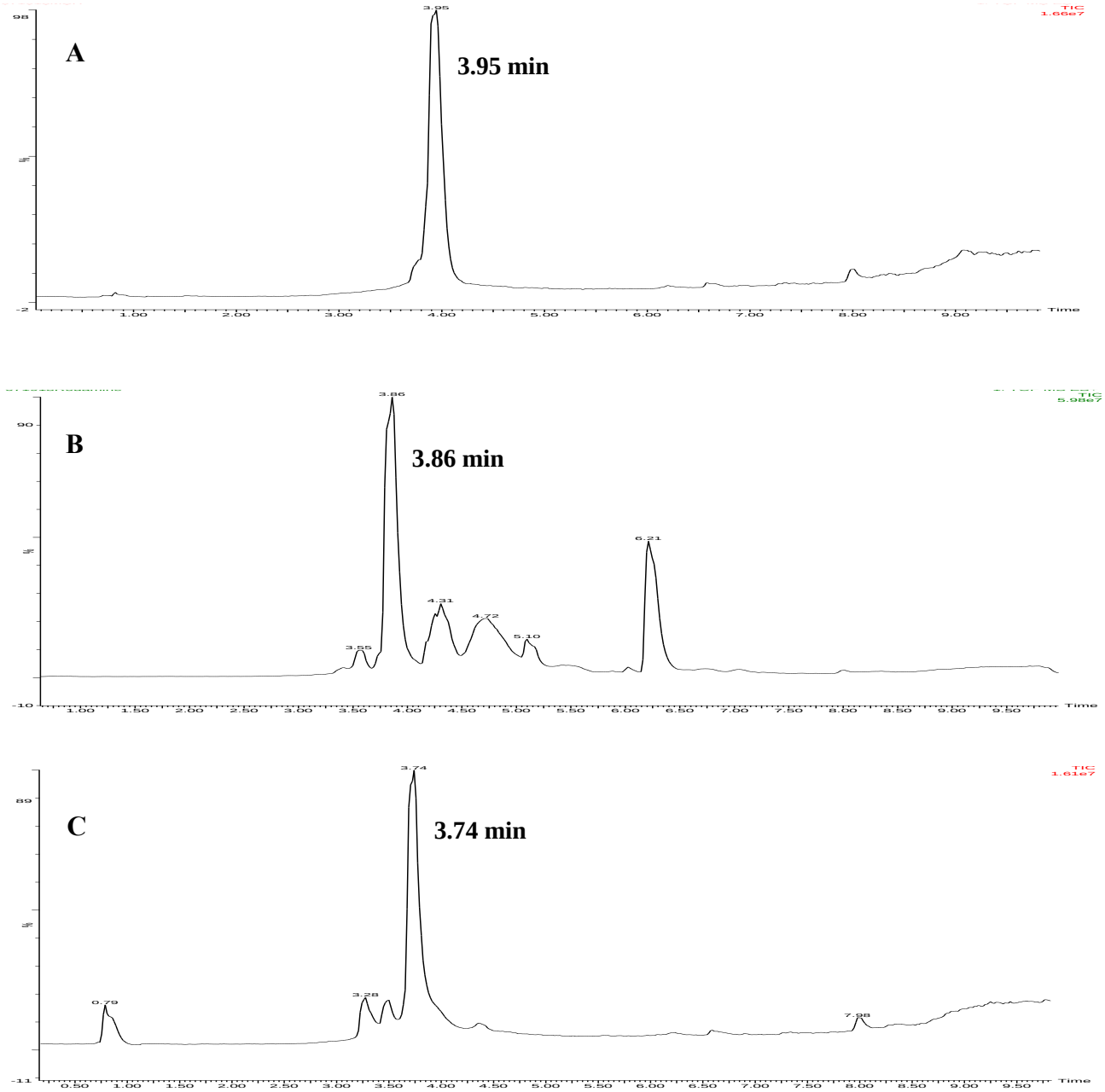


Figure 2.2. HPLC chromatogram of peptides 1 (A), 2 (B) and 3 (C) within retention times of 0 and 10 min. Retention times for peptides **1**, **2** and **3** were 3.95 min, 3.86 min and 3.74 min respectively. X-axis of the chromatograms indicates time (min) and Y-axis indicates relative intensity (%).

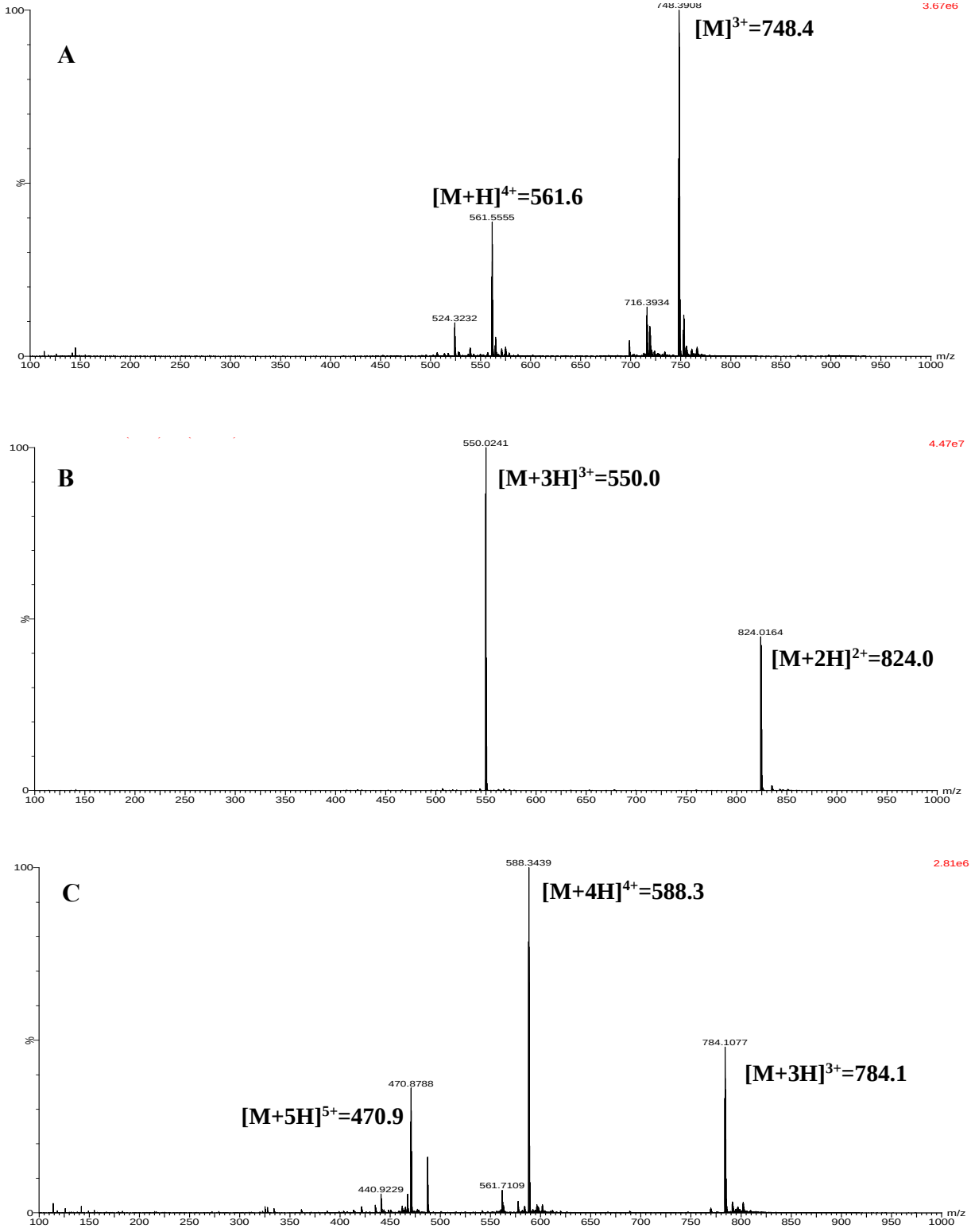
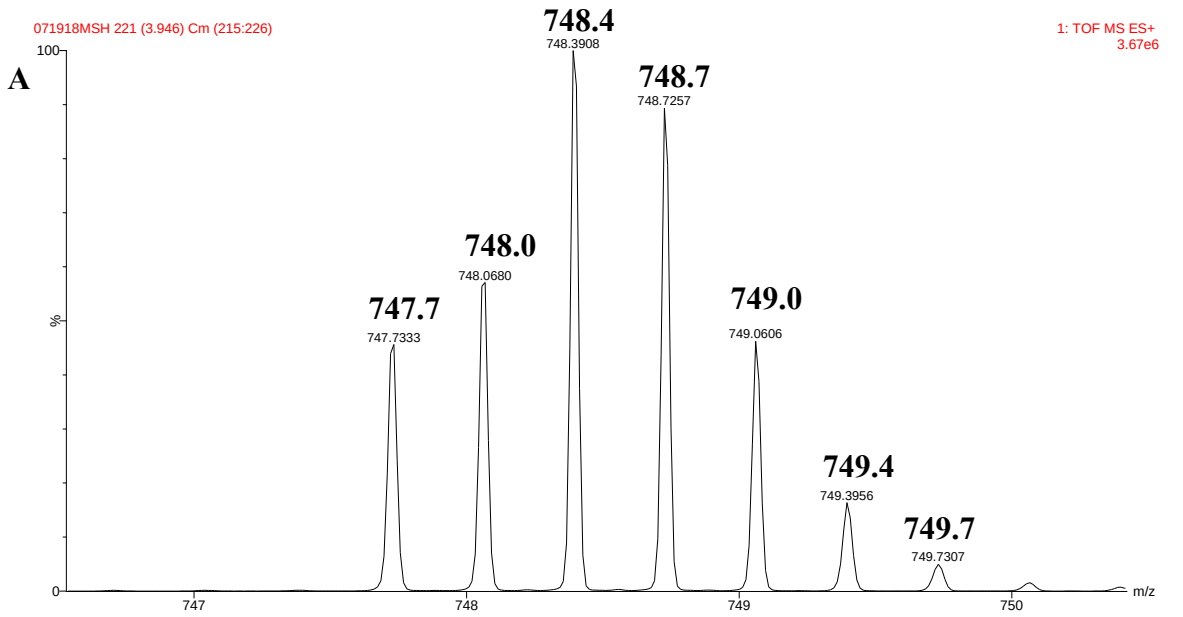


Figure 2.3. ESI+ mass spectra of peptides **1** (A), **2** (B), and **3** (C), in the 100-1,000 m/z range.


A
071918MSH 221 (3.946) Cm (215:226)
1: TOF MS ES+
3.67e6
100
%
0
747.7
747.7333
748.0
748.0680
748.4
748.3908
748.7
748.7257
749.0
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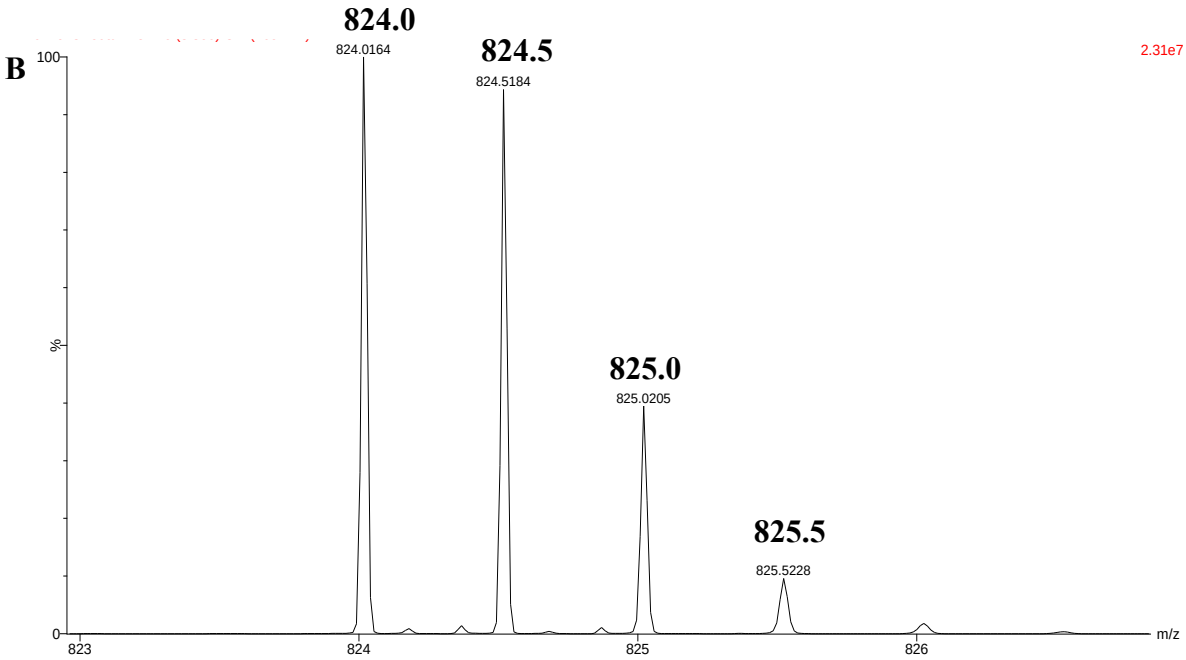
749.4
749.3956
749.7
749.7307
747
748
749
750
m/z


B
2.31e7
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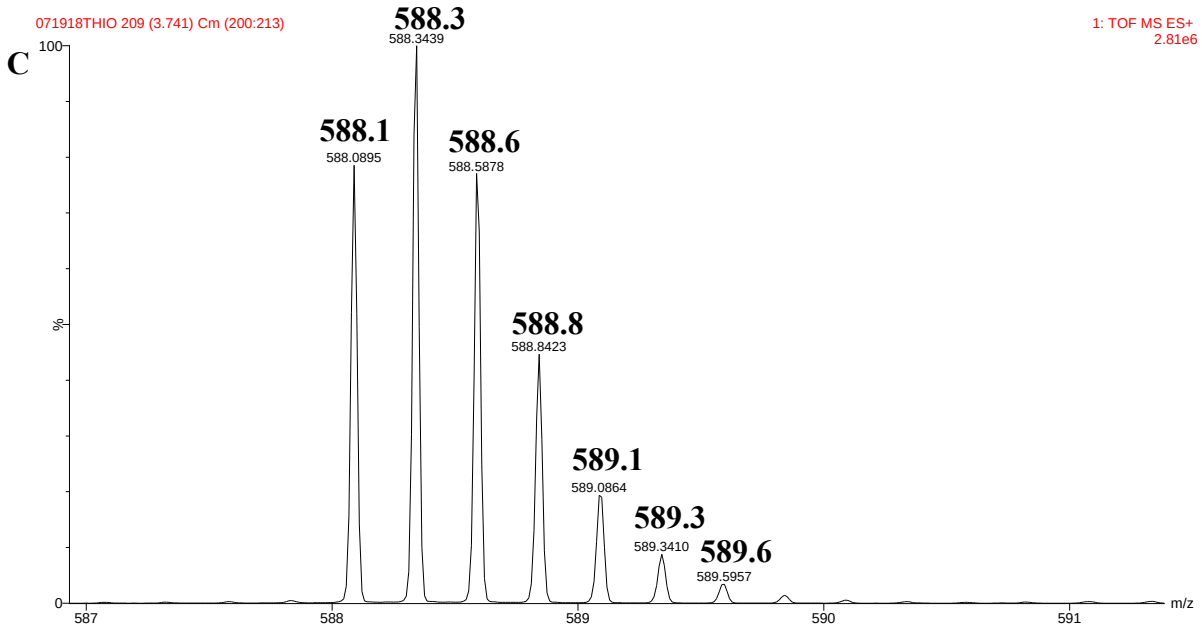

Figure 2.4. Isotopic distribution of major mass peak from mass spectrum using TOF-MS, for m/z peak of 748.4 for peptide **1** (A), 824.0 for peptide **2** (B), and 588.3 for peptide **3** (C). X-axis of the mass spectra indicates m/z and Y-axis indicates relative abundance (%).

Table 2.1. LC and MS data for peptide **1**, **2** and **3**

Peptide	Retention time (min)	Calculated m/z	Measured m/z from monoisotopic peak
1	3.95	$[M]^{3+} = 747.3$	$[M]^{3+} = 747.7$
2	3.86	$[M+2H]^{2+} = 823.9$	$[M+2H]^{2+} = 824.0$
3	3.74	$[M+4H]^{4+} = 589.3$	$[M+4H]^{4+} = 588.1$

2.3.2. Identification of cell lines expressing melanocortin receptor 1 (MC1R)

In order to identify cell lines that potentially expressed sufficient MC1R for further studies, we conducted immunocytochemistry (ICC) assays using three melanoma cell lines, WM-266-4, WM-115, and A-375 and a metastatic prostate tumor cell line, PC-3, as control. All of the cell lines tested herein were of epithelial origin. Anti-human MC1R polyclonal primary antibody raised in

rabbit, and anti-rabbit IgG goat secondary antibody coupled to the fluorophore DyLight 550 were used in the experiment. Cells were fixed but not permeabilized.

In a previous study, human and mouse melanoma cell lines exhibited MC1R expression at a level that was detectable in binding assays using radiolabeled [^{125}I]-NDP-α-MSH.[37] In our experiments, all three melanoma cell lines, WM-266-4, WM-115, and A-375, showed high levels of MC1R expression, as presented in Figure 2.5. PC-3, a cell line derived from a metastasized prostate tumor in bone, exhibited no detectable MC1R signal, providing evidence for the specificity of the primary antibody. Cells treated with only secondary antibody showed no detectable fluorescence and served as negative controls. We therefore selected WM-266-4 for use in peptide binding assays because of a history of use in our laboratory.

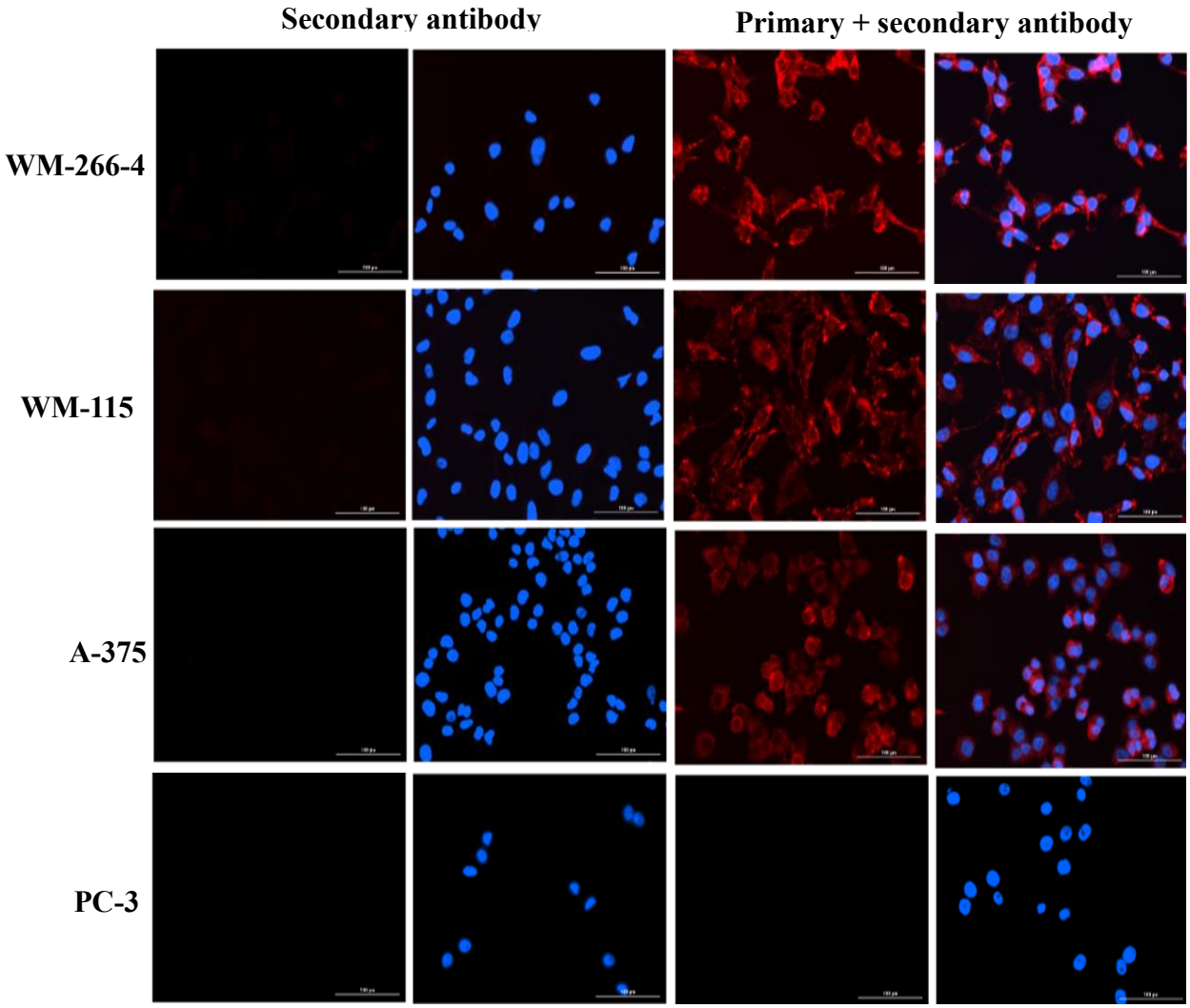

Figure 2.5. MC1R expression in melanoma cell lines (A-375, WM-115, and WM-266-4) and prostate cancer cell line PC3 as control. Fluorescence from DyLight 550-coupled secondary antibody (red) was detected in the RFP channel of a Cytation3 multimode plate reader. Hoechst 33342 was used to stain the nucleus (blue) and was detected in the DAPI channel. Samples (columns 3 and 4) were treated with primary and secondary antibodies and Hoechst 33342. Controls (columns 1 and 2) were treated with secondary antibody and Hoechst 33342 only and showed no detectable red fluorescence. Columns 1 and 3 correspond to RFP channel only, whereas 2 and 4 display the overlay of RFP and DAPI channels. Size bar = 100 μm.

2.3.3. Determination of binding affinities of peptides 1 and 3 in WM-266-4

We determined the dissociation constant (K_d) and maximum binding (B_{max}) of peptide **1** in WM-266-4 using a lanthaligand-based saturation binding assay (Figure 2.6.A). WM-266-4 cells (20,000 cells/well, n=3) were incubated with different concentrations of peptide **1**, starting at 2 nM and progressively increasing until saturation was achieved (~100 nM). In a separate set of wells (n=3), the experiment was repeated using co-incubation with a high concentration (5 μM) of non-labeled peptide **2** to determine nonspecific binding.

Since the WM-266-4 cell line expresses MC1R natively, unlike other studies with cell lines transfected to produce high levels of MC1R,[22] we used colder temperatures (4 °C) and longer incubation times to favor complete receptor saturation by ligand without internalization of the ligand-receptor complex.[38] Data points were fitted to a one site fit- total and nonspecific binding equation in GraphPad Prism software. Concentration of labeled ligand was plotted in the x-axis and fluorescence at 615 nm was plotted in the y-axis (see Figure 2.6.A). Subtraction of nonspecific binding from total binding was used to determine specific binding for peptide **1**. Europium counts at maximum binding was found as B_{max}=3,000 ± 460 AU, and dissociation constant was K_d= 9.4 ± 5.7 nM.

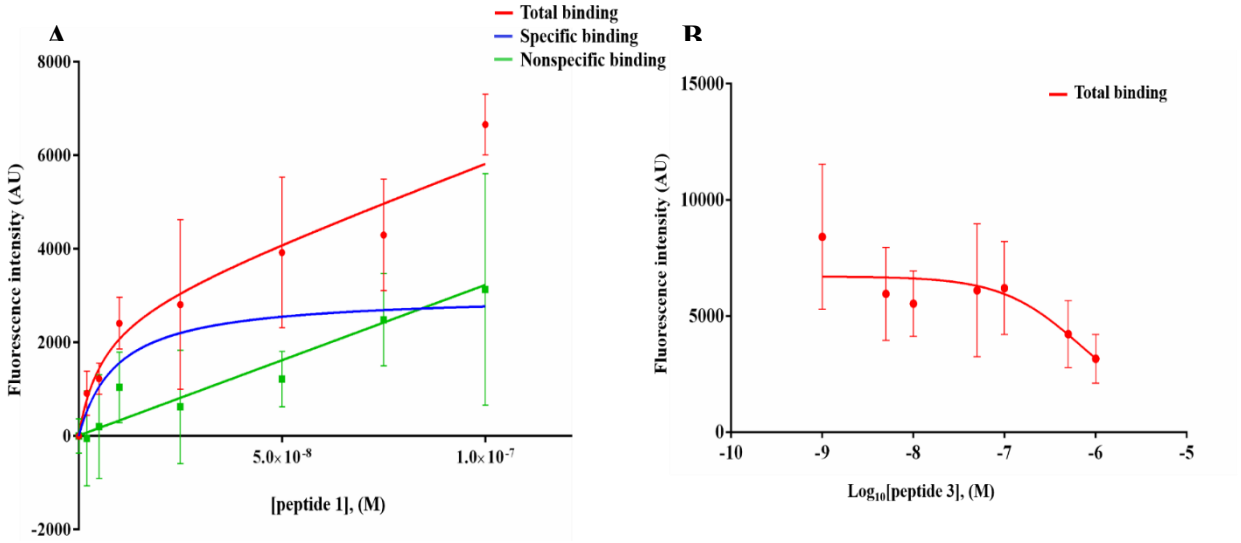

Figure 2.6. Determination of binding parameters of peptides **1** and **3** in WM-266-4 cell line. Time-resolved fluorescence (TRF) was measured in a BioTek Cytation3 multimode plate reader using excitation at 340 nm, emission at 615 nm, 400 µs delay time, 300 µs collection time, and gain of 200. (**A**) Saturation binding curve for peptide **1** in WM-266-4, using a specific binding curve to obtain A B_{max} of 3,000 ± 460 AU and K_d of 9.4 ±5.7 nM, with R^2 of 0.81. **(B)** Competitive binding curve for peptide **3**, with EC_{50} of 0.68 µM, and R^2 of 0.71.

A competition binding assay was performed to determine the affinity of peptide **3** for MC1R in the WM-266-4 cell line. The saturation concentration for peptide **1**, where all the MC1R receptors are occupied by the peptide, was determined to be 100 nM based on the data presented in Figure 2.6.A. WM-266-4 cells (20,000±2,100 cells/well, n=6) were incubated with different concentrations (1 nM to 1 µM) of non-labeled peptide **3** to compete with labeled peptide **1** (Figure 2.6.B). Similarly to the saturation binding assay, colder temperatures (4 °C) and longer incubation times were used to allow complete receptor saturation by ligand without internalization of the ligand-receptor. Data points were fitted using one site-fit logEC_{50} equation in GraphPad Prism software. 10–based logarithmic concentrations of peptide **3** were plotted in the x-axis and fluorescence at 615 nm was plotted in the y-axis. The EC_{50} value for peptide **3,** defined as the half maximal effective concentration or concentration to induce a 50% response by a compound, was calculated as 0.68 µM.

2.3.4. Estimation of expression level of MC1R using peptide 1 in WM-266-4

We estimated MC1R expression level (receptor copy number per cell) as reported previously.[32] Briefly, a calibration curve was prepared with different concentrations (0.195 pM to 1.25 nM) of peptide **1** in deionized water mixed with an equal volume of DELFIA enhancement solution to obtain a total volume of 100 µL per well in a 96 well plate (Figure 2.7).

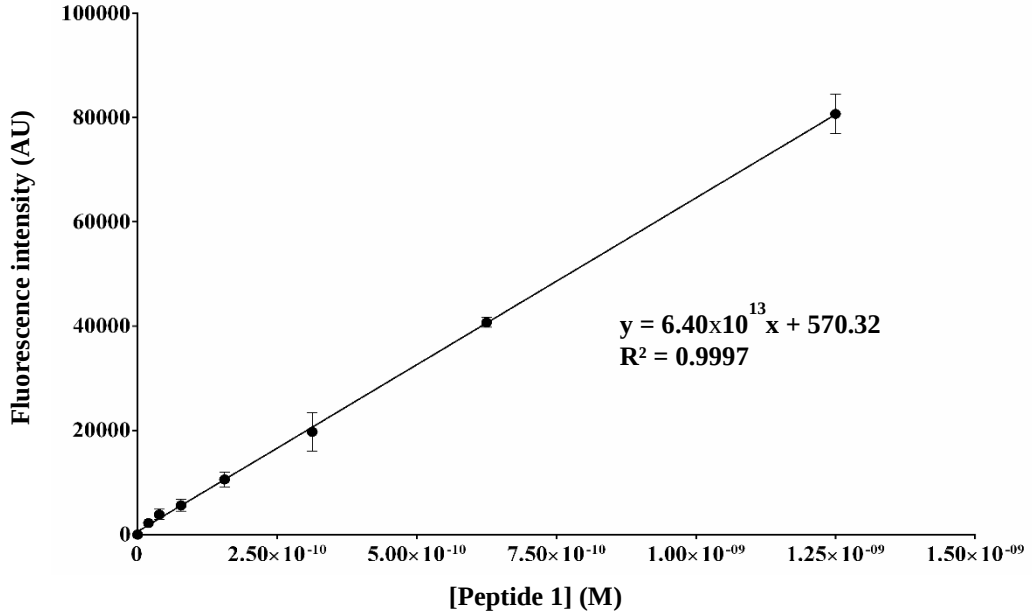

Figure 2.7. Calibration curve of peptide **1** in diluted DELFIA enhancement solution. Time-resolved fluorescence (TRF) was measured in a BioTek Cytation3 multimode reader with excitation at 340 nm and detection at 615 nm, 400 µs delay time, 300 µs collection time and gain of 200.

From the calibration curve, a straight line equation correlating fluorescence intensity (y) and europium concentration (x) in a well was obtained. Using the maximum specific binding found from Figure 2.6.A (B_{max}=3000 ± 460 AU) and linear regression analysis, the europium concentration was found to be 3.80 ×10^{-11} M.

It was expected that each molecule of peptide **1** would possess one equivalent of europium based on its chemical structure. This value was confirmed by comparing the concentration of peptide found by HPLC and the number of europium(III) molecules associated with the same amount of peptide based on DELFIA analysis. Since each labeled peptide bound to one receptor copy, we determined the receptor number present in one well. In turn, the average number of cells per well was measured using a hemocytometer and found to be 20,000 ± 2,100 cells/well (n=6). These results were then used to calculate the receptor number per cell. The equation is given in section 2.5.4.

The total number of ligands and consequently receptors, per well was calculated to be 2.29×10^9 and the number of receptors per cell was thus determined to be 115,000 ± 17,000. A previous report that used the same technique found 240,000 ± 60,000 MC1R receptor copies per cell in the colon cancer cell line HCT116 transfected with MC1R,[33] indicating that there was good expression even from the native gene in WM-266-4. Given that NDP-α-MSH can bind to other MCRs with different affinities, the total number of receptors reported herein might include other MCRs rather than MC1R exclusively. Further experiments could elucidate the specific number of MC1R receptors per cell by using a ligand with higher specificity for the title protein as reported previously,[39] or by blocking with MC1R-specific antibodies raised against the same binding site as MSH(7). However, such assays are beyond the scope of this study.

2.4. Conclusions and future directions

In this study, we successfully synthesized and characterized two analogs of [Nle^4, D-Phe^7]-α-MSH to target melanocortin receptors. Furthermore, we demonstrated an application of a europium-labeled peptide **1** to estimate the native level of receptor expression in a cancer cell line using a highly sensitive TRF method. Detection technologies based on europium-labeled peptides are more stable, faster, easier, and safer than radioligand binding assays while showing a high signal-to-noise ratio.[30] Although lanthanide-labeled ligands are not fluorescent, organic fluorophores can be covalently coupled to lanthanide chelates and act as antennas. These antennas absorb excitation light and transfer energy from an excited singlet state to a triplet state of the lanthanide ions, thus

emitting photons over a long timescale, which can be detected by TRF.[30] Thus, peptide **1** can also be utilized to study the oligomerization, internalization, and downstream signaling pathways of melanocortin receptors.

Peptide **3** can serve as a multifunctional ligand for targeting melanocotin receptors in several ways. The azide group present next to the MSH(7) sequence could serve as a chemical reporter through highly biocompatible, fast, selective, and stereospecific reactions such as Staudinger ligation with phosphines, copper(I) catalyzed azide-alkyne Huisgen cycloaddition, and copper-free strain-promoted azide-cyclooctyne addition (altogether known as "click chemistry").[40-42] Furthermore, these reactions are bioorthogonal, i.e., they can occur inside of living system without interfering with native biochemical processes, which allows probing into the cell with minimal disruption or noise. Consequently, peptide **3** could be readily tagged *in situ* and *in vivo* with a range of commercially-available cycloalkyne–decorated probes, including biotin or streptavidin, luciferin, fluorescent proteins (e.g., GFP or RFP), fluorescent organic dyes (e.g., Alexa Fluor or Cy5), radioactive isotopes, or contrast agents (e.g., gadolinium(III) chelates for magnetic resonance imaging).[43,44]

In addition to the azide group at Lys(N_3), peptide **3** was functionalized with four thiol (-SH) groups at the N-terminal position by coupling four cysteine branches to the G2 dendrimer. Thiol groups can be tagged with probes, such as those mentioned above, via facile reactions in mild conditions by forming thioethers with iodoacetamides, maleimides, benzylic halides and bromomethylketones substrates.[45] Additionally, dendritic thiol branches of peptide **3** can conjugate to thiol-functionalized nanoparticles by forming disulfide bonds which opens a wide window of highly sensitive microscopy and imaging techniques and tissue-directed delivery.[46] Along with these, thiol-containing moieties can also be adsorbed onto silver, gold, or other noble metals by sulfur-metal coordinate covalent bonds, a basic principle used to build self-assembled monolayers (SAM) in surface patterning, molecular electronics, and biosensors.[47]

2.5. Experimental section

2.5.1. Solid phase peptide synthesis (SPPS)

N-*α*-Fmoc-protected amino acids, ethyl (E)-2-cyano-2-(hydroxyimino)acetate (Oxyma Pure), *N*,*N*′-diisopropylcarbodiimide (DIC), hydroxybenzotriazole (HOBt), *O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), and *N*,*N*′-diisopropylethylamine (DIEA) were purchased from Novabiochem® (Billericia, MA), Chem-Implex International, Inc. (Wood Dale, IL) or Protein Technologies, Inc. (Tucson, AZ). Tentagel S RAM resin was purchased from Novabiochem®. Protected forms of amino acids with reactive side chains were used as follows: Ser(tBu), Tyr(tBu), Glu(OtBu), His(Trt), Arg(Pbf), Trp(Boc), Cys(StBu), Lys(Boc), Lys(N_3), 2,3-diaminopropionic acid (Dap) and 6-amino hexanoic acid (Aha). Protected amino acids were purchased from Novabiochem® (Billericia, MA). Diethylenetriaminepentaacetic acid (DTPA) was purchased from TCI chemicals (Portland, OR) and europium(III) chloride hydrate ($EuCl_3.6H_2O$) from Alfa-Aesar (Ward-Hill, MA). Reagent grade *N*,*N*′-dimethylformamide (DMF) and dichloromethane (DCM), dimethyl sulfoxide (DMSO), formic acid, tetrahydrofuran (THF), triisopropylsilane (TIS), thioanisole (TA), ethanedithiol (EDT), bromophenol blue, ammonium acetate, HPLC grade water and acetonitrile were purchased from Fisher (Fair Lawn, NJ), Sigma-Aldrich (St. Louis, MO) or Alfa-Aesar (Ward-Hill, MA) and were used without further purification. Sep-Pak® C-18 cartridges were purchased from Waters (Milford, MA). Fritted syringes were obtained from Torviq (Oro Valley, AZ).

All peptides were synthesized manually using 2 to 20 mL plastic syringe reactors fitted with a frit. Solid phase peptide synthesis was performed in Tentagel S RAM resin (loading: 0.24 mmol/g) by using N-α-Fmoc protecting groups and DIC/OxymaPure or HCTU/DIEA activation. The resin was swollen in DCM overnight and washed with DMF and DCM. Fmoc was routinely cleaved with 20% piperidine in DMF (2 min), followed by DMF wash (2×) and 20% piperidine in DMF (18 min). Resin was washed with DMF (3×), DCM (3×), bromophenol blue in 0.1 M HOBt/DMF, and finally with DMF and DCM. Amino acids and other acids, e.g. Fmoc-6-aminohexanoic acid, were coupled by using preactivated OxymaPure esters in DMF, which were generated *in situ* by mixing

3 eq. of acid, 3 eq. of OxymaPure, and 3 eq. of DIC and allowing their reaction to occur for 5 min. Bromophenol blue left in the resin after the washing step allowed for qualitative and continuous monitoring of reaction progress. After a 1 h reaction, the resin was rinsed with DMF and DCM, and preactivated HCTU ester of the acid (2 eq. of acid, 2 eq. of HCTU, and 2 eq. of DIEA in DMF for 5 min) was added for 1 h. Then, the resin was washed with DMF (2×) and DCM (2×), and tested for unreacted -NH_2 using the Kaiser test. If positive, a second coupling was performed using preactivated HCTU ester. Any unreacted amines on the resin were capped by using excess acetic anhydride:pyridine 1:1 for 1 min. The resin was then washed with DMF (3×) and DCM (3×) and the procedure described above was repeated for the next amino acid until all amino acids were coupled.

Cleavage from the resin was achieved by injection of 10 mL/g resin with 90% trifluoroacetic acid (TFA), 2.5% water, 2.5% triisopropylsilane (TIS), 2.5% thioanisole (TA), and 2.5% ethanedithiol (EDT) and stirring for 3 h at room temperature. Crude peptides were removed from the syringe by filtration, concentrated by evaporation under a stream of air, precipitated in ice-cold diethyl ether, centrifuged, washed several times with ether, and lyophilzed to yield white solid powders that were stored at −20 °C. The final compounds were analyzed and purified by preparative HPLC and characterized by HPLC-UV/Vis ESI-MS and TOF-MS spectrometry. For HPLC, 0.1% formic acid in water was used as solvent A and 0.1% formic acid in acetonitrile was used as solvent B, whereas the gradients were optimized for each peptide.

DTPA was coupled to the peptide in the resin using the following steps. 20 eq. of DTPA anhydride (final conc. 0.6 M) and 40 eq of HOBt were dissolved in dry DMSO in a glass scintillation vial by heating to 50 ^{o}C, followed by stirring for another 20 min at room temperature. The resin was added to this mixture and microwaved twice at 60 ^{o}C for 30 min to allow complete coupling. The resin was washed in the following sequence: dimethylsulfoxide (DMSO) (3×), Tetrahydrofuran (THF) (3×), 20% water in THF (3×, 5 min), 10% water and 10% DIEA in THF (2×, 5 min), 20% water in THF (2×, 1 min), THF (3×), and finally DCM (3×). The peptide was cleaved from the resin as described above.

Europium(III) was chelated to the DTPA coupled-peptide and purified using the following steps. The peptide was dissolved in 0.1 M aqueous ammonium acetate solution (pH 8.0) and 3 eq. of europium(III) chloride hydrate ($EuCl_3.6H_2O$) was added to the solution. The reaction mixture was stirred at room temperature until complete chelation was confirmed by HPLC-MS, which occurred after 3 h. To prevent europium from cleaving off from the chelate during HPLC, TEA/AcOH buffer was added to all solvents. 0.1% TEA/AcOH in water (pH 6.0) was used as solvent A, whereas 10% solvent A in acetonitrile adjusted to pH 6.0 with acetic acid was used as solvent B. HPLC was performed changing the proportion of solvent A from 10 to 90% over 40 min. Purification of europium(III)-chelated compound was performed in Sep-Pak® C-18 cartridges (360 mg). The cartridge was preconditioned with 5 column volumes each of acetonitrile, methanol and water. The reaction mixture was loaded onto the cartridge and washed multiple times with water to remove excess europium salt. The column was washed (2×) with two column volumes of 5, 10, 20, 30, 50, and 70% aqueous acetonitrile and the fractions were checked by HPLC-MS. The 30% acetonitrile fraction contained most of peptide **1**, while little product was found in 50% fraction. Both fractions were collected, mixed, lyophilized and stored at −20^{o}C.

2.5.2. Cell culture

Melanoma cell lines A-375, WM-115, and WM-266-4 and metastatic prostate tumor cell line PC-3 were obtained from the American Type Culture Collection (Manassas, VA). Eagle's modified essential medium (EMEM), Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS) and antibiotic-antimycotic solution (100X) were procured from Corning (Manassas, VA), while 2.5% trypsin solution was obtained from Quality Biological (Gaithersburg, MD). Phosphate buffered saline (PBS) tablets and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Aldrich (St. Loius, MO). CELLSTAR® T75 cell culture flasks were procured from Greiner Bio-One (Monroe, NC). Nunc 96 well colorless assay plates, Greiner Bio-One 96 well clear bottom black plates, centrifuge tubes, and pipette tips were purchased from VWR (Radnor, PA).

All experiments involving biohazardous materials were conducted under Biosafety Level 2 containment and approved by the Virginia Tech Institutional Biosafety Committee (IBC protocol 14-043). Cell lines were grown as previously reported under the conditions recommended by the

supplier. DMEM and EMEM media were supplemented with 10% FBS and 1% of 100X antibiotic. Cells were incubated in complete medium (DMEM for A-375 and PC-3, or EMEM for WM-266-4 and WM-115) at 37°C in a 5% CO_2 incubator. Cells were subcultured at 80-90% confluency by using 0.25% trypsin/0.53 mM EDTA. Cells were used before passage 15 to decrease phenotypic drift. Cell counting was performed in an InCyto C-Chip improved Neubauer hemocytometer and seeded into T75 flasks for general culture, colorless 96-well plates for immunocytochemistry and clear bottom 96 well black plates for binding assays.

2.5.3. Immunocytochemistry

Solutions of 4% paraformaldehyde in PBS were obtained from ChemCruz Biotechnology, Inc. (Dallas, TX). Bovine serum albumin (BSA) was purchased from Sigma Aldrich (St. Loius, MO). Anti-MC1R primary antibody raised against human proteins using rabbit as host was purchased from Alomone labs (Jerusalem, Israel). Goat anti-Rabbit IgG (H+L) cross-adsorbed secondary antibody tagged with DyLight 550 was purchased from Thermofisher Scientific (Rockford, IL).

Immunocytochemistry was performed in cultured melanoma cell lines WM-266-4, WM-115 and A-375, using metastatic prostate tumor cell line PC-3 as control. Antibody dilutions were prepared in 2% bovine serum albumin (BSA) in PBS, a 1:200 dilution was used for primary antibodies and a 1:50 dilution for secondary antibody as per vendor's recommendation. Cells were grown as described above, seeded at 10,000 cells/well in a 96-well plate and allowed to attach for 24 h. Upon aspiration of media, cells were fixed by incubation with 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were incubated with diluted primary antibody for 1 h, rinsed with 2% BSA in PBS (3×), incubated with DyLight550-tagged secondary antibody for 1 h, rinsed with PBS (3×), and incubated with 2 μg/mL Hoechst 33342 in PBS for 5 min for DNA counterstaining. Then the cells were rinsed with PBS (3×). Negative controls were treated under the same conditions as all other samples, except they were incubated with BSA instead of primary antibodies.

Cells were imaged using a Biotek Cytation3 Cell Imaging Multi-Mode Reader. Conditions for image acquisition (light intensity, integration time, and camera gain) and processing (contrast and

brightness) were optimized. We made sure that no fluorescence was detected in negative controls and signal was only coming from stained samples. Imaging and image processing conditions were kept constant across cell lines and samples to allow qualitative comparison.

2.5.4. Binding assays

DELFIA enhancement solution was purchased from Perkin Elmer (Waltham, MA), and BSA and Tris buffered saline tablets were obtained from Sigma Aldrich (St. Louis, MO). BSA was added in Tris-NaCl solution to prepare DELFIA wash buffer (50 mM Tris, 15 mM NaCl, 2% BSA), whereas ligand solutions were prepared in ligand binding media (0.2% BSA in EMEM). All binding assays were performed using the WM-266-4 melanoma cell line.

For saturation binding assays, cells were seeded in a 96 well black plate at 20,000 cells/well and incubated for 24 h in an incubator set at 37 ^{o}C and 5% CO_2 to allow attachment. Upon aspiration of media, cells were washed once with ligand binding media and 50 µL of binding media or peptide **2** (nonlabeled ligand) were added per well, accordingly. Cells were incubated for 30 min at 4 ^{o}C and 50 µL of increasing concentrations of peptide **1** (labeled ligand) were added. Cells were incubated for 2 h at 4 ^{o}C and washed with DELFIA wash buffer (3×). 100 µL of DELFIA enhancement solution was added to all wells and incubated on a plate shaker for 1 h (250 rpm) in dark. Time-resolved fluorescence (TRF) readings were taken in BioTek Cytation3 instrument using excitation at 340 nm, emission at 615 nm, 400 µs delay time, 300 µs collection time, and a gain of 200.

Competition binding assays were performed following the same protocol, except that peptide **1** (labeled ligand) was added first, at 50 µL/well, and incubated for 30 min at 4^{o}C. Then increasing concentrations of peptide **3** (nonlabeled ligand) (50 µL/well) were added to all wells and incubated for 2 h at 4 ^{o}C. Washing and reading were performed as described above.

For the europium calibration curve, peptide **1** (prepared in deionized water, final concentration 0.195 pM to 1.25 nM) was mixed with an equal volume of DELFIA enhancement solution in a 96

well plate to obtain a total volume of 100 μL per well. The plate was incubated on a plate shaker for 1 h at 250 rpm protected from light. TRF readings were taken as described above. The following equation was used to correlate the europium concentration to receptor number per cell:

$$R=C_{Eu}\times E_{R\text{-}Eu}\times V_{sol}\times N_A \qquad \text{(Eq. 1)}$$

where R is the number of receptor copies per well, C_{Eu} is the molar concentration of Eu per well as determined by DELFIA, $E_{R\text{-}Eu}$ is the stoichiometric ratio between the number of receptor copies and the equivalents of Eu, Vsol is the volume of solution in liters (10^{-4} L in this case), and N_A is Avogadro's number (6.022×10^{23} units/mole). $E_{R\text{-}Eu}$ was calculated as 1 based on the 1:1 ratio between ligand and receptor,[33] and the presence of an average of 1 equivalent of Eu per peptide molecule, as determined from DELFIA and coinjection of sample with H-Trp-OH as described in the discussion.

2.6. References

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3. CONCLUSION

From grades of fever in microbial infection to β-amyloid plaques in Alzheimer's disease, biomarkers contribute to a profound history of disease diagnosis, risk assessment, progression monitoring, and efficient therapeutic interventions. Due to their replicability and reproducibility even in small subject groups, they pose an enormous role as surrogate endpoints to substitute real endpoints (e.g. death) in medical research, drug development and clinical trials of complex diseases (e.g. cardiovascular and neurological disorders, cancer, and AIDS). Proteins, whether tissue bound (e.g. ER in breast tumor and CD4+ T-cell in AIDS) or secreted in body fluids (e.g., PSA in serum in prostate cancer and LDL cholesterol in heart disease), are the most accessible and optimizable set of biomarkers that are the closest indication of the spatial and temporal events occurring in the course of a disease.

Unfortunately, capturing and analyzing most of the proteome using the available techniques and translating already-discovered techniques to clinics are still challenging. Beyond the detection of a particular biomarker, understanding its expression pattern in different populations, tissues, or stages is vital for research on complex diseases. In this thesis project, we addressed this issue and envisioned a novel technique occupying cell surface receptors, a major class of protein biomarkers.

Cell surface receptors, the signaling proteins embedded in the plasma membrane, possess extracellular ligand binding sites, which make them ideal diagnostic targets employing antibodies, peptides or small molecules that mimic their native ligands. We designed an *in-situ* technique using multifunctional thio-dendrimeric peptides that optimally bind to their receptors and, upon attachment to silver nanodiscs (AgNDs) and detachment from cells, might imprint a putative expression profile (i.e., receptor number and pattern) onto the nanodiscs. Upon imprinting, peptide thio-dendrimers can be further tagged with gold nanoparticles (AuNPs) in their azide handles. Since AuNPs have different optical properties than AgNDs, those bound to already organized thio-dendrimers will generate high contrast images when observed under electron microscope. We discussed the design and possible experiments to establish a proof-of-concept of this technique in

the first chapter of the thesis, using a melanocortin receptor 1 (MC1R), a G-protein coupled receptor (GPCR) as a model cell surface receptor.

In the second chapter, we demonstrated the synthesis, chemical characterization, and whole-cell binding assays of the peptide ligands to target MC1R, which completed the first set of goals to prove the aforementioned technique. We utilized [Nle^4, D-Phe^7]-α-MSH, or NDP-α-MSH, a high affinity tridecapeptide analog of the native melanocortin ligand α-MSH, an agonist of MC1R that binds to other melanocortin receptors (MC2R-MC5R) with various affinities. Along with MSH(7) (peptide **3**), a heptapeptide analog of NDP-α-MSH, we also synthesized a europium-tagged analog of NDP-α-MSH (peptide **1**) and a non-labeled analog of NDP-α-MSH (peptide **2**). We examined the surface expression of MC1R in three melanoma cell lines and a prostate cancer cell lines using immunocytochemistry and selected WM-266-4 melanoma cell line for further experiments. We determined the expression level of MC1R in this cell line using europium labeled peptide **1** via DELFIA, which could be used to correlate the number of the same receptor on imprinted nanodiscs. Finally, we determined the binding constant for peptide **3** on WM-266-4, which is necessary to demonstrate its optimal binding to the cells prior to imprinting. These initial results matched with our expectations, which verified the technique to be a potential one in biomarker research and therefore to be moving forward with next steps.

The idea of *in-situ* nanoimprinting of cell surface receptors, or as we termed it, a "nanocamera," still needs to be curated and validated to become a useful technique in academic research, in high-throughput bio-tech industries, or in clinical diagnostics. There is a plethora of new and potential techniques being published every day in biomarker research, but very few of them could meet the requirements for their implementation. Our technique occupies peptides that mimic the native ligands, which can be synthesized by solid phase peptide synthesis (SPPS), a cost-effective way for both small labs and large industries. In addition to their affordability, these dendrimeric peptides are small and could be easy to modify (e.g., by changing the number or composition of amino acids) to generate optimal simultaneous binding simultaneously to the specific receptors and the AgNDs without twisting among themselves. This is an exceptional advantage of this technique compared to large and biologically complex antibody-based methods, and could be used

to gain insight into receptor dimerization, conformational change, and their underlying mechanisms, which are fundamental events in GPCR signaling. Utilization of two different metal nanoparticles, AgNDs as an immobilization surface for thiols and AuNPs as detection probes, is another highlight of this technique. Metal nanoparticles have been used widely and applauded for their low-cost, tunability to various sizes and shapes by fast chemical reactions, less toxicity than radioisotopes, strong crosslinking to peptides and biomolecules, easy handling, and excellent resolution in different modalities of detection methods. Additionally, this technique avoids harsh or extensive sample processing steps, thus helping to preserve the putative receptor expression profile on tissues and imprint.

Combining all these aspects, *in-situ* nanoimprinting could be a sensitive, cost effective, and fast technique with huge potential to be utilized on its own or integrated with other techniques to develop newer and more efficient devices. One possible application would be the development of a mechanical device for the study of solid tumors. Upon penetrating to the site of interest, the device could automatically attach to the tumor and detach biologically inert strips (e.g., nanodiscs) coated with specific peptides. The device would collect multiple samples from various tumor sites at different time points. Subsequent analysis of the imprinted strips would showcase the expression profiles of specific proteins and allow their comparison. Thus, this technique could take us one step closer to personalized medicine via personalized diagnostics.