DESIGN AND DEVELOPMENT OF METAL-PEPTIDE NANOSCALED MATERIALS

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A Dissertation
Submitted to the Graduate College of Bowling Green
State University in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

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ABSTRACT

Michael Y. Ogawa, Advisor

The work presented in this dissertation demonstrates how de-novo designed peptides having the double stranded \( \alpha \)-helical coiled-coil conformation can be organized into molecular assemblies by metal coordination. The design of these peptides explores the positioning of a strong metal binding site, 4-pyridylalanine, within the hydrophilic portion of the peptides in order to coordinate the metal complexes into well-defined geometries.

The effect of the peptide’s sequence on the stability of the formed metal-peptide complex has been shown through the reaction of Pal14 and Pal14n with ethylenediamine platinum (II). In particular, it was found that peptides based on an IEALEGK repeat do not form stable metal complexes. In contrast, peptides based on an IAALEQK repeat do form stable platinum complexes which in water assemble into globular structures that are 40 nm in diameter. Incorporation of a covalent crosslink into the Pal14n coiled-coil structure (Pal14C19nox) forces this peptide to form linear assemblies after the reaction with the platinum complex.

The last chapter of this dissertation reveals that multiple metal binding sites on the hydrophilic exterior of the two-stranded coiled-coil Pal14Pal21n forces the formation of cyclic tetramers, rather then a polymeric product. It was also found that the conformational “plasticity” of the non-covalent coiled coil Pal14Pal21n is necessary for the formation of the platinum-peptide tetramer. In contrast, the covalently “secured” coiled-coil structure of Pal14C19Pal21nox restricts conformation changes within the molecule, which inhibits the platinum complex from orienting the peptide ligand in order to form the tetramer structure.
This dissertation is dedicated to my parents,

Nadegda and Valery Tsurkan and

to my sister and brother, Alyona and Mitya Tsurkan.
I would like to thank my family for the support they have given me throughout my life. Without their unwavering faith in my abilities, and their high expectations, I would have fallen short long ago.

I would like to thank my advisor, Dr. Michael Ogawa, for his never-ending patience throughout this learning process. His calm demeanor, and his willingness to try my ideas has allowed me to excel as a scientist, and has taught me lessons about science and the scientific process that will serve me for the rest of my career. His tough critiques of my work may have pushed me to what I thought was the edge of my sanity, but have shown me how much I truly can handle, and has made me a more cautious, strong chemist.

I would like to thank my committee Prof. George S. Bullerjahn, Prof. Pavel Anzenbacher and Prof. Sheryl Coombs for their service for the past few years. Their eagerness to assist me on my journey is much appreciated. I would also like to thank Prof. Douglas C. Neckers for his agreement to join my committee last minute.

Finally, I would like to thank everyone else who has helped to make my experience here at BGSU successful. The faculty and staff in the photochemistry department have helped me tremendously. The use of equipment in Dr. Neckers and Dr. Castellano’s labs has been instrumental to my work. Dr. Kintsle has been a role-model to me, and I admire very much his teaching style and his attitude towards the learning process.

Throughout my time at BGSU, I have met many people who have made an impact on my life, and their friendships and have helped me through good times and bad. My good friend Sarah, and her steady comfort and encouragement have assisted me, and her tireless attention to
detail while proof-reading my dissertation has helped me, and you - the reader, immensely. My friends Doug and Jody have been with me from the beginning of my time here, and I will always be grateful for their friendship.

My special thanks go to my friends and co-workers for creating friendly working environment.
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CHAPTER I: INTRODUCTION

I.1 INTRODUCTION AND BACKGROUND

I.1.1 Statement of the problem

Proteins which contain metal ions as an essential part of their activity are present in all biological systems. The most important and extensively studied of these is the photosystem, which is the main source of energy and carbon in nature.\(^1\) Due to complications with general energy sources, such as limitations in the amount of fossil fuels, such as oil and coal, and ecological problems with nuclear energy sources, many efforts have been made to prepare an artificial photosystem as an alternative energy source. One way to prepare this system is to mimic nature by designing an artificial metalloprotein containing photochemical activity. However, a major problem with this approach is designing a molecular structure that can facilitate long-lived charge separation as a key process in the control and regulation of photochemical energy conversion.\(^2,^3\) In nature, photosystems I\(^4,^5\) and II\(^6,^7\) both consist of many peptide subunits joined together, and are distinguished as antenna complexes and reaction centers.\(^8-10\) Figure I.1 shows the schematic view of photosystem I-II where antenna complexes absorb light and transfer energy to the reaction center which initiates charge separation. Chlorophylls are the major light-harvesting protein complexes in structural organization of the antennas.\(^11\) Their reaction center is an organized array of metaloproteins constructed for photo-induced charge separation and initializes the electron transport chain for the conversion and storage of solar energy.\(^12,^13\) Recently, progress has been made in the development of unnatural molecular complexes which mimic the light-harvesting activity of chlorophylls in nature.\(^14\) The
preparation of a non-natural reaction center is still under development because of the complexity of the structure-mechanism relation.

Figure 1.1. Schematic view of light energy conversion into electric current by assemblies of protein in photosystem I-II.

The French scientist Becquerel was the first to discover the photo-electric effect in 1830. The conventional solar cell in its modern view first appeared in 1954 and was based upon semiconductor solid structures. For the next 50 years, solar cells were upgraded many times but fundamental restrictions in the efficiency of these system for solar energy conversion has forced scientists towards engineering new photoactive chemical systems. The first attempt to mimic nature in its ability of light collection and conversion was attempted through development of dye-sensitized solar cells. In the dye sensitized solar cells, chromophore is transferred into an exited state by light absorption, which further initializes electron flow through “charge-
separation”. Many of these systems are based on the principals of supramolecular chemistry using chemical transformation of discrete molecules. This strategy generally utilizes the bonding properties of metal complexes and organic ligands to construct molecular assemblies for energy conversion. Water is commonly used as the substrate in these systems for reduction, which allows storage of solar energy in hydrogen molecule. Similar to natural photosystems, in artificial photosystems the ligands (dye) have the role of antennas and the metal cores usually form a photo-reactive center through charge separation\textsuperscript{14,22}, as shown in Figure I.2:

![Figure I.2. A general schematic view of light energy conversion by an artificial photosystem into electric current. (Adapted from reference 27)](image)

The development of a donor-acceptor pair is one of the main challenges for an efficient charge separation in these artificial photosystems. Many various donor-acceptor pairs have been proposed for such a system. Among them, systems based on the ruthenium(II) tris(di-imine) and related ruthenium(II) bis-(terpyridyl) systems derivatives have become the most common.\textsuperscript{23} Since 1972, when reductive electron-transfer quenching of [Ru(bpy)\textsubscript{3}]\textsuperscript{2+} was discovered\textsuperscript{24}, the amount of work dedicated to the application of photochemical properties of ruthenium
complexes has grown exponentially. The design of a stable chemical architecture for integration of appropriate donor and acceptor pairs at specific positions is common for all of these works.

Several alternatives to the ruthenium complexes were proposed. The di- and triimine complexes of platinum(II) using acetylide ligands are an example of alternatives to the ruthenium complexes. The main advantages of these platinum (II) chromophores are the excited-state energies, which are higher than those found for the ruthenium complexes, and photo-induced charge separation, which is much faster than the reverse charge recombination reaction.

Another alternative to the ruthenium complexes utilizes multi-electron chemistry in metal complexes with two-electron mixed valencies. Water oxidation is a four electron process while the Ru(II)-Ru(III) transition is a one electron event. Therefore, one advantage of using platinum complexes for water splitting is the two electron process of the Pt(II)-Pt(IV) transition. In other studies, it has been shown that two-electron mixed valency complexes of iridium and rhodium, can achieve two and four electron transformations. Therefore, these systems could potentially perform a water reduction process in one step. Unfortunately, the efficiency or stability of these artificial photoactive assemblies is still far below the efficiency of the natural ones. Controlling the distance between suitable electron donor and electron accepter pairs is crucial for the efficiency of photo-induced charge separation in all of these systems. Proteins, which have a well defined structure, can be used to control the distance between the donor and electron accepter pairs, and therefore engineer a photo-reactive center.

The de novo design of synthetic proteins has significantly broadened the area of protein design and engineering, and are mainly aimed at the relationship between sequence, structure
and function. Much work in this field has focused on the design of artificial proteins, incorporating various chemical activities which can be made through rational synthesis. In development of an artificial photosystem, the modulation of redox activity and study of electron transport of a natural cofactor in de-novo designed peptides is of special interest. Incorporation of a photoactive metal complex such as hemes or ruthenium-bipyridine within a well design protein scaffold could mimic the natural protein assemblies in their photochemical energy conversion abilities. One way to construct this protein scaffold is to utilize the coordination properties of transitional metal complexes to arrange de novo designed peptide subunits into high ordered molecular structures; the geometry of which would be dictated by the metal center. Thus, these bioinorganic materials can be used as a framework for the engineering of artificial photosystems with the properties of the natural one. In other words, combining the principles of supramolecular coordination chemistry with de novo protein design is a rational way to engineer the structural arrangement of natural photoactive protein assemblies.

I.1.2 Supramolecular coordination chemistry

Supramolecular chemistry is a fast-growing field of science that focuses on the production and manipulation of materials with nanometer-scale dimensions. In order to reach this goal, non-covalent interactions, such as coordination bonds, hydrogen bonds and Van der Waals interactions are used to self-assemble molecular subunits into structures of well defined geometry.

There are several definitions of the term “self-assembly”, which describes the production of nanomaterials in the field of supramolecular chemistry. The fullest overview of this term has been organized in recent review papers and includes several principles:
a) Self-assembling units are held together by labile, non-covalent interactions;

b) The assembling of subunits into larger aggregates is selective, where the subunits bind cooperatively to form the most stable aggregate;

d) The aggregates are discrete rather than infinite. This means that self-assembled supramolecules are normally favored thermodynamically over oligomeric or polymeric systems, profiting from enthalpic rather then entropic effects.

c) The aggregates can be recognized by their properties that differ from those of the individual components;

Investigators within the field of supramolecular coordination chemistry have exploited the fact that coordination compounds are formed by highly directional metal-ligand bond interactions. It was found that the incorporation of appropriate bridging ligands into a transition metal complex allows various coordination units to be linked together in an assembly with discrete geometrical modes such as squares, grids, boxes, rods, tubes, etc. The use of coordination bonds in the self-assembly of these structures is very useful, not only because of the various molecular geometries which can be reached through metal coordination, but also because of their relatively high bond strengths. The energies of coordination bonds are approximately 10-30 kcal/mol per interaction, which are between those of strong covalent bonds in carbon-based macrocycles and weaker hydrophobic and electrostatic interactions in biological systems. The lability of coordination bonds results in the formation of a thermodynamically favored molecular assembly by promoting a self-repairing mechanism that disfavors the formation of weak complexes. On the other hand, the high strength of coordination bonds increases the stability of
the formed structures and in some cases can be used as a “molecular key” to stabilize the formed structure.

![Figure I.3](image)

**Figure I.3** The concept of the supramolecular self-assembly of square macrocyclic mediated by a protected, square-planar transition metal.

The design of the macrocyclic structure can be seen in molecular square formation, where a transition metal complex of square planar geometry coordinates linear bridging ligands (Figure I.3). This example clearly illustrates how combining a linear, bidentate ligand with a metal complex of square planar geometry can produce a macrocyclic, square supramolecular assembly, which is the thermodynamically favored product. This strategy is typical for the formation of two dimensional assemblies\(^5\), in which all metal centers are located in one plane of the supramolecular structure. The variety of possible two dimensional geometries which can be reached through metal coordination is shown in Figure I.4:
Formation of molecular assemblies is the ultimate goal of researchers in the field of supramolecular chemistry. Since the beginning of the 1990s, scientists have made progress in the formation and characterization of two dimensional molecular structures, and new works in this area continuously appear in literature.\textsuperscript{56-58} The attention of researchers has now shifted to the design of more complicated three dimensional structures that diversify the properties and potential applications of supramolecular self-assemblies.\textsuperscript{59} The formation of three dimensional assemblies is usually reached by use of metal complexes or “naked” metal ions in conjugation with “three dimensional” ligands, in which multiple coordination sites are located in different

![Table and Diagram]

**Figure I.4.** Possible two dimensional structures which can be formed with a mix of ligands and metal complex geometries. (Copied from reference 45, with the permission of ACS)
planes of the molecule. The variety of possible three dimensional geometries which can be reached through metal coordination of the “three dimensional” ligands is shown in Figure I.5:

**Figure I.5** Possible three dimensional structures which can be formed by appropriate mix of ligands and metal complexes geometries. (Copied from reference 45 with permission of ACS)

Applying similar principles to the design of new bimolecular assemblies can result in the formation of new materials with potentially unique properties. Peptides and proteins with well defined, stable secondary structures such as $\alpha$-helices or $\beta$-sheets can be used as a three dimensional ligands within this approach. Incorporation of a strong metal-binding site into the peptide backbone could lead to the ability of such a molecule to be coordinated by metals into well organized molecular assemblies. Therefore, the use of peptide molecules as a ligand within
this approach is highly innovative and valuable for construction of new types of molecular assemblies.

**I.1.3 Molecular assemblies**

The design of new molecular self-assemblies is considered to be the most powerful strategy for the development of new materials of nanometric scale and currently attracts much interest. In comparison with classical organic or polymer synthesis, the main advantage of this approach is the possibility of designing a variety of relatively simple molecules which would self assemble into highly organized, complicated structures. Moreover, recent progress of supramolecular chemistry in the formation of complex, three dimensional structures has led to the construction of materials with an inbuilt functionality, which allows the design of systems to perform useful mechanical, electrochemical, photochemical, or enzymatic chemistry. Among of these activities, the advantages of processes which are induced by light, such as availability and manipulability, make the photochemical activity of molecular assemblies especially interesting.

There are two principal ways to control of the properties of molecular assemblies that show different functionality after light irradiation. The first group is the system in which supramolecular organization is controlled by the photoproperties of the assemblies. These activities are usually reached by incorporation of a photo-isomerisable molecule into the active part of the assemblies. The classic example of such photo-isomerisable molecules is azobenzene. It has been shown that a thiol-reactive azobenzene cross-linker can be incorporated in a peptide by reaction of cysteine (Cys) residues (Figure I.6.). Geometrical changes in such molecules after light irradiation will stabilize (i, i+3) or disturb (i, i+11) the peptide
conformation depending on the design. The possibility of using both cis and trans forms of azobenzene as a structure to stabilize peptide conformation, and the relative insensitivity of these structures to amino acid sequences is used to induce and control various functions of molecular assemblies. This includes everything from the control of self-organization of molecular assemblies to the coordination or release of guest molecules in host-guest complexation.

Figure I.6. Schematic models of the trans and cis conformations of the azobenzene cross-linker for i, i+3 (left) and i, i+11 (right) peptides of α-helix structure. The peptide backbones are represented by a silver ribbon. The cysteine side chain and cross-linker atoms are colored according to atom type: carbon = green, nitrogen = blue, oxygen = red, and sulfur = yellow. (Copied from reference 69 with the permission of Elsevier)

Another group of photoactive assemblies are molecular systems in which photoactivity is controlled through supramolecular organization. The control of the photoreaction is a complicated process compared to ground state reactions. The mechanistic pathway of a reaction in the ground state can be controlled by changing easily accessible external factors such as temperature or use of an appropriate catalyst. In contrast, the photochemical reaction pathway goes through very short-lived excitation states, the activation barrier of which is not significant.
Nevertheless, the control of photoreactions, such as energy or electron transfers, can be reached through the manipulation of the distance between chromophores. The distance between the chromophores is determined by the supramolecular organization of photoactive assemblies. Photosystems I and II are great examples of such systems in nature, where the energy of the photo-induced charge separation transforms into a water reductoin process instead of being reversed. This transformation is caused by supramolecular organization of these complexes. It has been shown that damage of such complex molecular organization in photosystem II leads to the loss of water reduction activity even in the presence of a charge separation event.\textsuperscript{13, 79, 80} Therefore, development of molecular assemblies for the control of photoactivity by molecular organization is particularly important for the systems in which photoproperties are difficult to control.\textsuperscript{78} A particularly active area of research within this strategy is the design and synthesis of supramolecular architectures for the transformation of light energy.\textsuperscript{2, 63}

It has been noted that most of the photoactive assemblies which have been studied for light collection and conversion have been constructed based on metal- coordination binding principles. Pd/Pt or Re can be used as metal centers in complexes with various organic ligands for the construction of molecular framework in such assemblies.\textsuperscript{14} Applying similar concepts, but substituting organic ligands on bio-molecules such as peptides can result in a new strategy for production of novel nanometer-scale materials containing photochemical activity. The formation of metal-peptide complexes can generally be considered from two different points of view. The first is from inorganic chemistry, in which the metal-peptide reactions occur between metals and appropriate metal binding groups within the peptide sequence. These reactions are usually well known and go to the most thermodynamically stable product. The second is the biochemical point of view. Here, non-covalent interactions of biomolecules in general, and peptides in
particular, with transitional metals has been intensely investigated during the last several decades.\textsuperscript{81, 82}\ Therefore, for the successful design and synthesis of metal-peptide framework and further development of photoactive systems, it is important to understand the chemistry of metal-peptide complex formations.

\section*{I.1.4 Metal-containing proteins}

When preparing non-natural metal-containing proteins, it is especially important to understand the forces which influence the final metalloprotein structure. Coiled-coil proteins have been used as a model for studying these effects.\textsuperscript{83, 84-90} There are two extreme conditions where these forces were studied. In the first, final metalloprotein structure is defined by peptide scaffold.\textsuperscript{82, 83, 91, 92}\ Here the peptide defines the final coordination geometry of the metal. For example, the binding Hg\textsuperscript{2+} to the hydrophobic interior of three-stranded coiled-coil peptide forces the Hg\textsuperscript{2+} to adopt trigonal planer geometry which is unusual for this ion.\textsuperscript{91}\ The second condition is where the metal coordination properties determine the final folding of metalloprotein,\textsuperscript{93, 94} which can be seen in the binding of Cu\textsuperscript{+} to the C16C19 peptide.\textsuperscript{93}\ This peptide contains two cysteine residues at specific predesigned positions and exists within a random coil conformation. However, binding the metal ion produces a dramatic conformational change to a four stranded coiled-coil containing a tetranuclear copper cluster (Figure I.7.). In all of the examples above, peptide coordination by metal systems depends on the initial design of the peptide scaffold. In particular, the energy of metal-ligand binding sets in motion many weak peptide-peptide interactions which are responsible for the final protein folding. Therefore, all of these studies consider the combined forces of metal-ligand binding and internal peptide interactions which influence final metalloprotein folding. This leaves a gap in the studies of
metal-peptide interactions where metals individually coordinate proteins which are not initially designed to fold together.

![Figure I.7. Computer generated model of the Cu(I) adduct of C16C19-GGY. The etalloprotein exists as a four-stranded-helical bundle which contains a cyclic Cu(I)₄S₄(N/O)₄ cofactor (adapted from reference 105).](image)

Other peptide chemists have made significant strides towards incorporating unnatural amino acids into de-novo designed proteins.⁹⁵,⁹⁶ These investigations seek a better understanding of metalloprotein functions, but aim at expanding possible applications for these systems. There are two main directions of these studies. The first is de-novo design or modifications of natural proteins by the incorporation of unnatural amino-acids or non-natural metal-containing cofactors.⁹⁷,¹⁰⁰ The purpose for these studies is to produce enzymatic or other activities in the investigated proteins. Another direction is studying peptide “micro structures”, when small peptide molecules are utilized as simplified models for studying metal-peptide interactions. Most
of this research is focused on the stabilization of peptide microstructures.\textsuperscript{101-104} The variety of turn/loop-motifs which can be obtained in this manner can potentially be precursors for the construction of new metal-peptide materials. The intramolecular metal-peptide interaction under investigation in all of these studies leaves a gap in the studies of the formation of intermolecular metal-peptide complexes.

Nature utilizes many weak non-covalent interactions to hold polypeptides in stable three dimensional structures. We have shown that these weak interactions can potentially be replaced by a single kinetically inert bond to produce new protein structures.\textsuperscript{105} In this work we explored the directional bonding properties of coordination compounds to orient synthetic proteins for the formation of metal-peptide assemblies in a new manner. The development of such assemblies is very promising for creating a protein framework in which the positioning of metal groups is well defined. Because the distance between the metal groups is controlled, such assemblies could be used for “bottom-up” design of metal-peptide complexes for studying photo-induced charged separation.
I.2. STRUCTURAL DESIGN

The structural design of the metalloproteins studied in this dissertation combines the principles of supramolecular coordination chemistry with *de novo* protein design in order to produce new types of metal-peptide nanoassemblies. This design is inspired by the success of coordination chemistry in the construction of molecular metalo-organic, macrocyclic structures, some of which have photoactive properties.$^{14}$ We are trying to construct new bioinorganic assemblies based on metal-coordination binding principles. The formation of Pd/Pt or Re complexes with various peptide ligands for the construction of molecular frameworks is the first step in the design of such assemblies. There are two main criteria which must be reached for successful design of such structures:

1. **Engineering of peptide ligand with well defined metal-to-metal geometries**

2. **The formation of the complex through non-covalent, reversible interactions**

A well defined geometry of the peptide ligand is necessary for the peptides to be oriented by metal coordination into high ordered molecular assemblies. Among the various possible geometries, linear metal-to-metal geometry is the simplest and easiest to design. Concurrently, the formation of non-covalent, reversible bonding is the key component for the synthesis of such assemblies. Such interactions can be achieved through engineering labile metal-ligand coordination bonds in the metal center or non-covalent peptide-peptide interaction. Metal-ligand interaction can be easily manipulated by changing the metal binding center. In contrast, engineering of stable non-covalent peptide-peptide bonding forces is a challenge. Recent
achievements in the bottom-up design of natural coiled-coil dimer motifs made this possible. Therefore, in the design of metal-peptide complexes for the formation of metal-peptide assemblies, both inorganic and biochemical approaches for the formation non-covalent interaction need to be considered.

Figure 1.8. Computer generated model of a metal-peptide complex with two metal complexes engineered in positions opposite each-other (left). Knob into holes model of coiled-coil structure (right) (adapted from reference 40).

The work of our research groups utilizes the metalation reaction of de-novo designed peptides for investigating electron-transfer in proteins. These studies use proteins of well defined geometry for the positioning of various metal complexes
within their structure. The known geometry of these peptides allows for the positioning of metal complexes with different oxidation potentials at a distance where electron transfer rate can be studied. Among the most well-studied of such peptides are the synthetic, two-stranded \(\alpha\)-helical coiled-coils, which were modeled after a common protein dimer motif found in biology. These structures consist of an inter-twining of two \(\alpha\)-helices to form a left-handed supercoil\cite{115,116}, and have recently been applied in the design of new supramolecular architectures\cite{117-119}. Figure I.2.1 shows an example of a metal-peptide complex which was studied by our group. In this coiled-coil peptide, two metal complexes are positioned opposite each-other for the study of electron transfer through the hydrophobic peptide interior. Importantly, this design makes the coiled-coil peptide act like a metal ligand with linear metal-to-metal geometry. Therefore, this peptide was applied in this design as a ligand, which can be coordinated by metal complexes into metal-peptide assemblies.

\(\alpha\)-helical coiled-coil structures are formed through the organization of non-covalent interfaces which can be simply shown as the “knobs into holes” packing of hydrophobic side-chains (Figure I.8.). Hydrophobic interactions are the main driving forces responsible for oligomerization of the peptides\cite{120}. The amino acids sequence of synthetic coiled-coil peptides can be based on a seven residue heptad repeat\cite{121} as shown in figure I.9. This repeat can be denoted as: \((abcdefg)\text{n}\), where positions a and d of each heptad are occupied by hydrophobic amino acids thus forming a hydrophobic core. An interesting feature of these structures is that their oligomerization states can be changed by subtle alterations of the packing interactions occurring within their hydrophobic cores, even through single amino acid substitutions. Positions b, c, f, e and g are filled by residues which are exposed to a hydrophilic environment. Amino acids at the e and g positions have the ability to form salt bridges, which further stabilize the
Amino acid residues at positions b, c and f form the hydrophilic part of the molecule, and have the smallest effect on the conformation and oligomerization state of coiled coil peptides. This gives scientists some freedom in engineering various functional groups by the substitution of amino acids residues within the hydrophilic part of the structure. Within this portion of the peptide, incorporation of a metal bonding site in the f position allows straight line positioning of the metal binding sites and is necessary for direct coordination of a metal complex.

Figure 1.9. Schematic views of synthetic coiled coil. Top: primary amino acid sequence of the two helices of the coiled coil. Left: helices in parallel orientation. Right: helical wheel diagram of the coiled coil; view down helical axes of the coiled coil (adapted from reference 120).
in the design of directed metal complexes. Histidine does not form directed bonds with metal complexes as well because it has two nitrogen atoms, both of which can form coordination bonds. In order to create coordination bonds between the peptide and metal complex, a strong metal-binding site with the properties of the formation of direct a coordination bond was necessary to include into the peptide structure. For this purpose, we utilized the coordination properties of an unnatural amino acid 4-pyridylalanine. This amino acid contains a para-substituted pyridine ring which is known to form strong, highly directional coordination bonds with a variety of metal complexes.

All the peptides used in this work form double-stranded parallel α-helical coiled-coils. Two principally different amino acid sequences were used to design an α-helical coiled-coil structure. The de-novo design of the first peptide sequence is based on a naturally occurring GCN4 leucine zipper. Among the natural peptides which form coiled-coil structures, GCN4 is one of the most studied and was a model for de-novo design a variety of proteins. The second peptide sequence is an artificial peptide sequence. It was recently shown that peptides of this sequence form one of the most stable α-helical coiled-coil structures. The original peptide sequence is based on IEALEGK heptad repeat and is named Pal14. This peptide has been extensively used by our group to prepare a variety of metal-substituted, two-stranded α-helical coiled-coils. Another alternative is a new peptide (Pal14new), whose sequence is based on IAAL EQK heptad repeat. This peptide has been shown to form very stable two stranded α-helical coiled-coils. In both cases, the non-natural amino acid 4-pyridylalanine (Pal) was placed at position 14 of these sequences, which is the most solvent-exposed position of the second heptad repeat. This modification was made in order to incorporate a strong metal binding
site into the middle of the peptides which were expected to form side-to-side assemblies after metal bonding.

Both $\alpha$-helical coiled-coil peptides based on the original and new sequences are used as bridging ligands in the reaction with square planar metal complexes. In particular, $[\text{Pt}(\text{en})\text{X}_2]$ or $\text{fac-}[\text{Re(CO)}_3]$ complexes were used to join the peptide molecules in geometries dictated by the steric demands of the metal center, where en = ethylenediamine. Complexes of platinum(II) and Re(I) are known to form highly stable metal-pyridine bonds which do not readily dissociate under mild conditions and simplifies the characterization of the metal-peptide products.\textsuperscript{132-135} Pt(en) and Re(CO)$_3$ cores have been extensively used by researchers in the field of coordination chemistry to prepare a variety of higher ordered molecular structures with some interesting properties.\textsuperscript{47, 53, 54, 56-58, 61, 134-139}

Because the designed assemblies consist of two well distinguished parts, such as a metal center and peptide ligands, two different strategies for the formation of metal-peptide assemblies were considered. The first is the formation of a metal-peptide complex which then will form assemblies through the formation of coiled-coil structures. The other is the formation of the assemblies through the coordination of peptide ligands by a metal complex. Within both of these strategies, the effect of multiple metal bonding sites on the structure of the formed assemblies is of interest as well. Therefore this work was divided into three parts based on the coordination properties.

In the first part of this work, we explored the possibility of using non-covalent coiled-coil forces to construct high order structures. In particular, we focused on the production of a stable metal-peptide complex which can self-assemble through the formation of coiled coils. The peptides used in this design have only one metal binding site and therefore can be considered as
a mono-dentate ligand. Nevertheless, the ability of such peptide to form a stable dimeric structure drives the formed metal-peptide complex into high molecular weight assemblies of different shapes. The schematic view of this strategy is shown on the figure I.10:

![Self-assembly of metal-peptide complexes](image)

**Figure I.10.** The strategies of producing of nano-assemblies through the formation of non-covalent coiled-coil structure.

In the second part of the work, the principles of supramolecular chemistry were applied for the synthesis of self-assembled structures, which are based on peptide ligands. Here the driving force of the assembly formation is the reaction of the metal complexes with 4-pyridylalanine. In particular, we use coiled-coil peptides as ligands to form a high order self-assembly through the coordination of metal complexes. In order to reach this goal, covalently crosslinked coiled-coils were used to prevent dissociation of the coiled-coil peptide structure.
This modification allows the coiled-coil peptide structure to form a stable linear bridging ligand. The schematic view of this strategy is shown in figure I.11:

**Pre-crosslinked coiled-coils as bridging ligand**

![Diagram](image)

**Figure I.11.** The strategies of producing of nano-assemblies through the formation of non-covalent coiled-coil structure.

The final part of this dissertation explores the use of multiple metal binding sites in the hydrophilic exterior of a two-stranded coiled-coil to manipulate the spatial organization of peptide assemblies. Incorporation of multiple metal binding sites decreases the degree of freedom of the resulting metal-peptide complexes, which forces the formation of a cyclic, enthalpy driven product rather than polymeric, entropy driven products.

The work in this PhD project focuses on the construction of well defined molecular frames through metal coordination of peptide molecules. Understanding metal-peptide...
interaction and contribution of metal ions to the stability of protein structures are the main goals of our research. The work to be described is necessary to better understand metal-protein interactions and their applications for the preparation of new metal-peptide structures.
CHAPTER I REFERENCES:


15. Becquere´l, E., Comptes Rendues 1839, 6, 561.


CHAPTER II: EXPERIMENTAL

II.1 Materials

The reagents pyridine, chloropentaammine ruthenium (III) chloride, dichloromethane, and β-mercaptoethanol were purchased from the Sigma-Aldrich Company (St. Louis, MO). Dichloro(ethylenediamine)platinum (II) and palladium (II) chloride were purchased from Alfa Aesar (Ward Hill, MA). The Fmoc-protected L-amino acid derivatives including 4-pyridylalanine (PAL), 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide (HBTU), anhydrous 1-hydroxybenzotriazole (HOBt), piperidine and diisopropylcarbodiimide were purchased from Peptide International Inc. (Louisville, KY). HPLC purity methanol and acetonitrile were purchased from EMD chemicals (Gibbstown, NJ). All reagents were used as received without further purification.

II.2 General Methods

All peptide purification and peptide metalation reactions were monitored by reverse-phase HPLC analyses on either a preparative reversed-phase Vydac C-18 column (10µM particle size, 22×250 mm) or a semipreparative Vydac C-18 column (10 µM particle size, 10×250 mm). A linear gradient of water/acetonitrile containing 0.1 % (v/v) trifluoroacetic acid was used as the mobile phase as shown in Figure II.2. (red line). In some cases a water/methanol mixture containing 0.1 % (v/v) trifluoroacetic acid was used as the mobile phase for purification of metal-peptide complexes (Figure II.2. (blue line). The HPLC runs were performed over 90 min using the flow rate of 2 ml/min for
semipreparative and 5 - 6 ml/min for preparative columns. For HPLC separations, the monitoring wavelengths were set a wavelength range of 210-262 nm. A two-pump system (Waters Model 515) equipped with a Waters Model 996 diode array detector/spectrophotometer having a 1-cm path length cell was used. The collected peptides were lyophilized and the purity of the collected peptide was verified by analytical HPLC, MALDI-MS and ESI-MS. Two stranded α-helical coiled-coil conformation for apopeptides were confirmed by circular dichroism spectroscopy (CD) and high performance size exclusion chromatography (HPSEC) experiments. Absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. MALDI-TOF mass spectroscopy was performed on Bruker Daltonics Omniflex MALDI-TOF spectrometer. Electrospray ionization mass spectrometry was performed on Shimadzu LCMS 2010A. $^1$HNMR and $^1$HCOSY spectroscopy was performed on Bruker Avance 300. Circular dichroism spectra were obtained using Aviv model 202-01 DS circular dichroism spectrometer (Lakewood, NJ) equipped with a thermoelectric temperature controller. Fluorescence spectra were obtained with a single photon counting spectrofluorimeter from Edinburgh Analytical Instruments (FL/FS 900). $^1$

![Figure II.2. Gradient of water/acetonitrile and water/methanol used in HPLC experiments.](image-url)
II.3 Peptide synthesis

All peptides were synthesized using solid-phase methods on an Applied Biosystems Model 433 A peptide synthesizer by standard Fmoc-chemistry. The 0.25 mmol scale protocol with a N-terminal capping protection strategy by acetic anhydride was used. Activation was achieved by 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide (HBTU), and 1-hydroxybenzotriazole (HOBt) in DMF. Deprotection of the amino acid side chains and cleavage from the resin was performed by reaction with a mixture of trifluoroacetic acid (TFA) (82.5% v/v), phenol (5% v/v), dithiothreitol (DTT) (2.5% v/v), thioanisole (5% v/v) and water (5% v/v) for 2.5 hours at room temperature. The crude peptide was then precipitated in cold anhydrous diethyl ether, collected by vacuum filtration and dried under vacuum. Final purification was achieved by preparative reversed-phase C18 HPLC as described in the General Methods section.

II.4 Formation of covalent Cys-Cys crosslinking

Oxidation of cysteine residues was performed by iodine in acetic acid/water mixture (1:1). 5 mg of iodine were dissolved in 5 ml of glacial acetic acid and added to 5 ml of water containing 40 mg of peptide. The reaction mixture was stirred at 40°C for 45 minutes. To stop the reaction, 20-30 ml of water/acetonitrile (1:1) were added to the flask. The reaction mixture then was evaporated several times from the mixture of water/acetonitrile to remove excess iodine. The solid residue was dissolved in H₂O and purified by HPLC. The formation of crosslinked peptide was proven by MALDI-MS, SDS-PAGE and ESI_MS molecular weight observation.
II.5 Synthesis of Dichloro(ethylenediamine) palladium (II)

Synthesis of dichloro(ethylenediamine) palladium (II) was performed as described earlier. \(^2\) 0.5 g PdCl\(_2\) was dissolved under reflux in the mixture of 10 ml H\(_2\)O and 3 ml of HCl. The clear solution was filtrated and NaOH was added until the pH of the mixture become within pH 2-3. An equal molar amount of ethylenediamine was added drop wise to warm PdCl\(_4\)\(^2\). After the pH was adjusted to pH 2-3 by HCl, the solution color changed from red to yellow. Yellow crystals formed after keeping the reaction mixture overnight in the hood under room temperature. The precipitate was filtrated and washed with H\(_2\)O. Dinitro(ethylenediamine)palladium (II) was synthesized by reaction with an equal molar amount of silver nitrate in H\(_2\)O.

II.6 Synthesis of dinitro(ethylenediamine) platinum (II)

Similar to the synthesis dinitro(ethylenediamine)palladium (II), Dinitro(ethylenediamine)platinum(II) was synthesized by reaction of dichloro(ethylenediamine)platinum(II) with an equal molar amount of silver nitrate in water.

II.7 Synthesis of metal-peptide complexes

The metal peptide complexes were prepared by incubating Pt(en)(NO\(_3\))\(_2\) or [NEt\(_4\)]\(_2\)[ReBr\(_3\)(CO)\(_3\)] with the peptides. The platinum-peptide reaction was carried out in D\(_2\)O at 60°\(\text{C}\) for one week. The rhenium-peptide reaction was carried out in methanol at 40°\(\text{C}\) for one week.
II.8 Circular dichroism spectroscopy

All the circular dichroism spectra were obtained in double distilled water (ddH$_2$O) or phosphate buffers of different concentration (10-50 mM KH$_2$PO$_4$/ 20-100 mM KCl, pH 7). A rectangular 1 mm or 5-mm cell was used. The spectra were obtained as an average of 5 sec readings using 1nm wavelength step. Mean residue molar ellipticities were calculated according to the equation:

$$[\theta] = \frac{[\theta]_{\text{obs}}}{(10* l \cdot c \cdot n)}$$

where $[\theta]_{\text{obs}}$ is the observed ellipticity measured in degrees, $l$ is the path length of the cell in centimeters, $c$ is the molar peptide concentration, and $n$ is the number of amino acid residues in the peptides.

Calculation of the helicity of the apopeptides and peptide molecules in metal-peptide complexes is made based on suggestion that 100% helicity is $34'000 \text{ deg cm}^2 \text{ dmol}^{-1}$

The 100% helix can be calculated from the equation:

$$-40000 \times [(n - 4)/n]$$

where $n$ is the number of residues.\(^3\)\(^4\) The concentration of apopeptides was determined using the molar extinction coefficient of para-substituted pyridine for phosphate buffer (pH 7) $\varepsilon$ (256 nm) = 1995 M$^{-1}$cm$^{-1}$ and for water (pH < 5.0) $\varepsilon$ (256 nm) = 3000 M$^{-1}$cm$^{-1}$. The concentration of metal-peptide complex was determined using the molar extinction coefficient of the para-substituted pyridine $\varepsilon$ (250 nm) = 3500 M$^{-1}$cm$^{-1}$ for platinum-peptide complexes and $\varepsilon$ (262 nm) = 4000 M$^{-1}$cm$^{-1}$ for rhenium-peptide complexes.
II.9 Conformational analysis

The conformational stability of the peptides was determined from GdnHCl denaturation studies which were performed by monitoring the change of ellipticity at 222 nm with increasing GdnHCl concentrations. The experiment was carried out in phosphate buffer (50 mM PO4, 100 mM KCl, pH 7.0) with 0-8 M concentration of GdnHCl.

Analysis of GdnHCl denaturation curves was performed using a two-state unfolding model to determine the fraction folding. The fraction folding ($F_f$) can be calculated from the equation:

$$F_f = ([\theta]_{obs} - [\theta]_d)/([\theta]_f - [\theta]_d)$$

where $[\theta]_{obs}$ is the observed molar ellipticity measured in degrees, $[\theta]_f$ and $[\theta]_d$ are the ellipticities of the folded and denatured states, respectively.

The free energy of unfolding was calculated according to the equation:

$$\Delta G = -RT\ln(2c F_u^2/F_f)$$

where $R$ is the molar gas constant, $T$ is the temperature in Kelvin, $c$ is the peptide concentration, and $F_u$ is the fraction unfolded which is denoted as:

$$F_u = 1 - F_f$$

Free energy of unfolding was calculated based on assumption of a linear relationship between $\Delta G$ and [GdnHCl]. The value of $\Delta G^{H2O}$ was determined by linear extrapolation method using equations:

$$\Delta G = \Delta G^{H2O} - [\text{GdnHCl}]$$

The difference in the free energies of unfolding of two peptides ($\Delta G$) was calculated as described before by subtracting $\Delta G$ values of a pair of peptides at the $[\text{GdnHCl}]_{1/2}$ of the peptide.
II.10 SDS-PAGE gel electrophoresis

SDS-PAGE electrophoreses were run on a mini-PROTEAN II Cell using a Bio-RAD power PAC200 power supply. For the SDS-PAGE experiments a discontinuous gel system was used having a 4% stacking gel (containing acrylamide (37.5:1), 0.5 M Tris pH = 6.8, ddH2O, APS, and TEMED) and a 16.5% resolving gel (containing 40% acrylamide (37.5:1), 3 M Tris-Cl 0.3% SDS pH = 8.45, glycerol, ddH2O, APS, TEMED) The plates were prerun for 10 minutes at 20 mA (for 2 plates) with a sample buffer containing 0.1% SDS; 0.2 M Tris-Cl pH = 8.9 and a running (bottom) buffer containing 0.1% SDS; 0.1 M Tris base, 0.1 M tricine.

The gels were stained with Coomassie blue containing 0.25% Coomassie R-50, 50% methanol, and 7.5% glacial acetic acid for 10-30 minutes, until the gel would turn dark blue. The gel was then placed in a destaining buffer contained 10% methanol and 10% glacial acetic acid for overnight.

II.11 High performance size exclusion chromatography (HPSEC)

HPSEC experiments were performed on a Superdex 75 (molecular weight determination rang is 3-70 kDa for globular proteins) or Superdex 200 (molecular weight determination rang is 10-300 kDa for globular proteins) Amersham Pharmacia Biotech columns connected to a Waters Model 515 pump equipped with Waters Model 996 diode array detector. The peptide samples were eluted using 50 mM KH2PO4/ 100 mM KCl, pH7, with 0.2-0.4 ml/min flaw rate, and monitored at a wavelength range of 210-262 nm. To keep reproducibility of the results, purification of the columns using 0.5 M sodium
hydroxide was performed after each 15-20 runs. The estimation of molecular weight was performed on calculation of $K_d$.

The $K_d$ can be calculated from the equation$^8,9$:

$$K_d = \frac{(V_{act} - V_0)}{(V_t - V_0)}$$

Where $V_{act}$ is the elution volume of the solute, $V_0$ is void volume of the packing (obtained from the elution time of blue dextran) and $V_t$ is the total accessible volume of the column (obtained from the elution time of b-mercaptoethanol).

### II.12 Atomic force microscopy

Atomic force microscopy (AFM) experiments were performed at the AFM Core Facility at the Davis Heart and Lung Research Institute and Biomedical Engineering Center, Ohio State University using a Digital Instrument Multimode AFM in tapping mode equipped with MicroMasch NSC15 probes. Two different techniques (evaporation and incubation-rinsing) were used to prepare samples for the experiments.

In the evaporation technique, samples were prepared at concentration ranging from 0.1 mg/ml to 0.001 mg/ml in water. Two microliters of the solution were spotted onto freshly peeled muscovite mica and air-dried before imaging. In the incubation-rinsing technique, samples were prepared by depositing 100 μl of a sample solution on freshly cleaved mica. After incubation for 10 min, the samples were rinsed with filtered water and dried under a steam of nitrogen. Concentrations of samples in this AFM experiment were in the range from 0.1 mg/ml to 0.05 mg/ml. All data was analyzed by WSxM Develop 10.0 software and transferred into digital (JPEG) files.
II.13 TEM microscopy

Transmission electron microscopy (TEM) experiments were performed at the Medical University of Ohio on a Philips CM 10 transmission electron microscope. The samples of different concentration (100-5 mM) were applied onto Collodion coated grids for 1 minute and dried. The dried samples were negatively stained for 30-60 seconds with 0.5% uranyl acetate.

II.14 Light scattering

The light scattering experiments were carried out on a static laser scattering Waytt miniDAWN instrument and a quasi elastic light scattering detector Waytt QELS. The change of reflective index during the experiment (dn/dc) was monitoring by in-line reflective index detector Optilab ret. All the detectors were set up in-line with a Shimadzu LC-10AT liquid chromatograph equipped with a Shimadzu SPD-10AV UV-Vis detector having a 1-cm path length cell.

The value of dn/dc for apopeptides and metal-peptide complexes was determined by plotting the absolute value of the reflective index of the samples with known concentration against the peptide concentration, and can be determined by:

$$n_{\text{sample}} = (\text{dn/dc}) \cdot c_{\text{sample}} + n_{\text{H2O}}$$

This assumes a linear relationship between change of reflective (dn) index and concentration of the samples (dc). An alternative for that was the determination of dn/dc values from the signal of the in-line UV-Vis spectrometer by recalculation of the UV signal into the sample concentration. All light scattering data was analyzed using Astra V software.
CHAPTER II REFERENCES:


CHAPTER III: FORMATION OF METAL COMPLEXES WITH PEPTIDE AS MONODENTATE LIGANDS

III.1 STRUCTURAL DESIGN

III.1.1 Introduction

The following part in this dissertation describes the formation of new metal-peptide assemblies through non-covalent peptide-peptide interactions. The presented approach is focused on the formation of a stable metal-peptide complex, which will further form the assemblies through the formation of α-helical coiled-coil structures. The peptides used in this structural design have a single metal binding site, and are therefore a mono-dentate ligand. Nevertheless, the ability of such peptides to form a stable dimeric structure drives the formed metal-peptide complex into high molecular weight assemblies of different shapes. The schematic view of this strategy is shown in figure III.1:

Figure III.1. Strategy the formation of metal-peptide assemblies
III.1.2. Design of peptides ligands

The peptide used in this work forms non-covalent two-stranded α-helical coiled-coils.\textsuperscript{1-6} This design was inspired by the naturally occurring GCN4 leucine zipper.\textsuperscript{7-13} The amino acid sequence of this peptide is based on a seven residue repeat\textsuperscript{1,2,6} (heptad), which can be denoted as (abcdefg)n, where positions \textit{a} and \textit{d} of each heptad are occupied by hydrophobic amino acids (isoleucine and leucine) forming a hydrophobic core. The amino acid residues at \textit{g} and \textit{e} positions (lysine and glutamic acid) form inter-chain salt bridges, which further stabilize the coiled coil structure and are crucial for maintaining the parallel orientation of the peptide. The amino acid residues at positions \textit{b}, \textit{c}, and \textit{f} are exposed to a hydrophilic environment. The \textit{b} position is occupied by glutamic acid residues, which were expected to increase solubility of the peptide. The amino acids residues at \textit{c} and \textit{f} positions are alanine and glycine. Also in this design, the non-natural amino acid 4-pyridylalanine (Pal) is placed at position 14 by substituting one of the glycine amino acid residues in the sequence.\textsuperscript{14} This modification was made in order to incorporate a strong metal binding site into the peptide molecule. Together, this design makes the peptide sequence an IEALEGK heptad repeat which we named Pal14. The design, sequence, and computer model of the peptide is shown in figure III.2:
Figure III.2. Left: Schematic views of the helical wheel chromatogram of the coiled coil; view down helical axes of the coiled coil with pyridine rings at f position. Right: computer generated model of Pal14 peptide in coiled-coil form. Bottom: amino acid sequence of Pal14.

The solid phase method described in the experimental procedure was used to prepare the 30-residue Pal14 polypeptide. The purity of the peptide was confirmed by the appearance of a single peak in the analytical HPLC run. The presence of pyridylalanine amino acid residues in the peptide molecule was proven by a characteristic absorption band at 256 nm in the UV spectrum of the synthesized peptide molecule (Figure III.3 A and B). The accuracy of the synthesis was shown by MALDI mass spectroscopy (Figure III.3 C). MALDI-MS reveals a single peak of the molecular ion (calc’d: 3298.89; obs’d: 3298.81).
Figure III.3. Characterization of apopeptide Pal14: The purity of the peptide was confirmed by analytical HPLC (A), UV spectrum (B) and MALDI-MS (C).

III.1.3. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is used to evaluate the secondary structure of peptides. The CD spectrum of the apopeptide consists of minima at 208 and 222 nm showing that the peptide exists within an α-helical conformation (Figure III.4. (left)). The intensity at 222 nm is used to determine the α-helical content\textsuperscript{15,16} which is 57.3\% ($\theta_{222} = -19.400$ deg cm\textsuperscript{2} dmol\textsuperscript{-1}) for Pal14. The ratio of $\theta_{222}/\theta_{208}$ equals 1.02 for the apopeptide falls within the range generally considered to be indicative of a coiled-coil conformation.\textsuperscript{17} Temperature dependence of CD
signal at 222 nm of the apopeptide shows that the melting point of Pal14 conformation is about 70°C, and is completely denaturated at temperatures above 80°C (Figure III.4. (right)). Together, these results show that the apopeptide exist as a stable, two-stranded α-helical coiled-coils in the absence of metal ions.

Figure III.4. Left: Circular dichroism spectrum of Pal14 in water at 25°C. Concentration of Pal14 is 1x10⁻⁴ mole/L. Right: Temperature dependence of CD signal at 222 nm

III.1.4. Conformational analysis

The stability of the α-helical conformations of the apopeptide was studied by guanidinium chloride (GdnHCl) titration.¹⁸⁻²⁰ Denaturation dependence of peptides with increasing concentrations of GdnHCl allows for estimation of the conformational stability of peptides. This method is more reliable in comparison to others, such as temperature or urea denaturation. Free energy of the coiled-coil unfolding was determined by extrapolating the linear factor in the change of the free energy with increasing concentration of guanidinium chloride to
the y axial (Figure III.5. left). The full description of the procedure is given in the Chapter II. The
denaturation curve and the free energy of the apopeptide’s unfolding are shown in Figure III.5:

![Denaturation plot and free energy of folding](image)

**Figure III.5.** Left: denaturation plot of Pal 14 (85 μM) with increasing concentration of
 guanidinium chloride. Right: The free energy of Pal14 coiled coil folding with increasing
 concentration of guanidinium chloride.

The denaturation midpoint for Pal14 was determined as 2.5 M GdnHCl and \( \Delta G_{\text{H}_2\text{O}} = 7.3 \)
 kcal/mole. The data for Pal14 coiled coil folding is summarized in Table III.1. For comparison,
the previously studied H21(30-mer)\(^1\), which has similar peptide sequence, has been shown toexist as a two-stranded coiled-coil with a monomer-dimer dissociation constant of \( K_d = 1.5 \pm 0.4 \)
μM has \( \Delta G_{\text{H}_2\text{O}} = 7.31 \) kcal/mol which is similar to that of Pal14. Together, these results show that
Pal14 forms stable two-stranded α-helical coiled-coils, and therefore can be used as a metal
ligand for the formation of non covalent metal-peptide assemblies.

**Table III.1.** Conformational stability data for Pal14.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Helicity in H(_2)O (%)</th>
<th>[GdnHCl](_{\text{m}})</th>
<th>Slope (m)</th>
<th>( \Delta G_{\text{H}_2\text{O}} ) (kcal)</th>
<th>( K_d ) (app)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pal14</td>
<td>57.3</td>
<td>2.5</td>
<td>0.674</td>
<td>7.3</td>
<td>4.1 x 10(^{-6})</td>
</tr>
</tbody>
</table>
III.2. RHENIUM PEPTIDE COMPLEX

III.2.1. Complex of Re as the metal core

The rhenium complex $[\text{NEt}_4]_2[\text{ReBr}_3(\text{CO})_3]$ was examined for its ability to form the metal-peptide complex. The Re complex has an octahedral geometry in which the two labile bromine ligands are 90° to each other and can be easily substituted by stronger ligands such as pyridine. This property makes the $[\text{NEt}_4]_2[\text{ReBr}_3(\text{CO})_3]$ complex a good target for our design. Re(CO)$_3$ cores have been extensively used by Hupp and co-workers to prepare square molecular structures which have a variety of interesting properties.

Aliphatic para-substitutions in the pyridine ring do not strongly change its reactivity or the UV spectrum. The UV spectrum of Pal14 is similar to the spectrum of pyridine, and has one absorption band centered at 256nm. This makes the metal-pyridine reaction an appropriate model reaction to study the formation of the metal-peptide complexes. The UV spectrum of Re(CO)$_3$BrPy$_2$ was used as a model to observe the change in the spectrum of the apopeptide during the reaction (Figure III.6. (left)). The UV spectrum of Re(CO)$_3$BrPy$_2$ consists of an absorption band at 264 with a shoulder at 300 nm, which are the ligand-centered and metal-to-ligand-charge-transfer bands respectively. Therefore, the formation of the coordination bond between Re(CO)$_3$ core and pyridine ring within the peptide molecules can be identified by this characteristic change in the UV spectra of the pyridyl ligand.

To create the designed metal-peptide assemblies, Pal14 was reacted in methanol at 37 °C with equalmolar amounts of $[\text{NEt}_4]_2[\text{ReBr}_3(\text{CO})_3]$. These conditions previously were used to form the tricarbonyl rhenium(I) complexes of phosphine-derivatized amino acids. Figure III.6. (right) shows the UV spectra of apopeptide Pal14 and the reaction mixture of Pal114 with
rhenium complex. After the metal binding, the spectrum of the reaction mixture shows a new absorption band centered at 264 with a shoulder appearing at 300 nm which is similar to the spectrum of Re(CO)₃BrPy₂. This spectrum confirms the formation of the metal-peptide conjugate, and confirms that the reaction of the complex formation between the Re(CO) core and the peptide ligands can be monitored by UV spectroscopy.

**Figure III.6.** Left: UV/Vis spectrum of Re(CO)₃BrPy₂. Right: comparison of the UV/Vis spectrum of apopeptide before (black) and after (red) binding to Re(CO)₃ core.

### III.2.2. SDS-PAGE experiment

The formation of rhenium-peptide complexes was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The denaturizing condition of this experiment allows for separation of peptide molecules by molecular weight. This technique is found to be accurate in determining molecular weights within the range of 0.5-200 kDa with an estimated error about 10%.
The apopeptide exists as a monomer species with a molecular weight of approximately 3.3 kDa under the denaturing conditions of the SDS-PAGE experiment. On the other hand, the molecular weight of the di-substituted metal-peptide product corresponds to the molecular weight of two peptide molecules crosslinked together by rhenium complex, which is about twice bigger than the apopeptide alone, about 6.5 kDa. Therefore, the formation of the di-substituted platinum-peptide complex can be monitoring by SDS-PAGE experiment.

The formation of the rhenium-peptide complex is clearly seen in the SDS-PAGE experiment by the appearance of a new band with a molecular weight 6.5 kDa (Figure III.7). The Pal14 apopeptide (first band) is used as an extra standard for monitoring the di-substituted metal-peptide complex formation. The intensity of the bands in SDS-PAGE allows for determination of relative product concentrations. The intensity of the two bands which correspond to the apopeptide and the di-substituted metal-peptide complex are similar for the rhenium-Pal14 reaction mixtures. This suggests equal amounts of the apopeptide and di-substituted metal-peptide complex species in the reaction mixtures.

Figure III.7.SDS-PAGE experiment of the reaction mixture of the rhenium complex with Pal14.
III.2.3. Circular dichroism spectroscopy

Circular dichroism spectroscopy is a valuable tool for the characterization of secondary peptide structures in solutions. The spectrum of the rhenium-Pal14 reaction mixture in methanol consists of negative bands at 208 and 222 nm indicating \( \alpha \)-helical peptide conformation. The ellipticity ratio of \( \frac{\theta_{222}}{\theta_{208}} \) of this spectrum is 0.85 which is indicative of a monomeric \( \alpha \)-helical helix\(^2\) (Figure III.8.). This spectrum shows that alcohol solvents maintain the \( \alpha \)-helical structure, but disturb the hydrophobic interactions of the \( \alpha \)-helical coiled-coil structure. Therefore it was concluded that Pal14 exists as a monomer \( \alpha \)-helical helix in methanol.

![Figure III.8. Circular dichroism spectrum of the rhenium-Pal14 reaction mixture in methanol.](image-url)
The circular dichroism spectrum of Pal14 is solvent dependent; the peptide exists as a monomeric \( \alpha \)-helical helix in methanol, which is considered to be a denaturant. However, this peptide forms coiled-coils in aqueous solutions and therefore the formation of metal-peptide assemblies should be characterized under aqueous non-denaturizing conditions, such us the conditions of high performance size exclusion chromatography (Chapter II).

### III.2.4. Size exclusion high performance chromatography

High performance size exclusion chromatography (HPSEC) was found to be very useful in the characterization of non-covalent peptide-peptide complexes in solution.\textsuperscript{27,28} This technique is especially useful for the characterization of the assemblies, the low bonding energy of which does not allow application of other techniques such as SDS-PAGE and mass spectroscopy. An example of this assembly is a non-covalent \( \alpha \)-helical coiled coil.

![Figure III.9. Left: HPSEC experiment of Pal14. Right: Calibration graph for SE Superdex 75 in phosphate buffer pH7.](image)
The di-substituted rhenium-peptide complex was expected to assemble into high molecular weight structures through the formation of a coiled-coil. Characterization of these self-assembled products was obtained by HPSEC experiments which were performed under non-denaturing conditions (see Chapter II). The molecular weight was determined by comparison to a calibration graph of the peptides of known molecular weights (Figure III.9 (right)). The HPSEC experiment for the apopeptide shows the presence of assemblies with the mass of a peptide dimer (Mw = 6.6 kDa) which corresponds to a coil-coiled structure (Figure III.9.). This elution time is used as a reference in the following HPSEC experiments.

The HPSEC chromatogram of the rhenium-Pal14 reaction mixture shows the presence of a small shoulder which is not present in the sample of the apopeptide alone (Figure III.10.). This shoulder, which appears earlier than the peak of the apopeptide at 30 min in the chromatogram, indicates the presence of a non-covalent high molecular weight (Mw > 6.6 kDa) species in the solution. However, the main product in this reaction mixture is unreacted apopeptide or mono-substituted rhenium-Pal14 complex which are unable to form metal-peptide assembles. The intensity of the shoulder in comparison to the intensity of the peak of the apopeptide reveals the presence of a di-substituted Re-Pal14 complex that is about 15% of the reaction mixture. This is three times less then that determined in the SDS-PAGE experiment. An attempt to collect and characterize this high molecular weight fraction was not successful.
Figure III.10. HPSEC of reaction mixture of the rhenium complex with Pal14. Phosphate buffer pH7 Superdex 75

III.2.5. Reverse phase high performance liquid chromatography

The formation of the rhenium-peptide complex was monitored by reverse-phase high performance liquid chromatography (HPLC). The HPLC data for the rhenium-Pal14 reaction mixture shows two new peaks appearing at slightly longer retention times than that of the Pal14 starting material (Figure III.10.). Both products display new absorption bands at 264 and 300 (shoulder) nm, which is similar to the UV- spectrum of the related rhenium-pyridine complex (Figure III.6.). After 4 days of reaction, the peaks having the intermediate retention time dominated the chromatogram, and was assigned to a metal-peptide complex in which one or two Pal-14 peptides are coordinated to the rhenium center.
The purification of the rhenium-peptide reaction products was performed by the collection of the fraction in the HPLC experiment. The further characterization of the collected samples by reinjection into HPLC revealed the presence of only apopeptide. Any attempts to characterize the collected fractions by HPSEC and SDS-PAGE experiments show the similar results. Together, these results indicate a low stability of the separated rhenium-Pal14 complexes, which mostly decompose under the conditions of HPLC.
III.2.6. Cross-linking by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

Covalent crosslinking is a valuable technique for the characterization of non-covalent protein complexes. Therefore, more information about the high molecular weight structures formed by the metal-peptide complexes was expected to be obtained by trapping these non-covalent assemblies through covalent crosslinking.

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was chosen as the crosslinking agent. The chemistry of EDC peptide crosslinking is the formation of a covalent (peptide) bond between carboxyl and amino groups in side chains of amino acids residues, and usually happens only between associated peptide molecules.\textsuperscript{29,30} There are 4 lysines and 8 glutamic acid groups in each molecule of Pal14. The amino acid residues, which form salt bridges between the g and e positions in the peptide molecules, are the aim of the crosslinking reaction.

Figure III.12. SDS-PAGE experiment for crosslinked pure Pal14neutral peptide and reaction mix of Pal14neutral by EDC. Lane 1: protein markers, Lane 2: Pal-14 (3.3 kDa), Lanes 3-6: corner reaction mixture crosslinked with EDC.
Reaction conditions for EDC cross-linking are methanol-water solution with 20 minutes of incubation. The SDS-PAGE analysis of the crosslinked reaction mixture shows the presence of several discrete high molecular weight species (Figure III.12.). Two bands in this mixture have a higher intensity in comparison with others. The molecular weight of these bands are somewhere in between 25 and 40 kDa. The domination of the species with these molecular weights suggests some organization in the formation of the assemblies.

III.2.7 Summary

The di-substituted rhenium-Pal14 complex was formed and has shown to assemble into high molecular weight species through the formation of coiled-coils. Unfortunately, the instability of the formed complex, which is shown by HPLC and HPSEC, prevents purification and characterization of the metal-peptide assemblies. Both, HPLC and HPSEC are performed in aqueous solutions, and therefore the instability of the rhenium-peptide complex can be explained by hydrolysis of the rhenium-pyridylalanine bond. Changing the solvent from methanol to water was expected to stabilize the secondary structure of the peptide ligands, and increase the rate of the reaction. However, the rhenium-pyridylalanine complex barely forms in aqueous conditions, which suggests a low stability of the Re-Pal14 bond in water. These properties of the Re complex forced us to find an alternative metal complex that is stable under aqueous conditions for the coordination of the peptide ligands.
III.3. PLATINUM PEPTIDE COMPLEX

III.3.1. Introduction

Ethylenediamine platinum (II) chloride was chosen as an alternative to the rhenium complex. Ethylenediamine platinum (II) is a variant of cisplatin, where the amino groups are substituted by ethylenediamine. Cisplatin is one of the most studied inorganic compounds due to its biological activity. This complex has square planar geometry and coordinates ligands at 90° angles. This complex also forms highly stable coordination bonds with pyridyl nitrogen atoms. Because this bond does not readily dissociate under mild conditions, we utilized it to simplify purification and characterization of the expected metal-peptide products. The chelating effect of ethylenediamine, which is a bidentate ligand, was expected to increase the stability of the platinum complex, and prevent the formation of unwanted products through substitution of the amino groups. To increase the solubility of the platinum complex, the chloride ligands were substituted by nitro groups.

Similar to the rhenium complex, ethylenediamine platinum (II) is used as an inorganic core to create coordination bonds with the peptide metal-binding site, pyridylalanine. The metal peptide complex was prepared by incubation of equal molar amounts of Pt(en)(NO$_3$)$_2$ and Pal14. The concentration of the apopeptide was about 3x10$^{-2}$ mole/liter. The reaction was carried out in H$_2$O at 60°C in one week. For some studies, the reaction was carried out in the presence of Mg$^{2+}$ ion. H$^1$-NMR, NMR-COSY and SDS-PAGE were used to monitor the propagation of the reaction.
III.3.2. Square planer complex of Pt as the metal core

Like the rhenium-peptide reaction, the reaction of [Pt(en)X₂] with pyridine was used as a model to study the formation of the metal-peptide complex. However, the platinum (II)-pyridine bond formation was investigated in more detail.

Monitoring the reaction between Pt(en)(NO₃)₂ and pyridine by UV spectroscopy reveals no large changes in the shape of the absorption spectrum. However, there is a small decrease at 256 nm and a small increase at 240 nm, with a shoulder appearing at 270 nm (Figure III.13.). Nevertheless, these changes are strong enough to confirm the formation of the metal-peptide conjugate of the platinum (II) complex with pyridyl ligands. These results also permit determination of the concentration of the apopeptides and metal-peptide complexes by using the extinction coefficient of pure pyridine at 256 (ε= log3.3) and the Pt-pyridine complex at 250 nm (ε = log3.5).

![Figure III.13. The reaction ethylenediamine platinum (II) with pyridine by UV spectroscopy.](image)

**Figure III.13.** The reaction ethylenediamine platinum (II) with pyridine by UV spectroscopy.
The reaction between ethylenediamine platinum (II) and pyridine is slow, and can be monitored using NMR spectra by examining the chemical shifts of the pyridyl protons signals upon metal binding. The formation of the platinum-pyridine complex is manifested by the appearance of new signals from the mono and di-substituted products. Proton assignment is based on the splitting of the signals and their integrated intensity. The signals of the para ($p$) and meta ($m$) protons are triplet, and have a relative integrated intensity of one and two protons respectively. The signal of the ortho ($o$) protons is a doublet, and has a relative integrated intensity of two protons. The full assignment of the pyridine protons signals before and after platinum binding is shown in Figure III.14.

The mono-substituted platinum-pyridine complex (the signals $o'$, $p'$ and $m'$) forms first, but binds the second pyridine ring quickly, and nearly disappears at the end of the reaction. The reaction is almost complete in 4.5 hours, which can be seen in the $^1$HNMR spectrum where the pyridyl signals of the complex Pt(en) with two pyridines (the signals $o''$, $p''$ and $m''$) have more than 90% of the total signal integration. The chemical shifts of all protons are shown in Table III.2.

<table>
<thead>
<tr>
<th>Table III.2. $^1$H-NMR chemical shifts of pyridine and Pal14 before and after metal coordination.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H NMR signals</td>
</tr>
<tr>
<td>Pyridine</td>
</tr>
</tbody>
</table>
Figure III.14. Reaction of ethylenediamine platinum (II) with pyridine by $^1$HNMR spectroscopy, where "o" are the signal of ortho protons; "m" are the signal of meta protons; "p" are the signal of para protons in the pyridine ring.

The mono substituted platinum-pyridine complex forms during the reaction, but the pathway of the reaction leads to the most thermodynamically stable product: the di-substituted platinum-pyridine complex. The formed platinum-pyridine bond is kinetically inert under ambient conditions (25°C), which suggests irreversibility of the reaction and stability of the formed platinum-peptide complexes.
III.3.3. SDS-PAGE gel electrophoresis

Formation of the platinum-peptide complex was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Pal14 exists as a monomer species with a molecular weight of approximately 3.3 kDa under the denaturing conditions of SDS-page. Similar to the rhenium-peptide reaction, the molecular weight of the di-substituted platinum peptide corresponds to the molecular weight of two peptide molecules crosslinked together by a platinum center (6.5 kDa). Therefore, the propagation of the reaction is marked by the appearance of the band with a molecular weight of 6.5 kDa corresponding to the molecular weight of a di-substituted metal-peptide complex, which is Pt(en)(Pal14)$_2$ (Figure III.15).

Surprisingly, Pal14 does not react completely with the platinum complex. Monitoring of the reaction mixture of ethylenediamine platinum (II) with Pal14 (Figure III.15.) with time reveals the presence of both the di-substituted platinum-peptide complex and apopeptide, even after 8 days of reaction.

![Figure III.15. Monitoring of the formation of the platinum complex with Pal14 by SDS-PAGE experiment.](image-url)
The band intensity, which allows relative determination of the sample concentration, of the apopeptide and di-substituted platinum-peptide complex bands are similar for both the platinum-Pal14 and rhenium-Pal14 reaction mixtures. This suggests equal amounts of monomer and di-substituted metal-peptide complex species in both reaction mixtures.

III.3.4. NMR spectroscopy

NMR spectroscopy and X-ray crystallography are the most commonly used techniques for the characterization of supramolecular structures in coordination chemistry. In the current system, the $^1$HNMR signals of the pyridine ring in pyridylalanine are located in the aromatic region, which is separated (> 4 ppm) from the aliphatic proton signals in the peptides and proteins. The signals of the pyridyl protons overlap with the signals of the amide protons in the peptide backbones. However, the amide protons in the peptide backbones are usually in exchange with protons of polar solvents, and therefore their signals should disappear if the solvent is deuterated. This makes the NMR signals of pyridyl protons in Pal14 an appropriate indicator of the metal-peptide complex formation.

The formation of the platinum-peptide complex was monitored by H$^1$-NMR spectroscopy. The treatment of Pal14 by D$_2$O does not completely annihilate the signals of amide NH protons in the aromatic region of the $^1$H-NMR spectrum. Nevertheless, the well-dispersed lines of ortho and meta pyridyl protons of apopeptide are clearly seen in the apopeptide NMR spectrum (Figure III.16.). The chemical shifts of the pyridyl protons in D$_2$O are similar for Pal14 and pyridine. This suggests a high similarity in the molecular environment, and subsequently in the metal coordination reactivity. Because of the similarity of pyridine and 4-pyridylalanine, the NMR signals of apopeptide at 8.7 ppm and 8.0 ppm were assigned to the signals of the ortho and
meta pyridyl protons respectively. The cross peak between these signals in the $^1$HCOSY spectrum reveals their coupling with each-other, and supports the assignment of the pyridyl protons in the apopeptide. (Figure III.17. left)

**Figure III.17.** Formation of the platinum-Pal14 complex by H$^1$ NMR-COSY experiment, spectrum is taken in D$_2$O.

The formation of the platinum-Pal14 complex appeared as new signals of pyridine protons in the down field of the NMR spectra. The Pal14 NMR signals of both the ortho and meta pyridyl protons are shifted upfield by 0.25 ppm and 0.52 ppm respectively after the metal binding. All chemical shifts of the pyridyl protons for the apopeptide and pyridine before and after metal coordination reaction are shown in Table III.3:
Table III.3. $^1$H-NMR chemical shifts of pyridine and Pal14 before and after metal coordination.

<table>
<thead>
<tr>
<th>$^1$H NMR signals</th>
<th>ppm ortho protons before metal coordination</th>
<th>ppm ortho protons of mono coordinated product</th>
<th>ppm meta protons of di coordinated product</th>
<th>ppm meta protons of mono coordinated product</th>
<th>ppm meta protons of di coordinated product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>8.61</td>
<td>8.67</td>
<td>8.71</td>
<td>7.68</td>
<td>7.56</td>
</tr>
<tr>
<td>Pal14</td>
<td>8.7</td>
<td>8.45</td>
<td>8.0</td>
<td>7.54</td>
<td>7.48</td>
</tr>
</tbody>
</table>

The new signals in the NMR spectra of the platinum-peptide reaction mixture were assigned to the di-substituted platinum-peptide complex due to the observation of this complex in the SDS-PAGE experiment. Similar to the results of the SDS-PAGE experiment, the NMR spectra of the platinum-Pal14 reaction mixture show the presence of signals for both the di-substituted platinum-peptide complex and apopeptide even after 8 days of reaction (Figure III.16). Concurrently, the NMR-COSY experiment of the reaction mixtures reveals the presence of two different cross peaks. One peak corresponds to the coupling of the pyridyl protons of the apopeptide and the other peak to the di-substituted platinum-peptide. (Figure III.17).

III.3.5. Circular dichroism spectroscopy

According to non-covalent assembly design, the formation of the coiled coil structure is necessary for assembling the formed di-substituted metal-peptide complexes, and can be characterized using circular dichroism (CD) spectroscopy. In water, the CD spectrum of the platinum-Pal14 reaction mixture consists of negative bands at 208 and 222 nm; which is characteristic of $\alpha$-helical peptide conformation$^{17,34}$ (Figure III.18.). The ellipticity ratio of this spectrum is $[\theta]_{222}/[\theta]_{208} = 1.0$ which indicative of coiled-coil conformation.$^{17}$ This shows that the
formed metal-peptide complex can form coiled-coils, and therefore assemble into high molecular weight structures.

**Figure III.18.** Circular dichroism spectrum of the platinum-Pal14 reaction mixture in water.

### III.3.6. Size exclusion high performance chromatography

The di-substituted platinum-peptide complex was expected to assemble into high molecular weight structures through the formation of a coiled-coil. Like the rhenium-peptide reaction, characterization of these self-assembled products is obtained by HPSEC experiments, which are performed under non-denaturing conditions (see Chapter II). The HPSEC for the reaction of ethylenediamine platinum (II) with Pal14 shows the presence of several platinum-peptide assemblies of different sizes. The peak of apopeptide and assemblies of 2 and 3 di-substituted platinum-peptide complexes are clearly seen and illustrated with arrows in Figure
III.19. (left). The formation of these platinum peptide assemblies, which have molecular weights of about 14 kDa and 20 kDa, are explained by the presence of an apopeptide impurity, which terminates the formation of the lager metal-peptide assemblies. The molecular weights of these assemblies are determined by comparison of the Kd value to the calibration graph for this column (Figure III.9).

The largest peak has the smallest elution time, 19 minutes, and therefore corresponds to the largest assemblies in the reaction mixture, making it of interest. These high molecular weight platinum-peptide assemblies travel with the front (void volume) of the size exclusion (SE) column, limiting the determination of their size and relative mass. Therefore, we can assume the molecular weight of the assembly is greater than 70 kDa, as this is the upper range of the SE column.

Purification of the high molecular weight platinum-peptide assemblies was attempted by collecting the fraction in the HPSEC run followed by reinjection onto the SE column. Unfortunately, this experiment did not give the expected result. The HPSEC chromatogram for the repurified Pt-peptide assemblies still shows the presence of low molecular weight species (Figure III.19. (right)). The molecular weights of these species (apopeptide, 14 kDa and 20 kDa) are the same as those removed when purifying the reaction mixture. The simplest explanation for this result is remnants of apopeptide in the collected fraction.
In order to get the full picture of platinum-peptide assembly formation, the reaction was monitored by HPSEC using a Superdex 200 column with a range of 10-300 kDa. In the beginning of the reaction of Pal14 and the platinum ethylenediamine, the HPSEC chromatogram shows only the presence of assemblies with the mass of a peptide dimer, which corresponds to the coil-coiled structure of the apopeptide. As expected, with the formation of di-substituted platinum-peptide complex, the mixture of the platinum-peptide complexes assembles into larger molecular structures. These structures are capped by unreacted apopeptide molecules into a mixture of assemblies which have different sizes. The different sizes of these assemblies (apopeptide 2, 3, and 4 etc. assemblies of the platinum-peptide complexes) are responsible for the jagged shape of the HPSEC chromatogram. Like the results of SDS PAGE and NMR, the HPSEC experiment does not show a strong change in the reaction mixture, even after reacting...
for 8 days. The HPSEC chromatogram of this reaction mixture is similar to the previous chromatogram.

Figure III.20. Monitoring the formation of the rhenium complex with Pa114 by HPSEC experiment Phosphate buffer pH7 Superdex 200.
The results of the HPSEC experiments suggest the formation of large (greater than 70 kDa) platinum-peptide assemblies through the creation of a coiled-coil. Because unreacted apopeptide forms coiled-coils with the formed di-substituted metal peptide complexes in the Pt-peptide reaction mixture, these assemblies vary in size. The shape of these assemblies is most likely a linear structure, with platinum-peptide complexes forming the body and unreacted apopeptides at the terminal end. The presence of a small amount of large molecular assemblies in the HPSEC experiment for rhenium-Pal14 reaction mixture suggests a low stability of the rhenium-Pal14 coordination bond under HPSEC experiment conditions. Purification of the di-substituted platinum-peptide complex from unreacted apopeptide is needed for the characterization of the formation platinum-peptide assemblies.

III.3.7. Reverse phase high performance liquid chromatography

HPLC of the platinum-Pal14 reaction mixture shows results similar to the rhenium-Pal14 reaction. The HPLC chromatogram shows the appearance of three peaks with slightly different retention times (Figure III.21.). The retention time of the second peak is identical to the retention time of Pal14 in and the UV spectrum of this peak matches that of the apopeptide. Both the first and third peaks have the UV-Vis spectral characteristics of a platinum-pyridylalanine bond, which is a single band at 250 nm with a shoulder at 270 nm. Therefore, these products are most likely mono and di substituted platinum-Pal14 complexes.
III.3.8. Purification of di-substituted platinum-peptide complex

Preparative HPLC is used to separate the di-substituted platinum Pal14 complex from unreacted apopeptide. After injection of the reaction mixture into HPLC, three fractions with different retention times were collected and immediately analyzed by gel electrophoresis (Figure III.22.). SDS-PAGE shows that the reaction mixture contains an apopeptide monomer (peak 2) and platinum peptide complexes which correspond to mono (peak 3) and di-substituted (peak 1) platinum-Pal14 complexes (Figure III.22.(right)). However, this result can be reached only in the case of immediate SDS-PAGE after the collection of HPLC fractions. Otherwise, the first fraction, which corresponds to di-substituted platinum-peptide complex, appears on the gel as a mixture of two compounds, with the molecular weights of the di-substituted platinum-peptide.
complex and apopeptide. These results indicate instability of the di-substituted platinum-peptide complex and its fast decomposition to monomer species.

**Figure III.22.** Left: Separation of the reaction mixture of ethylenediamine platinum (II) with Pal14 by HPLC. Water-acetonitrile linear gradient 0.1% TFA Right: Analysis of each fraction in the HPLC experiment by SDS-PAGE.

The results of the SDS-PAGE show that the di-substituted platinum-peptide complex and apopeptide were the main products in the reaction. The inconsistency of the peak intensity in the HPLC chromatogram, in which the di-substituted platinum-peptide complex has the smallest intensity (about 15% of the reaction mixture), is probably due to the decomposition of the platinum-Pal14 complex under the highly acetic conditions of HPLC.
III.3.9. Stability of original platinum-peptide complex

The results of the HPSEC and HPLC experiments demonstrate the instability of the di-substituted platinum-peptide complex. To better clarify this observation, a time resolved SDS-PAGE experiment was carried out. The purified di-substituted platinum-peptide complex was incubated in a phosphate buffer (pH7) at room temperature for various times and loaded onto the gel. (Figure III.23.) The disappearance of the band which corresponds to the di-substituted platinum-peptide complex structure, and the appearance of the band of monomer species, are seen with time. The di-substituted platinum-peptide complex has completely disappeared after 8 hours of incubation in the phosphate buffer.

Figure III.23. SDS-PAGE analysis of the purified metal-peptide dimer complex after incubating in phosphate buffer (pH7) at room temperature for various times
III.3.10. Modeling of the di-substituted platinum-peptide complex

The driving force of the formation of the metal-peptide di-substituted complex is the reaction of ethylenediamine platinum (II) with pyridylalanine. To the best of our knowledge, there are no examples of unstable platinum-pyridyl complexes under ambient conditions. Therefore, the instability of the platinum-pyridylalanine bond in the di-substituted platinum-peptide complex can only be explained by a strong repulsion within the complex. Modeling was expected to show the source of this repulsion.

Pal14 contains negatively charged glutamic acid residues in the b position of its sequences. There are two opposite orientations in which the peptide ligands are close to each other (Figure III.24.). In the parallel orientation of the peptide ligands, the negatively charged groups of the glutamic acid amino acid residues are located on opposite sides of the peptide complex, but still interact at the top of the molecule (the close proximity of the acetic groups are shown by arrows). In the anti-parallel orientation of the peptide ligands, all eight negatively charged groups are located near to each other, making this conformation highly unlikely. The models of the di-substituted platinum-peptide complex show that electrostatic repulsion could be the cause of the instability of the di-substituted platinum-peptide complex.

The presence of magnesium ion is well known as a stabilizer of negative coulomb repulsion in DNA oligamers, and is necessary for the formation of DNA assemblies. However, when the reaction of ethylenediamine platinum (II) with Pal14 was carried out in the presence of Mg\(^{2+}\) ion, there were no major changes in the composition of the reaction mixture. This shows that the charge residues in the peptide sequence probably are not the cause of the observed low stability.
Another possible reason of the instability of the di-substituted platinum-peptide complex is the effect of the low conformational stability on the formation of the complex. The apopeptide has 57.2% helicity which suggests that it is partially unstructured. It is difficult to predict the conformational dynamics of this molecule in the formed metal-peptide complex and therefore, the strong repulsion between the peptide molecules could be the reason for the low platinum-pyridylalanine bond stability of the bi-bonded platinum complex.

**Figure III.24.** Computer model of ethylenediamine platinum (II) complex with Pal14. Front and top view of the parallel (left) and anti-parallel (right) orientation of the peptide ligands in the platinum-peptide complex. The arrows show the strong coulomb repulsion in the platinum-peptide complex.
III.3.11. Summary

The di-substituted platinum-Pal14 complex is formed and assembles into high molecular weight species (> 70 kDa) through the formation of coiled-coils. Unfortunately, the instability of the formed platinum-peptide complex, as seen by SDS-PAGE and HPSEC, prevents purification and characterization of the formed platinum-peptide assemblies. Together, these results led to the design of an alternative coiled-coil peptide. In this design, the peptide has a formal neutral charge and higher conformation stability than pal14.
III.4. **DE-NOVO DESIGN AND CHARACTERIZATION OF NEW COILED-COIL PEPTIDE**

### III.4.1 Introduction

The design of the new peptide is focused on the formation of a stable metal peptide complex (Figure III.25.) in which the peptide sequence’s contribution to the stability of the metal-peptide complex was given a special attention. Because both electrostatic and steric repulsions are important for the stability of metal complexes, the design of the new peptide attempts to reduce these effects. In order to show the difference between the peptides of original and “new” design, the conformational stability of the “new” peptide compared with the characteristics of original one.

**Figure III.25.** Scheme of the platinum-peptide reaction.
III.4.2. Structural design of new peptide

There are three main forces contributing to the formation of a coiled coil structure. The first is hydrophobic packing forces, which are the main driving force of the coiled-coil formation. Among the possible hydrophobic amino acids residues, isoleucine and leucine at the $a$ and $d$ positions have the strongest attraction for the formation of the two-stranded coiled coil$^{35,36}$. The second force is the electrostatic interaction of the lysine and glutamic acid at the $e$ and $g$ positions, which also stabilize the parallel orientation of the peptides in the coiled-coils$^{36}$. The third force is the $\alpha$-helical propensity which influences the $\alpha$-helical secondary structure within the coiled coil. It was shown$^{36-38}$ that increasing alanine residues in the hydrophilic part of the peptide sequence increases the stability of the $\alpha$-helical coiled-coil. All of these factors were applied in the design of the new peptide which is named Pal14new (Pal14n).

The original molecular design of Pal14 includes negatively charged glutamic acid residues which give the peptide a formal negative charge. These acid residues are located in the $b$ position of the peptide backbone and are positioned there to increase solubility of the peptide. These negatively charged residues in Pal14new peptide were substituted by alanine residues. To keep the peptide soluble in aqueous solutions, the glycine residues at the $f$ positions were substituted by glutamine residues. Glutamine amino acid residues do not have a formal charge, but still keep the peptide molecule highly soluble. This makes the sequence of the peptide an IAALEQK heptad repeat. Similar to the design of Pal14, the non-natural amino acid 4-pyridylalanine (Pal) was placed at position 14 of Pal14new for direct coordination of metal complexes. The design, sequence and computer model of the peptide is shown in figure III.26:
III.4.3. Synthesis and characterization of Pal14new

Solid phase method described in the experimental procedure was used to prepare the 30-residue Pal14new (Pal14n) polypeptide. The purity of the peptide was confirmed by the appearance of a single peak in analytical HPLC. The presence of pyridylalanine amino acid residues in the peptide molecule was proven by the characteristic absorption band at 256 nm in the UV spectrum of the synthesized peptide molecule (Figure III.27 A and B). The accuracy of the synthesis was shown by MALDI and ESI mass spectroscopy (Figure III.27 C and D). MALDI-MS reveals a peak of the molecular ion (calc’d: 3279.98; obs’d: 3281.71) and a smaller peak with the molecular weight of the apopeptide bound to one atom of sodium (calc’d: 3302.98; obs’d: 3304.61) which is common for this technique. Concurrently, ESI-MS shows signals m/z =
1095, 821, and 657 which corresponds to the +3, +4, and +5 ions of a species with a molecular weight of 3280 (calc’d: 3279.98).

Figure III.27. Characterization of apopeptide Pal14n: The purity of the peptide was confirmed by analytical HPLC (A), UV spectrum (B), ESI-MS (C) and MALDI-MS (D).
III.4.4. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is used to evaluate the secondary structure of the apopeptides, which plays an important role in the stability of the formed di-substituted platinum-peptide complexes.

The CD spectrum of both original and new apopeptides consists of minima at 208 and 222 nm showing that the peptides exist within an \(\alpha\)-helical conformation (Figure III.28. (left)). The intensity at 222 nm is used to determine the \(\alpha\)-helical content\(^{15, 16}\) which is 57.3% for Pal14 \((\theta_{222} = -19.400 \text{ deg cm}^2 \text{ dmol}^{-1})\) and 91.2% for Pal14new \((\theta_{222} = -31.000 \text{ deg cm}^2 \text{ dmol}^{-1})\) in water. A value of \(\theta_{222}/\theta_{208} > 1\) for both apopeptides falls within the range generally considered to be indicative of a coiled-coil conformation\(^{17}\). The molecular weight of this assembly is shown to correlate with a peptide dimer, as determined by high performance size exclusion chromatography. Together, these results show that both apopeptides exist as a two-stranded \(\alpha\)-helical coiled-coils in the absence of metal ions. The low value of Pal14 helicity can be interpreted as a low stability of the \(\alpha\)-helical conformation which still exists within the coiled-coil structure.

Temperature dependence of the CD signal at 222 nm of the apopeptides shows that the “melting point” of Pal14 conformation is about 70°C. The peptide completely denaturates at temperatures above 80°C (Figure III.28. (right)). In contrast, Pal14new does denaturate substantially with increasing temperature. The apopeptide still exists as 55% \(\alpha\)-helical structure at 80°C. The beginning of the decomposition curve for this peptide appears only at temperature higher 85°C. (Figure III.4.2. (right)). Together, these experiments show a vast difference in the stability of the peptide’s coiled-coil structures. Nevertheless, the determination of binding constant of coiled-coil is necessary to quantify the conformational stability.
Figure III.28. Left: Circular dichroism spectrum of Pal14 (black) and Pal14new (red) in water. Concentration of Pal14n is $1.35 \times 10^{-4}$ mole/L and concentration of Pal14 is $1 \times 10^{-4}$ mole/L. Right: Temperature dependence of CD signal at 222 nm

III.4.5. Conformational analysis

The stability of the $\alpha$-helical conformations of apopeptides was studied by guanidinium chloride (GdnHCl) titration. The denaturation curve and the free energy of the apopeptides unfolding are shown in Figure III.29 and Table III.4.

Free energy of the coiled-coil folding was determined by extrapolating the linear factor in the change of the free energy with increasing concentration of guanidinium chloride (Figure III.30.). The full description of this procedure is given in the Chapter II. While Pal14 has a midpoint around 2.5 M GdnHCl and $\Delta G_{H2O}^{0} = 7.3$ kcal/mole, the denaturation midpoint for Pal14new has a midpoint around 7.5 M GdnHCl and $\Delta G_{H2O}^{0} = 13.8$ kcal/mole, which makes the difference in $\Delta G_{H2O}^{0} = 6.5$ kcal/mole. Together, these results show that the $\alpha$-helical coiled coil conformation of Pal14new has outstanding stability in comparison with the original peptide.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Helicity in H$_2$O (%)</th>
<th>[GdnHCl]$_{1/2}$</th>
<th>Slope (m)</th>
<th>$\Delta G^{H2O}$ (kcal)</th>
<th>$K_d$(app)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pal14</td>
<td>57.3</td>
<td>2.5</td>
<td>-0.674</td>
<td>7.3</td>
<td>4.1 x 10$^{-6}$</td>
</tr>
<tr>
<td>Pal14n</td>
<td>91.2</td>
<td>7.5</td>
<td>-1.115</td>
<td>13.8</td>
<td>6.9 x 10$^{-11}$</td>
</tr>
</tbody>
</table>

Figure III.29. The plot of the denaturation of Pal 14 (red) and Pal14new (blue) with increasing concentration of guanidinium chloride. The concentrations of peptide were within of 40-80 μM.
III.4.4. Kinetics of coiled-coil folding-unfolding

Recently, some examples the conformations of highly stable proteins have been shown to be due to a kinetic barrier in the unfolding process. Because of its exceptional stability, Pal14n coiled-coil was suspected to have a kinetic barrier. Two experiments were carried out to resolve this suggestion. The first is monitoring the change of CD signal at 222 nm with time in the presence of 6M GdnHCl. (Figure III.31. (left)) The CD signal did not show any significant change, even after three hours of monitoring. The second experiment, which is a temperature chromatogram of the CD signal at 222 nm, shows the reversibility Pal14n folding-unfolding conformational change (Figure III.32. (right)) Together these results disprove any presence of a kinetic barrier in the denaturation process of Pal14new.
Figure III.31. Kinetic study of Pal14n denaturation. Left: Change of CD signal at 222 nm with time. Right: reverse temperature chromatogram of the CD signal at 222 nm. The concentration of Pal14neuw was $8.5 \times 10^{-5}$ mole/L in a phosphate buffer solution of 6 M GndHCl pH=7.

III.4.5. Summary

Hydrophobic interactions are considered to be the main driving forces responsible for coiled coil oligomerization of the peptides. The hydrophobic interface and chain length of both new and original peptides are identical. Therefore, the difference in conformational stability can be explained by the difference in $\alpha$-helical propensity of the apopeptides. The original peptide has four glycine residues which are considered destructive to its $\alpha$-helix secondary structures. In contrast, the new peptide contains a high amount of alanine residues which are believed to induce $\alpha$-helical conformation. For example, substitution of one alanine for serine in similar peptide sequences gives a decrease in stabilization of 0.45 kcal/mole. Additionally, the coulomb repulsion between the glutamic acid residues at positions e and b in Pal14 contribute to the lower conformational stability of this peptide compared to Pal14new, in which the glutamic acid
residues at the b position are substituted by alanine. Together, these results show that Pal14n forms a two-stranded α-helical coiled-coil with a high stability, and therefore can be used as an appropriate ligand for the formation of non covalent metal-peptide assemblies.
III.5. PLATINUM COMPLEX WITH NEW COILED-COIL PEPTIDE

III.5.1. Introduction

An alternative peptide with a new sequence was designed and examined for the formation of a platinum-peptide complex. The very high stability of the α-helical conformation of this peptide was expected to stabilize the metal-peptide complex.

III.5.2. SDS-PAGE gel electrophoresis

Similar to the reaction of Pal14, the formation of platinum-Pal14n complex was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Pal14new under the denaturing conditions of the SDS-page experiment exists as a monomer species with a molecular weight of approximately 3.3 kDa. The molecular weight of the di-substituted platinum-peptide complex corresponds to the molecular weight of two peptide molecules cross-linked together by a platinum complex. The molecular weight of this complex is about twice as big as the apopeptide. Therefore, the formation of the di-substituted platinum-peptide complex can be monitored by SDS-PAGE.

Monitoring the reaction mixture revealed the disappearance of the apopeptide band with the molecular weight of 3.3 kDa and appearance of a new band with molecular weight around 6.5 kDa, which was assigned to the di-substituted platinum-peptide complex. The reaction of platinum with Pal14new was almost complete after four days, as seen by the product band on the gel in Figure III.32:
**Figure III.32.** Monitoring of the di-substituted platinum-Pal14n complex formation by SDS-PAGE

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**III.5.3. NMR spectroscopy**

The formation of the Pal14n-platinum complex was monitored by NMR spectroscopy and compared to the platinum-Pal14 reaction and the model pyridine reaction. Similar to Pal14, the treatment of a Pal14n by D$_2$O did not completely annihilate the signals of amide NH protons, but the well-dispersed lines of the ortho and meta pyridyl protons of the apopeptide are clearly seen in the NMR spectra (Figure III.33. (Top)). The cross peak between these signals in the $^1$HCO$\text{S}$ spectrum reveals their coupling to each-other and supports the assignment of the pyridyl protons in the apopeptide. (Figure III.33. (Bottom)). Importantly, the chemical shifts of the pyridyl protons are identical for both Pal14 and Pal14new which suggests similar environments of the pyridylalanine groups and therefore similarity metal coordination reactivity. All chemical
shifts of pyridyl protons of apopeptides before and after metal coordination reaction are shown in Table III.5:

**Table III.5.** $^1$H-NMR chemical shifts of pyridine, Pal14 and Pal14new before and after metal coordination.

<table>
<thead>
<tr>
<th>$^1$H NMR signals</th>
<th>ppm ortho protons before metal coordination</th>
<th>ppm ortho protons of mono coordinated product</th>
<th>ppm ortho protons of di coordinated product</th>
<th>ppm meta protons before metal coordination</th>
<th>ppm meta protons of mono coordinated product</th>
<th>ppm meta protons of di coordinated product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>8.61</td>
<td>8.57</td>
<td>8.71</td>
<td>7.68</td>
<td>7.56</td>
<td>7.51</td>
</tr>
<tr>
<td>Pal14</td>
<td>8.7</td>
<td>8.45</td>
<td>8.0</td>
<td>7.56</td>
<td>7.40</td>
<td></td>
</tr>
<tr>
<td>Pal14n</td>
<td>8.7</td>
<td>8.55</td>
<td>8.0</td>
<td></td>
<td></td>
<td>7.2</td>
</tr>
</tbody>
</table>

The formation of the metal-peptide complexes is verified by the appearance of new pyridine proton signals in the downfield region of the NMR spectra. A shift of the signals of pyridine protons after metal binding is expected from the reaction with Pal14 (Table III.5.). In this reaction, the formation of di-substituted platinum-Pal14 complexes is clearly demonstrated by the appearance of new signals at 8.45 ppm (ortho protons) and 7.48 ppm (meta protons). Therefore, the signals at 8.55 ppm and 7.2 ppm are assigned to the ortho and meta pyridyl protons of the di-substituted platinum-Pal14n complex. Concurrent monitoring of this reaction by COSY NMR also showed the presence of one cross peak between the signals of di-substituted platinum-peptide complex after incubation for four days. The results of these NMR experiments are consistent with the results of SDS-PAGE experiment.
Figure III.33. Top: The formation of the platinum complex with Pal14new by H\textsuperscript{1} NMR spectrum is taken in D\textsubscript{2}O. Bottom: The formation of the platinum complex with Pal14new by NMR –COSY spectrum is taken in D\textsubscript{2}O.
The observed chemical shifts of the pyridyl protons are similar for Pal14 and Pal14n. The signals of ortho pyridyl protons shifted 0.15 and 0.25 ppm, and the signals of meta protons shifted 0.8 and 0.52 ppm upfield for Pal14n and Pal14 after metal binding, respectively. However, the results of these reactions are different because Pal14 does not react completely with the platinum complex.

III.5.4. Mass spectroscopy

Two different ionization techniques (MALDI-TOF and ESI-MS) were used to analyze the platinum-peptide reaction mixtures by mass spectroscopy (MS). The MALDI experiment does not show a species with the molecular weight of a di-substituted metal-peptide complex for the reaction mixtures. This result can be explained by the low stability of the di-substituted platinum-peptide complex under the ionization conditions in MALDI-MS.

The ESI-MS of the platinum complex with Pal14new shows a mixture of signals, where the peaks at m/z = 1094, 821, and 657 correspond to the +3, +4, and +5 ions of a species which matches the molecular weight of apopeptide, and the peaks at m/z = 1376, 1147, 983, 860, and 765, which correspond to the +5, +6, +7, +8 and +9 ions of a species with an apparent mass of di-substituted platinum-peptide complex. Likewise, in the MALDI experiment, the signals of unreacted apopeptide correspond to the products of the di-substituted platinum-peptide complex decomposition.
Figure III.34. Top: ESI-MS of Pal14new. Bottom: ESI-MS of the reaction mixture of ethylenediamine platinum (II) with Pal14new

III.5.5. Circular dichroism spectroscopy

According to non-covalent assembly design, the formation of the coiled coil structure is necessary for assembling formed di-substituted metal-peptide complexes and can be characterised using circular dichroism (CD) spectroscopy. The CD spectrum of the Pal14n apopeptide in water consist of minima at 208 and 222 nm, demonstrating that the peptide exists in $\alpha$-helical conformation (Figure III.35.). The helical content of peptide can be determined by the signal at 222nm, and has a value of $\theta_{222} = -33.000$ deg cm$^2$ dmol$^{-1}$ which indicates that the apopeptide is almost 100% helical by comparison to the predicted value for a pure 30-residue $\alpha$-helix$^{15,16}$. The observed ratio of $\theta_{222}/\theta_{208} = 1.04$ is within the range indicating the formation of an
α-helical coiled-coil\textsuperscript{17}. In contrast to the CD of the apopeptide, the signal at 222nm of the platinum-peptide complex drops in absolute value. The CD signal of the platinum-peptide complex is $\theta_{222} = -14,700$ deg cm\textsuperscript{2} dmol\textsuperscript{-1}, which makes its helicity about 42 %. Importantly, the shape of the platinum-peptide CD spectrum is still indicative of coiled-coil structures with a ratio of $\theta_{222}/\theta_{208} \approx 1.12$, which is bigger than apopeptide alone. This behavior of CD spectrum was observed before for peptides\textsuperscript{44} and proteins\textsuperscript{45} which assemble into linear polymers through the formation of coiled coils. It was suggested that this behavior of CD spectrum is indicative of the formation of large molecular assemblies.\textsuperscript{42, 43}

\textbf{Figure III.35.} CD spectrum of apopeptide Pal14new (black) and the reaction mixture of ethylenediamine platinum (II) with Pal14new (red). Peptide concentration is 200-400 μM.
III.5.6. Size exclusion high performance chromatography

The di-substituted platinum-pal14n complex was expected to assemble into high molecular weight structures through the formation of a coiled-coil. The formation of the metal-peptide assemblies was monitored by high performance size exclusion chromatography (HPSEC). 27, 28

Monitoring the assemblies’ formation by size exclusion Superdex 200 with a range of 10-300 kDa shows results comparable to the previous experiment. In the beginning of the reaction, the HPSEC chromatogram shows the presence of one peak with the mass of a peptide dimer (6.5 kDa) complex corresponding to the coil-coiled dimer of Pal14new (Figure III.36.). As expected, with the formation of the platinum-Pal14n di-substituted platinum-peptide complex, the mixture of platinum-peptide complexes and unreacted apopeptide molecules assembles into larger molecular structures. Similar to the reaction of Pal14, on the second day of the reaction, the peak of apopeptide and assemblies of 2 and 3 di-substituted metal-peptide complexes can be seen in the chromatogram and are illustrated with arrows. The HPSEC data for the fraction of the reaction mixture after four days shows the presence of many metal-peptide assemblies of different sizes which appear as a wide peak and have a molecular weight range of 6.6-70 kDa. Like the platinum-Pal14 reaction, the formation of the assemblies of a low molecular weight was explained by the presence of impurities in the apopeptide or a metal-peptide monomer complex which terminates the formation of the final metal-peptide assemblies. At the end of the reaction, the HPSEC chromatogram shows the presence of only one peak with a retention time of 19 minutes. These high molecular weight platinum-Pal14n assemblies travel with the front (void volume) of the size exclusion (SE) column, thus limiting the determination of their size and
relative mass. This result shows that the formed metal peptide assemblies have molecular weight above 300 kDa.

Figure III.36. The formation of ethylenediamine platinum (II) with Pal14new by HPSEC. Phosphate buffer pH7 Superdex 200.

HPSEC shows the formation of large platinum-peptide assemblies through the formation of coiled-coils. This result is in contrast to the result for platinum-Pla14 reaction in which the presence of unreacted apopeptide could not be removed. We have suggested that the assemblies of Pal14new di-substituted platinum-peptide complex “finite” in size because they do not
precipitate, which is expected for “infinitive” polymer lines. The characterization of these assemblies by other techniques such as light scattering or covalent crosslinking is expected to clarify this suggestion.

### III.5.7. Cross-linking experiment

Covalent crosslinking is a valuable technique for the characterization of non covalent protein complexes. Covalent crosslinking is used to trap the formed high molecular weight metal-peptide assemblies in order to obtain their approximate molecular weight. Glutaraaldehyde was chosen as a crosslinking agent in this experiment.\textsuperscript{44, 45} Glutaraaldehyde forms covalent bonds between stericly close amino- and amido-groups which are widely present in the Pal14 sequence.

The crosslinking was carried out in a methanol-water solution for 20 min with varying amounts of glutaraldehyde. Analysis of this mixture by SDS-PAGE (Figure III.37.) shows the presence of two discrete species in the crosslinked reaction mixture. The molecular weights of these species are about 10 and 14 kDa, which correspond to three and four peptide molecules crosslinked to each other. The presence of the species with these molecular weights suggests some organization of the assemblies in the crosslinking reaction. In contrast, the solution of crosslinked Pal14 peptide does not show a significant amount of high molecular weight species in the absence of a metal complex.
Figure III.37. SDS-PAGE experiment for crosslinked pure Pal14new peptide and reaction mix of Pal14new by glutaraldehyde.

The molecular weights of the trapped platinum-peptide aggregates estimated by SDS-PAGE are much smaller then the molecular weight of the assemblies estimated by HPESEC. This inconsistency can be explained by the difference in the techniques, in which the results of the HPESEC experiment are more accurate because this experiment does not chemically modify the formed assemblies.

III.5.8. Light scattering

The intensity of light scatter (LS) is proportional to the size of the particle which allows its molecular weight determination if the dn/dc value is known for the sample.\textsuperscript{46-49} Moreover, structural properties of the particles in solution can be determined by comparison of static and dynamic LS experiments. The ratio between these two values is known to be indicative for the simplified shape of the particles.\textsuperscript{50, 51}
Static light scattering experiment shows the radius of the platinum-Pal14n assemblies are (MR) $18 \pm 4$ nm and the molecular weight of the platinum-Pal14n assemblies is 7000 kDa (Figure III.38.) Hydrodynamic radius is determined to be 13-18 nm. Together, these two methods show that the diameter of the metal-peptide assemblies is somewhere in between 26 and 44 nm. Importantly, the results of the LS measurements are independent of concentration within the range of 1-100 mg/ml. Dilution of the platinum-peptide sample 100 times decreased the hydrodynamic radius only in 20% (Figure III.39.).

![Figure III.38. HPSEC-LS for the platinum-Pal14n reaction mixture: molar mass and MS radius were determined by static light scattering. Hydrodynamic radius was determined by quasi elastic light scattering. Phosphate buffer pH7 Superdex 75](image)

Figure III.38. HPSEC-LS for the platinum-Pal14n reaction mixture: molar mass and MS radius were determined by static light scattering. Hydrodynamic radius was determined by quasi elastic light scattering. Phosphate buffer pH7 Superdex 75
Figure III.39. Hydrodynamic radius and conformational plots for the platinum-Pal14netral reaction mixtures with concentration 10 mg/ml (red) and 0.1 mg/ml (brown). Phosphate buffer pH7 Superdex 75.

The plot of the determined MS radius against the logarithm of molecular weights gives some structural information about the samples, and is useful for conformational analysis of the molecular assemblies. The slope of this graph differentiates between branch and linear polymers. For example, a slope value 0.33 is expected for the most compact, homogeneous, spherical molecules while slopes values higher than 0.6 are characteristic for linear polymers. The slope of the formed platinum peptide sample is 0.4, which suggests a branched structure of their assemblies. This value is somewhere in between of the values for a spherical (0.33) and
random coiled (0.5-0.6) conformations which allows us to suggest a non-ideal, spherical structure of the platinum-peptide assemblies.

The ratio between MR radius and hydrodynamic radius is another value which is indicative of the shape of the assemblies in solution.\textsuperscript{51} It was shown that this ratio is 0.75 for spherical molecules while values higher than 1.5 usually indicate linear structures. The ratio between MR radius and hydrodynamic ratio equal to 1 corresponds to the shape of hollow spheres. The platinum-Pal14n assemblies showed a MR-hydrodynamic radius ratio of 1.06, which suggests that their molecular shape is close to hollow spheres. Together these experiments suggest a globular structure for the platinum-Pal14n new assemblies, the shape of which is nearly spherical.

III.5.9. Transmission electron microscopy

Transmission electron microscopy (TEM) is a useful tool for the characterization of peptide assemblies\textsuperscript{42}. Because electrons passing through the sample have high energy (60-100kV), only atoms of heavy elements can be seen on the images. In order to visualize biomolecular samples consisting mostly of light atoms, staining with heavy atoms is necessary. Negative staining was used for visualization of the samples in TEM. In theory, the resolution of negative staining is high and can be up to 0.4 Å.

TEM examination of the Pt(en)(Pal14n)\textsubscript{2} samples revealed the presence of two different structures of the metal-peptide assemblies (Figure III.40.). The round assemblies were measured 30 ± 15 nm in diameter, and the wire-like assemblies were approximately 400 nm in length and
about 10 nm in wide. The assemblies on the images were badly resolved, thus complicating size
determination; however optimization of the conditions did not bring expected results.

Figure III.40. TEM images of globular assemblies obtained by negative staining from
samples having concentrations of 50 mg/L. These globules have diameters of 30-50 nm.
III.5.10. Atomic force microscopy

Atomic force microscopy (AFM) microscopy was applied to further quantify the three dimensional parameters of the platinum-peptide assemblies. In contrast to the TEM images, the AFM images were well resolved and easily reproducible. AFM microscopy shows three dimensional images of species on the surface, and therefore became the most common technique for characterization of peptide assemblies.\textsuperscript{53-57}

The samples of apopeptide were prepared and analyzed by AFM and used further as a reference for the samples of metal-peptide assemblies. AFM images of pure peptide Pal14new shown in figure III.41. were prepared using different techniques. Images A and C were obtained by incubation-rinse technique (0.5 mg/ml) and image B by evaporation. Images A and B look highly similar; the samples are well dispersed on the mica with little aggregation. The aggregations are larger in images using incubation-rinse technique, which can be attributed to unfiltered impurities. A lower concentration may potentially shows individual peptides, but will stretch the resolution limits of this instrument. Image C shows possible formation of a peptide film on the surface of the mica. This image is probably obtained from a place on the mica with a high local concentration of the peptide. Several layers of the peptide molecules on the surface are clearly seen. These layers appear as a fine flat surface with higher magnification. These images were made for comparison with the images of platinum-peptide assemblies.
Figure III.41. AFM images of pure peptide Pal14new. The images A and C were obtained by incubation-rinse technique and image B by evaporation.
The difference between AFM images for the pure peptide and the platinum-peptide complex are clearly seen. At a concentration of 50 mg/ml, the assemblies appear as highly packed globular structures (Figure III.42.). The image for the platinum-peptide complex shows the presence of multiple large aggregates with dimensions of 10-100 nm wide and 5-20 nm high. It can be seen that some of the assemblies are badly resolved because of the high concentrations of the samples on the surface. The observed dimensions of individual globules were 30-50 nm in diameter and 5-10 nm in height. These parameters are consistent with light scattering data.

A ten fold dilution of the sample shows that the metal-peptide assemblies still exist as randomly dispersed globular aggregates (Figure III.43.). In comparison with the higher concentrated samples, this image shows the presence of only a few large assemblies, (Figure III.44. B) which can be attributed to the low concentration of the platinum-peptide complex. The profile analysis shows that the height of a single assembly is 20-30Å, and the diameter is 10-20 nm (Figure III.45.). The images and the profile analysis reveal that the assemblies do not form a perfectly round shape on the mica surface.

At the same concentration of 5 mg/ml, some of the assemblies appear as wire-like structures (Figure III.46.). The profile analysis of these assemblies shows that their thickness varies and can be estimated from 4nm to 10nm. The height of the assemblies is between 4-8 Å, which is comparable with the dimension of a single peptide molecule lying on a flat surface. Together, these results show that wire-like structures are built through the assembly of the disubstituted platinum-peptide complexes in a ropelike array.
**Figure III.42.** AFM image of Pal14new complex with Pt(en)(NO$_3$)$_2$ water solution of 50 micrograms/ml. This image was obtained using incubation-rinse technique.

**Figure III.43.** Complex of Pal14new with Pt(en)(NO$_3$)$_2$ water solution of 5 micrograms/ml which is obtained by evaporation technique.
Figure III.44. A: Flat and 3-D images of Pal14new complex with Pt(en)(NO₃)₂. The images were obtained by incubation-rinse technique from a solution with a concentration of 50 micrograms/ml. B: Flat and 3-D images of diluted solution of Complex of Pal14new with Pt(en)(NO₃)₂ (water solution of 5 microgram/ml).

Figure III.45. Cross-wise profile of the metal-peptide Complex of Pal14new with Pt(en)(NO₃)₂ in water solution of 5 microgram/ml.
Figure III.46. Left: Wire-like aggregates of complex of Pal14new with Pt(en)(NO₃)₂. All these images were obtained by incubation-rinse technique from the solution of the platinum-peptide complex with concentration 5 micrograms/ml. Right: The profile of wire-like assemblies of Pal14new with Pt(en)(NO₃)₂.
The concentration at which non-covalent assemblies of peptides turn into monomers is the critical aggregation concentration (CAC).\textsuperscript{58} It seems that the wire-like assemblies are a transition state between the globular assemblies and individual platinum-peptide complexes. The wire-like shape of the assemblies appears at nano-molar concentrations, therefore the CAC for the platinum-peptide is somewhere below nano-molar concentration. This allows estimation of the order of the coiled-coil binding constant between the individual platinum-peptide complexes as $10^6$ molar or higher.

Two different techniques were used for the sample preparation. Both incubation-rinsing and evaporation (air-dry) techniques show the independence of the assemblies’ morphology from the sample preparation. This fact as well as the high reproducibility of the results makes AFM a most useful tool in the characterization of the formed platinum-peptide assemblies.

### III.5.11. Effect of different metals on the formation of the metal-peptide complex

Several different metal complexes were examined for the ability to form a stable complex with Pal14 and Pal14new. The metal choice for the experiment was based upon logic suggesting that increasing the energy of the metal-pyridine bond will increase the stability of the metal-peptide complexes. The Ni-Pd-Pt column in the periodic table of elements was examined by mixing the equal molar amount of peptides with metals.

A “naked” ion of Ni\textsuperscript{2+} did not show any stable products with any of the peptide ligands. Surprisingly, Pd(en)\textsuperscript{2+} which is known as a complex that forms stable metal-pyridine bonds, did not form any di-substituted metal-peptide complexes with the peptide ligands as well. The high liability of the coordination bonds, a property of the Pd-pyridine bond, is considered to influence the formation of the metal-peptide complexes. To examine this hypothesis, Ru(bpy)\textsubscript{2}\textsuperscript{2+} was
reacted with Pal14n. Ru(bpy)$_2^{2+}$ has been known to form a metal-pyridine bond which does not undergo rapid dissociation. After one week of monitoring the reaction mixture by SDS-PAGE, the experiment revealed the formation of di-substituted Ru-peptide complex (Figure III.47.). Under similar conditions during two weeks, the reaction of Pd(en)$_2^{2+}$ with Pal14new did not show any results.

Figure III.47. The reaction Ru(bpy)$_2^{2+}$ with Pal14n in D$_2$O by SDS-PAGE
III.5.12. Summary

The structural design used in this work combines the principles of supramolecular coordination chemistry with de novo protein design in order to produce new types of metal-peptide nanoassemblies. This strategy consists of two well distinguished parts which are a peptide ligand and metal coordination center. Therefore, two main contributions to the formation of metal-peptide complexes should be taking in consideration. First, is the reaction of ethylenediamine platinum (II) and a para-substituted pyridine which goes to the most thermodynamically stable product. In contrast to the formation of ethylenediamine platinum-pyridine complex, the reaction of ethylenediamine platinum (II) with the peptide ligands goes much slower. This observation can be explained by the presence of many functional groups in the peptide molecules, such as carboxylic acids and amino groups. These groups bind ethylenediamine platinum (II) much faster than pyridylalanine and must be considered in the reaction mechanism. Nevertheless, the pathway of the reaction leads to the thermodynamic product which is the platinum-pyridine bond. Changing the metal center shows the effect of metal-pyridylalanine bond properties on the stability of the metal-peptide complex.

Another contribution to the formation of metal-peptide complexes is the effect of peptide sequences on the ability such peptides to form the metal-peptide complex. Two coiled-coil peptides have been shown to form the platinum-peptide complex. However, the reaction of ethylenediamine platinum (II) with original peptide sequence leads to a reaction mixture which contains several metal-peptide assemblies of different size. Purification and characterization of such assemblies were complicated. On the other hand, the reaction of ethylenediamine platinum (II) with the new peptide produces metal-peptide assemblies without additional purification. In
order to design stable metal-peptide assemblies, these results suggest the importance of the effect of peptide sequence and metal core.

The di-substituted platinum-pal14n complex forms and self-assembles into high molecular weight species through the formation of coiled-coils. The structure of the formed assemblies on the surface and in solution was fully characterized by different techniques. The results of these experiments are summarized in Table III.6:

**Table III.6.** Diameter of Pt(en)-Pal14new assemblies as determined by different techniques.

<table>
<thead>
<tr>
<th></th>
<th>AFM</th>
<th>TEM</th>
<th>MALS</th>
<th>QELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt(en)(Pal14n)_2</td>
<td>40 ± 10 nm</td>
<td>30 ± 15 nm</td>
<td>36 ± 8 nm</td>
<td>32 ± 6 nm</td>
</tr>
</tbody>
</table>

The AFM and TEM microcopies showed that the metal-peptide assemblies have a globular structure with diameter about 40 nm on the surface. Concurrently, static and elastic light scattering experiments showed that the assemblies have a similar shape in solution with diameter about 35 nm. The conformational analysis of the LS data suggests the globular structure of the metal-peptide assemblies is close to the shape of a sphere. Together, these results allow modeling the structure of the platinum-peptide assemblies in solution and on the surface which is shown in the Figure III.48:
In contrast to the other experiments, the result of covalent crosslinking showed the presence of low molecular weights species. This result suggested some organization of the platinum-peptide assemblies in the reaction of crosslinking and led to the design of peptides with multiple binding sites in their backbone.
CHAPTER III REFERENCES:


IV.1. STRUCTURAL DESIGN

IV.1.1 Introduction

The following part of this dissertation describes the formation and characterization of new metal-peptide assemblies which use peptide ligands to bridge adjacent metal centers. In order to prevent non-covalent interactions within the formed metal peptide assemblies, covalently crosslinked peptides were designed. Therefore, the peptides to be used here exist as covalently crosslinked α-helical coiled-coils\(^1\) which have two metal binding sites on opposite surfaces of the coiled coil. Similar to the formation of non-covalent-metal peptide assemblies, Pt(en)(NO\(_2\))\(_3\)\(^2-4\) and [NEt\(_4\)]\(_2\)[ReBr\(_3\)(CO)]\(_3\)\(^5,6\) were expected to bind peptide ligands to form metal peptide polymers. Characterization of these metal peptide polymers was expected to show the effect of metal binding on the final structure of the formed assemblies. The schematic view of two possible geometries (linear and cyclic) is shown in figure IV.1:

![Figure IV.1 Strategy the formation of metal-peptide assemblies](image-url)
IV.1.2. Design of peptides ligands

The peptide design for this chapter has the same basic principle as the design of peptides in Chapter III. The amino acid sequence of these peptides are based on a seven residue heptad repeat\textsuperscript{7,9}, which can be denoted as: \((abcdefg)_n\), where positions \(a\) and \(d\) of each heptad are occupied by hydrophobic amino acids forming a hydrophobic core, \(g\) and \(e\) positions form inter-chain salt bridges, and positions “\(b\)”, “\(c\)”, and “\(f\)” are occupied by hydrophilic residues. According to the design, the non-natural amino acid 4-pyridylalanine (Pal) was placed at position 14.

Both peptides of “original” IEALEGK\textsuperscript{10-16} and “new” IAAEQK sequences were tested for the ability to form high molecular weight species. However, in both cases, a cysteine residue was placed at position 19 which occupies a \(d\) site in the hydrophobic core. This modification was made to engineer an inter-chain disulfide bond crosslink, which prevents dissociation of the coiled-coil structure.\textsuperscript{17} The sequences of the peptides used in this work form two-stranded \(\alpha\)-helical coiled-coils and are shown in Figure IV.2:

\[
\text{Ac-KIEALEGKIEALEPalKIEACEGKIEALEGKG-CNH}_2\quad\text{Pal-14C19}
\]
\[
\text{Ac-QIEALEQKIEALEPalKIEACEQKIEALEQKG-CNH}_2\quad\text{Pal-14C19n}
\]

**Figure IV.2.** Peptide sequences for CNG4 inspired Pal14C19 and “new” Pal14C19n peptides.
IV.1.3. Synthesis of the peptide ligand

The peptide Pal14C19 was synthesized by solid-phase techniques and purified by reverse-phase HPLC (Figure IV.3.). The molecular weight was proven by MALDI mass spectroscopy (Figure IV.4). MALDI-MS reveals the peak of the molecular ion (calc’d: 3509.04; obs’d: 3509.69) and a smaller peak with the molecular weight of the apopeptide bound to one atom of sodium (calc’d: 3532.04; obs’d: 3531.95), which is a common byproduct of this technique. The cysteine group could not be oxidized by air or flow of oxygen; most likely due to it is location within the hydrophobic core of the coiled-coil. The disulfide crosslinked peptide, Pal14C19ox, was prepared by oxidation of the cysteine residues with I2 and also purified by HPLC. The retention time of Pal14C19ox in the HPLC purification decreased in comparison to the non-oxidized peptide. It is thought that the hydrophobic part of the molecule faces each other in the oxidized dimer, leading to a corresponding decrease of hydrophobicity and HPLC retention time. Analysis of Pal14C19ox by MALDI-MS shows the presence of the oxidized peptide (calculated: 7016.08; observed: 7018.32) in addition to a smaller amount of the reduced peptide monomer (calculated: 3509.04; observed: 3510.10), which may be due to the presence of a double charged molecular ion or to the thermodecomposition of the disulfide bond of cysteine crosslinking (Figure IV.4.).
Figure IV.3. Analytical HPLC experiments for Pal14C19 before (left) and after (right) oxidation. H$_2$O/AcN linear gradient 0.1% TFA

Figure IV.4. MALDI-MS spectra of Pal14C19 before (left) and after (right) oxidation.
The ESI-MS experiment of Pal14C19 shows a mixture of signals (Figure.IV.5.), where the peaks at m/z = 1171, 878, 703, and 586 correspond to the +3, +4, +5 and +6 ions of a species with a molecular weight of 3510, which corresponds to the molecular weight of the apopeptide (calculated: 3509.04). The ESI-MS of oxidized Pal14C19ox shows the peaks at m/z = 1170, 1003, 878, 781, 703, 639, and 586, which then correspond to the +3, +4, +5, +6, +7, +8 and +9 ions of a species with an apparent mass of the peptide dimer (calculated: 7016.08). The difference between the ESI-MS spectra for reduced (Pal14C19) and oxidized (Pal14C19ox) forms of the peptide are the new signals, which appear in ESI-MS after oxidation, and correspond to the odd charges of the formed peptide dimer.

Figure.IV.5. ESI-MS spectra of Pal14C19 before (left) and after (right) oxidation.
IV.1.4. Circular dichroism of the peptide ligand

Circular dichroism (CD) spectroscopy is used to evaluate the secondary structure of the apopeptides and to show the difference between reduced and oxidized forms. The CD spectrum of both reduced (Pal14C19) and oxidized (Pal14C19ox) forms of the apopeptide consist of minima at 208 and 222 nm showing that the peptide exists within $\alpha$-helical conformation as shown in Figure IV.6:

![Figure IV.6. Left: Circular dichroism spectrum of Pal14C19 (black) and Pal14C19ox (red) in water. Concentration of Pal14C19 is 1.12x10^{-4} mole/l and concentration of Pal14C19ox is 1.11x10^{-4} mole/l. Right: Temperature dependence of the CD signal at 222 nm.]

The intensity at 222 nm was used to determine the $\alpha$-helical content which is 56.1% ($\theta_{222} = -19,000$ deg cm$^2$ dmol$^{-1}$) for Pal14C19 and 71.8% ($\theta_{222} = -24,400$ deg cm$^2$ dmol$^{-1}$) for Pal14C19ox in water. The low helicity value of the reduced form can be interpreted as a low stability of the $\alpha$-helical conformation which is interrupted by the presence of a polar cysteine residue in the hydrophobic part of the molecule. The ratio of $\theta_{222}/\theta_{208} = 1.10$ for Pal14C19ox is
indicative of the $\alpha$-helical coiled-coil conformation. In contrast, the reduced form of the apopeptide has a ratio of $\theta_{222}/\theta_{208} = 0.99$ which is a little low for a coiled coil conformation.

Temperature dependence of CD signal at 222 nm of the apopeptides shows that the “melting point” of the reduced form is about 40°C. Pal14C19 completely denaturates at temperatures above 55°C (Figure IV.6.(right)). Which suggests the reduced form of the apopeptide may exist in an $\alpha$-helical conformation without the formation of coiled-coil structure. In contrast, Pal14c19ox does not reveal such strong denaturation with an increase in temperature. The oxidized apopeptide has a “melting point” of about 80°C. The beginning of the decomposition curve for this peptide appears only at temperatures higher than 80°C. Together, these experiments clearly show a drastic difference in the stability of the reduced and oxidized peptide coiled-coil structures. Pal14C19ox apopeptides exist as two-stranded $\alpha$-helical coiled-coils in the absence of metal ions.
IV.2. FORMATION AND PURIFICATION OF PLATINUM-PEPTIDE COMPLEX.

IV.2.1. Synthesis of metal-peptide assemblies of Pal14C19

Two metal complexes \([\text{NET}_4]_2[\text{ReBr}_3(\text{CO})_3]\) and \(\text{Pt(en)(NO}_3)_2\) were examined for the ability to form metal peptide assemblies with the covalently cross-linked coiled coil peptides. The conditions of these reactions were similar to the conditions for the reactions with Pal14 and Pal14new; 40°C in methanol for the Re-peptide reaction and 60°C in water for the Pt-reaction. The reaction of the Pal14C19ox with \(\text{Pt(en)(NO}_3)_2\) did not show any visible changes in the reaction vessel. In contrast, the reaction of Pal14C19ox with equal molar amounts of \([\text{NET}_4]_2[\text{ReBr}_3(\text{CO})_3]\) in methanol at 37 °C showed some unexpected results. After reacting for a week, a gel-like substance appeared at the bottom of the microfuge tube (10mg/ml) which could be resolubilized upon shaking (Figure.IV.7.). This behavior is characteristic of stimuli-responsive polymer gels\textsuperscript{23, 24}. Namely, this observation assumes the presence of unstable polypeptide chains weak enough to be destroyed by stress, yet able to reform upon removal of the stress to the sample\textsuperscript{25}.

![Figure.IV.7. Images of stimuli-responds polymer gels in solid and liquid states.](image-url)
IV.2.2. SDS-PAGE experiment

The SDS-PAGE experiment has been used for the characterization of metal-peptide complexes (see Chapter III). The SDS PAGE shows that Pal14C19ox apopeptide has a molecular weight of 7 kDa, which is two times that of Pal14C19, due to covalent crosslinking which prevents the dissociation of the coiled-coils. Therefore, any species in the metal-peptide reaction mixtures which appear 7 kDa above the band of apopeptide in gel-electrophoresis, are considered to be products of this reaction.

The reaction of Pal14C19ox with Re revealed several products with molecular weights different than the apopeptide alone (Figure.IV.8.). These products with molecular weights of 7, 14, 21 and 28, kDa correspond to one, two, three, and four peptide molecules bound together through the coordination of the rhenium core. The intensity of bands in SDS-PAGE experiments corresponds to the amount of the compounds which were loaded onto the gel. This allows estimation of the relative concentration of the formed products. The fraction of unreacted peptide has the strongest intensity and therefore is the main product in this reaction (about 50%). This result resembles similar reactions of Pal14 with Re (see Chapter III.2.).

Figure.IV.8. SDS-PAGE experiment of reaction mixture of the rhenium (I) complex with Pal14C19ox.
Similar to the rhenium-peptide reaction, the SDS-PAGE experiment for the platinum-peptide reaction mixture shows a series of bands with molecular weights about 7, 14, 21, 28, 35 and 42 kDa, which correspond to one, two, three, four, five and six peptide molecules bound together by platinum ethylenediamine (Figure IV.9.). In contrast to the Re reaction, the band intensity of the first three products, which are apopeptide, two, and three peptide molecules bound together by platinum, are very similar. It seems that Pt(en)(NO$_3$)$_2$ forms more stable metal-peptide complexes when compared to rhenium-peptide complexes. Together, these results show the advantages of utilizing the platinum complex when producing high molecular weight metal-peptide assemblies.

![Figure IV.9. SDS-PAGE experiment of reaction mixture of ethylenediamine platinum (II) with Pal14C19ox.](image)

**Figure IV.9.** SDS-PAGE experiment of reaction mixture of ethylenediamine platinum (II) with Pal14C19ox.

**IV.2.3. Mass spectroscopy**

MALDI-MS experiments were performed to help characterize the composition of the metal-peptide reaction mixtures.$^{26}$ Due to the presence of multiple charged species, ESI mass spectroscopy could not be applied for the characterization of these assemblies. However, the
ionizing conditions of MALDI produce mono charged species, therefore allowing visualization of molecular ions. This experiment in tandem with SDS-PAGE was expected to reveal the formulation of the metal peptide assemblies in the reaction mixtures. MALDI-MS spectra for both Re-peptide and Pt-peptide mixtures consist of multiple broad signals as seen in Figure.IV.10 and Figure.IV.11.

The MALDI experiment for both rhenium and platinum-peptide reaction mixtures show the presence of several signals spaced 7.25 apart, in which the signal of apopeptide has the largest intensity. The peaks of high molecular weight species are wide which does not allow determination of their exact molecular weight. Nevertheless, all of the peaks in these mass spectra are divisible by the molecular weight of the apopeptide bound to one metal complex (7.25 kDa), with the highest ratio m/z around 27.5 kDa corresponding to four peptide molecules bound together by metal centers.

![Figure.IV.10.](image)

Figure.IV.10. MALDI-MS experiment for reaction mix of Pal14C19ox with the rhenium (I) complex.
The MALDI-MS experiments show a high similarity of the reaction mixtures for rhenium and platinum. However, the molecular weight of the largest metal peptide assemblies is 27.5 kDa which differs from the result of SDS-PAGE for platinum-peptide reaction mixture. Previous results show that rhenium and platinum bonds with peptides based on GCN4 sequences are not stable in MALDI-MS experiments (see Chapter III). Therefore, it has been suggested that the high molecular weight species which are seen in the gel for the platinum-peptide reaction do not survive under the denaturizing conditions of MALDI-MS.
IV.2.4. High performance size exclusion chromatography

The high performance size exclusion chromatogram (HPSEC) of the rhenium-peptide reaction mixture shows three well distinguished peaks which correspond to apopeptide, two and three peptide molecules bound by rhenium complexes. In addition to these peaks, there is a shoulder with an elution time > 25 min which corresponds to assemblies with molecular weights higher then 21 kDa (Figure.IV.12. (left)). The intensity of these peaks shows that the apopeptide is the biggest fraction in the reaction mixture, which is consistent with the results of MALDI and SDS-PAGE experiments.

The HPSEC experiment for the platinum-peptide reaction reveals peaks which are not well resolved (Figure.IV.12. (right)). Four peaks can be seen in the chromatogram and correspond to apopeptide, two, three and four peptide molecules coordinated by platinum complexes. In contrast to the rhenium-peptide experiment, the biggest fraction in this HPSEC diagram corresponds to high-molecular weight species. This result is inconsistent with MALDI-MS and SDS-PAGE data for platinum-peptide reaction mixture, in which the biggest fraction correspond to the unreacted apopeptide.

**Figure.IV.12.** HPSEC experiments for reaction mix of Pal14C19 ox with rhenium (I) (left) and platinum (II) (right) complexes. Phosphate buffer pH7 Superdex 75
The conditions of HPSEC allow separation of non-covalent molecular assemblies of α-helical coiled-coil peptides.\textsuperscript{27, 28} In contrast, the conditions of SDS-PAGE and MALDI-MS experiments can show species which have covalent or coordination bonding. Therefore, the differences between the results of HPSEC, SDS-PAGE and MALDI-MS suggest that the reaction of ethylenediamine platinum (II) with Pal14C19ox form high molecular weight platinum-peptide structures, the metal-peptide bonding energy of which is the energy of non-covalent peptide interaction.

IV.2.5. Separation and characterization of the formed metal-peptide assemblies

The formation and characterization of pure metal-peptide assemblies is a main interest of this work. Therefore we had to develop a method for separation the products of platinum-peptide and rhenium-peptide reactions. The application of high performance size exclusion liquid chromatography (HPLC) for separation of the formed products did not separate the metal-peptide assemblies neither for rhenium nor for platinum reaction mixtures. The HPLC chromatogram showed a mixture of peaks which were difficult to resolve.

As an alternative to HPLC, HPSEC was used to separate the formed metal-peptide assemblies. The fractions were collected and their purity was analyzed by HPSEC and SDS-PAGE experiments. The collection of the fraction in the HPSEC chromatogram for the rhenium-peptide mixture followed by reinjection of this fraction onto the SE column showed a high instability of the purified Re-peptide assemblies in which the product of decomposition, apopeptide Pal14C19ox, was the main fraction. This result was anticipated because of the low stability observed in the similar structure, di-substituted rhenium-Pal14 complex under HPSEC experiments conditions (see Chapter III.2.).
Figure IV.13. Left: HPSEC experiments for reaction mix of Pal14C19ox with ethylenediamine platinum (II) Phosphate buffer pH7 Superdex 75; Right: SDS-PAGE analysis of collected fractions in the HPSEC experiment.

The assemblies formed in the platinum-Pal14C19ox reaction have a higher stability than similar rhenium-peptide assemblies under HPSEC experiment conditions, and were successfully purified. Loading the collected fraction onto gel allowed their characterization by SDS-PAGE (Figure IV.13.). The gel electrophoresis showed that the second and third fractions, which correspond to platinum-peptide’s dimer and trimer products, are well purified. In contrast, fractions 4 and 5 showed the mixture of Pt-peptide assemblies of different molecular weights in which platinum-peptide’s tetramer and pentamer products dominate. The difference in the size of these molecular assemblies decreases, with an increase in the overall molecular weight. As shown in the Figure IV.14, the size difference between apopeptide and a metal-peptide dimer complex is about two fold; in contrast the size difference between metal-peptide tetramer and pentamer complexes is only about 20%. Therefore, the difference between the purity of the first
three and the last two fractions results from the low resolution of high molecular weight species in the HPSEC experiment.

The analysis of collected fractions by HPSEC has shown that all of the purified samples exist as a single peak in the SE chromatogram (Figure.IV.15. (left)). The results of this experiment were plotted on a calibration graph of log(MW) against Kd, which can generally show the extent of size separation\(^9\) (See ChapterII). Samples with similar morphology but different molecular weights can be fitted on a line in this graph. The first four fractions have

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**Figure.IV.14.** Sizes of the first five products of the reaction of Pal14C19ox with rhenium or platinum complexes estimated by molecular modeling.
been plotted on the calibration graph, and a best fit line shows the size-log(MW) ratio correlates well with each-other (Figure.IV.15. (right)). In contrast, the fifth fraction is an outlier, and falls below the line. This observation can be explained in two ways. First, the shape of fifth assembly is globular in nature which gives a smaller ratio of size-Log(MW) in comparison to linear assemblies. This would suggest a difference in the morphology between first four and fifth fraction. Another possible explanation is the poor resolution of the column. Namely, the elution time of this assembly is close to the elution front (dead volume) of the SE column, in which the Kd value is distorted. AFM imaging for the fifth and fourth fractions was completed were expected to show the morphology of these complexes.

Figure.IV.15. Left: HPSEC experiments for purified assemblies of Pal14C19ox with ethylenediamine platinum (II) Phosphate buffer pH7 Superdex 75; Right: Calibration graph for SE Superdex 75 in phosphate buffer pH7.
IV.2.6. Atomic force microscopy

AMF microscopy was useful in the characterization of the non-covalent metal-peptide assemblies showing globular and linear morphologies (Chapter III.3), and therefore was expected to give informative images for platinum and rhenium reactions with crosslinked Pal14C19ox. Both incubation-rinsing and evaporation (air-dry) techniques were used to show the independence of the assemblies’ morphology from the sample preparation.

The samples of apopeptide were prepared and analyzed by AFM as a reference for the samples of metal-peptide assemblies. AFM images of Pal14C19ox are shown in Figure IV.16. Images A and C were obtained by incubation-rinse technique (0.5 mg/ml) and image B by evaporation. These samples were well dispersed on the mica with aggregations similar to that of the non-oxidized peptide (Chapter II.3). Like the non-oxidized peptide, Pal14C19ox shows the possible formation of a peptide film on the surface of mica (image C). There are several gaps (shown by arrows) on the mica which can be explained by gaps in the peptide film covering the surface. These images were made for comparison with the images of metal-peptide assemblies.
Figure IV.16. AFM images of pure oxidized peptide Pal14C19ox. These images are from different sources. Images A and C were obtained by incubation-rinse technique (0.5 mg/ml) and the image B by evaporation.
The AFM images of the fourth and fifth fractions of the HPSEC experiment of the platinum-peptide reaction mixture show the presence of many circular structures (Figure IV.17.). These metal-peptide assemblies are seen on the images obtained by the evaporation technique (A,C). The platinum-peptide complex of fraction 4 (images A,B) appear as circles with diameters of 100-400 nm. The height of these large, spherical particles is ~13 to 20 nm. A higher resolution phase image of these samples shows much smaller structures which could possibly be individual tetramers. The platinum-peptide complex of fraction 5 (image C) appears as non symmetrical circles of 100-200 nm in diameter. The dimensions of these assemblies are far above the estimated dimension of the single metal-peptide complex of these fractions, which is 5-8 nm (Figure IV.14). The height of the spherical particles is from 13 to 20 nm, which about three times higher than the dimensions of the single metal-peptide assemblies observed in SDS-PAGE and MALDI. This behavior of the platinum-peptide sample (n = 5) in AFM experiment could explain the low Kd-log(MW) ratio in HPSEC for its fraction. However, fraction 4, which is consistent with the other fraction’s Kd-log(MW) value, forms similar circular aggregates. Importantly, these structures are not seen in the HPSEC. This suggests there are some very weak interactions which force the metal-peptide assemblies to form these circular aggregates on the surface of mica.

The formation of similar, low in free energy formation molecular assemblies has been observed before.\textsuperscript{29,30} It has been shown that the formation of assemblies can be induced by hydrodynamic and surface effects if the samples obtained by evaporation technique. Specifically, water evaporates very slowly in comparison to the most organic solvents, and can induce the formation of circle structures. The appearance of such low energy of formation assemblies is very sensitive to the evaporation technique; therefore the images of the samples prepared by
incubation-rinse technique were examined by AFM on the formation of the circle assemblies.

The formation of such large aggregates is also observed in the AFM images for the sample which formed a peptide gel.\textsuperscript{30} Because the similar behavior of the metal peptide assemblies of fractions 1 and 4 show the formation of circular structures in AFM, they can potentially form a gel.

**Figure IV.17.** AFM images of ethylenediamine platinum (II) with Pal14C19ox have many spherical particles present. These images were obtained by evaporation technique (A-C).
The staining-rinsing technique assumes the attachment of the samples to the surface of mica before the final drying step. Importantly, the hydrodynamic effect during the evaporation of these samples should not effect the final aggregation of the samples. The samples which were obtained by this technique did not show difference in the aggregations of fractions 4 and 5.

AFM images of platinum complex with oxidized peptide which were obtained by incubation-rinse technique (images D, E) are difficult to interpret (Figure IV.18.). These pictures show the presence of some spherical aggregates which have some similarity to the aggregates obtained through evaporation techniques. The dimensions of the aggregates are 50 nm - 1 μm in diameter. However, these assemblies did not have irregular structures, and look more like the assemblies that form a film of single platinum-peptide complexes. The gaps, which are characteristics for the presence of peptide films on surface, are shown by arrows in the Figure IV.18.
Figure IV.19. AFM images of rhenium complex with oxidized peptide which were obtained by evaporation technique.

The AFM images of the rhenium-peptide reaction mixture, which were obtained by evaporation, show the presence of large circular aggregates (Figure IV.19.). These assemblies have a similar shape to the platinum-peptide assemblies, but are much larger in size. The estimated size of these assemblies varies from 400 nm to 4 μm in diameter. The heights of these spherical particles are from 10 to 15 nm, which is similar to the dimensions of platinum-peptide aggregates, but much higher than the dimensions of single metal-peptide assemblies which are observed in SDS-PAGE and MALDI for these samples. The difference in the aggregation state of the platinum-peptide and rhenium-peptide assemblies may explain the stress-response gel formation by the rhenium-Pal14C19ox reaction mixture and absence of any gel properties in the case of platinum-Pal14C19ox reaction mixture. Importantly, the rhenium-peptide reaction mixture shows the gel formation in methanol solution while platinum-peptide reaction mixture,
which is prepared in water, does not form a gel. The formed rhenium-peptide gel disappears when placed in water. Therefore the solvent plays important role in the gel formation.

IV.2.7. Transmission electron microscopy

The negative staining of the rhenium-peptide reaction mixture was analyzed by transmission electron microscopy (TEM). The application of this electron microscopy was found to be inconvenient for visualization of our metal-peptide assemblies (Chapter III). The metal-assemblies are not visible by themselves in TEM and required staining with heavy atoms which can potentially bind the metal-peptide complexes and change the morphology of the sample. Control of the sample’s concentration, which influences the morphology of the samples (See chapter III), is difficult during the staining process. Nevertheless, this microscopy was applied for characterization of the rhenium-peptide reaction mixture and revealed similar (100 – 500 nm circular assemblies) results to those observed in AFM experiment. The samples are prepared by negative staining with uranyl acetate, therefore the light part of the images correspond to the presence of metal-peptide molecules and the dark to their absence (Figure IV.20.). The formation of similar large aggregates for peptide gel structure has previously been shown.30 The observation of these aggregates in both AFM and TEM suggests that these aggregates are not an

Figure IV.20. TEM images of rhenium complex with oxidized peptide which were obtained by evaporation technique.
IV.2.8. Summary

Both the rhenium and platinum complexes react with Pal14C19ox to form multiple metal-peptide assemblies. The results presented by HPSEC, SDS-PAGE and MALDI show that metal-binding of crosslinked coiled-coil peptides can form up to five metal-peptide conjugates. The formed platinum-peptide polymer is shown to have a higher stability when compared with the products of the rhenium-peptide reaction.

The results of the platinum-Pal14C19ox assemblies’ formation are similar to the result of the reaction of the metal complexes with Pal14, in which the formation of high molecular weight species was not observed. In contrast to this reaction, rhenium-peptide complexes form a metal-peptide hydro-gel structure which shows stress-responsive physical properties.

Understanding the formation of stress-responsive gel by the reaction of rhenium with Pal14C19ox is still under development. The assembling energy of the aggregates, which form the gel, is so low that these structures can be broken by a mechanical shock. Interestingly, platinum-Pal14C19ox reaction forms similar aggregates to the rhenium-peptide structural assemblies which are observed in AFM images, but do not form a gel. The lower energy of rhenium-Pal14C19ox binding becomes relevant in the formation of large metal-peptide aggregates, and is suspected to be an important factor in such behavior.
IV.3. SYNTHESIS AND CHARACTERIZATION OF METAL_PEPTIDE ASSEMBLIES
OF PAL14C19new

IV.3.1. Introduction

Similar to the reaction of non-covalent peptides (Chapter III), the covalently crosslinked peptide based on “new” IAALEQK sequence Pal14C19ox was expected to form more stable complexes with Pt(en)(NO\textsubscript{3})\textsubscript{2}, and therefore was expected to form longer metal-peptide chains. The conditions of these reactions were similar to the conditions for the reaction Pal14C19ox, which is 60°C in water for the platinum-reaction. The formation of the platinum-peptide products was observed by a characteristic change in the UV-Vis spectrum, which is peak centered at 250 and shoulder at 270 (See Chapter III.1.3.). At the end of the reaction, a small amount of precipitate was observed at the bottom of the reaction vessel.

IV.3.2. Synthesis of the peptide ligand

Similar to the synthesis of Pal14C19, the peptide Pal14C19new was synthesized by solid-phase techniques and purified by reverse-phase HPLC (Figure.IV.21.). The molecular weight (calc’d: 3288.81) was determined by MALDI mass spectroscopy, which is shown in Figure.IV.23. The observed peak has MW 3306 which corresponds to the molecular ion of Pal14C19ox bound to one atom of potassium, which is common for this technique. Pal14C19nox, was prepared by oxidation of the cysteine residues with I\textsubscript{2} and also purified by HPLC. The difference in the retention time of oxidized and non oxidized peptide forms in the HPLC experiment is larger in the “new” peptide sequence in comparison to “original” one. The higher hydrophobicity of the “new” peptide can explain this observation.
Analysis of Pal14C19nox by MALDI-MS shows the presence of the signal of molecular ion of oxidized peptide (calc’d: 6575.62.08; obs’d: 6584.95) in addition to a smaller amount of the reduced peptide monomer (calc’d: 3288.81; obs’d: 3287.51), which can be due to thermal decomposition of the disulfide bond of cysteine crosslinking or the presence of double charged peptide molecular ion (Figure.IV.23. (left)). Importantly, the signal intensity of the oxidised form of the new peptide is much higher when compared to the similar signal of the Pal14C19ox. This result shows a better stability of the new peptide under ionizing conditions in MALDI-MS and therefore was expected to show higher stability under conditions of SDS-PAGE and HPSEC experiments as well.
IV.3.3. Circular dichroism of the peptide ligand

Similar to the peptide of “original” sequence, the CD spectrum of both reduced (Pal14C19n) and oxidized (Pal14C19nox) forms of the apopeptide consist of minima at 208 and 222 nm showing that the peptide exists within the $\alpha$-helical conformation$^{19,20}$ as it shown in Figure IV.24:
Figure IV.24. Left: Circular dichroism spectrum of Pal14C19n (black) and Pal14C19nox (red) in water. Concentration of Pal14C19 is 1.24x10^{-4} mole/l and concentration of Pal14C19ox is 1.08x10^{-4} mole/l. Right: Temperature dependence of the CD signal at 222 nm

The intensity at 222 nm is used to determine the α-helical content\(^{21, 22}\) which is 56.8% (\(\theta_{222} = -19,350\) deg cm\(^2\) dmol\(^{-1}\)) for Pal14C19n and 86.8% (\(\theta_{222} = -29,510\) deg cm\(^2\) dmol\(^{-1}\)) for Pal14C19nox in water. Similar to the peptide of “original” sequence, the low helicity value of the reduced form can be interpreted as a low stability of the α-helical conformation which is interrupted by the presence of a bulky cysteine residue in the hydrophobic core of the molecule.

The ratio of \(\theta_{222}/\theta_{208} = 1.15\) for the oxidized form of the apopeptide is considered to be indicative of a coiled-coil conformation. The reduced form of the apopeptide has a ratio of \(\theta_{222}/\theta_{208} = 0.97\) which is a little low for a coiled coil conformation.

In contrast to the peptide of “original” sequence, temperature dependence of the CD signal at 222 nm of the reduced and oxidized form of the apopeptides did not show a strong denaturation of the peptide with an increase in temperature. The helicity of the oxidized form of the apopeptide has a higher helicity in comparison to the reduced form, but the shape of the
temperature dependence curve is very similar. Together, these experiments show a strong
difference in the stability of α-helical coiled coil conformation of the apopeptides of “new” and
“original” sequence.

IV.4.3. SDS-PAGE experiment

As expected, the reaction of Pt(en)(NO$_3$)$_2$ with Pal14C19nox revealed the formation of
longer metal-peptide polymer chains when compared to the reaction with Pal14C19ox. The
difference in the propagation of the metal-peptide polymer formation is seen when monitoring
the reaction by SDS-PAGE (Figure.IV.25.). This difference appears in the formation of a
species of high molecular weight (n = 7 and higher) on the 8$^{th}$ day of the reaction of the platinum
complex with Pal14C19nox (Figure.IV.25. (right)). It is also seen on the gel for the paltinum-
Pal14C19nox reaction that the apopeptide and platinum-peptide dimer, which is the first formed
complex, almost disappears, and the reaction consists mainly of the high molecular weight
species. In contrast, the highest molecular weight of the products in the reaction of Pal14C19ox
with platinum is about 33kDa (n = 5) (Figure.IV.25. (left)), and the biggest fraction in this
reaction mixture is unreacted apopeptide.
Figure IV.25. Left: Monitoring of the formation of ethylenediamine platinum (II) with Pal14C19ox by SDS-PAGE experiment. Right: Monitoring of the formation of the platinum complex with Pal14C19nox by SDS-PAGE experiment.

The SDS-PAGE experiment clearly shows the ability of a new peptide to form high molecular weight species in the reaction with ethylenediamine platinum (II). The large platinum-Pal14C19nox complexes, which have a molecular weight above 27kDa, appear as a tight series of bands. Resolving these species is difficult in microgel electrophoresis, but can be reached by applying higher potentials or full scale electrophoresis gel if necessary.

IV.3.5. Mass spectroscopy

The MALDI-MS spectrum of platinum-Pal14C19nox mixture exists as many signals, with the highest signals around 50kDa (Figure.IV.26.). This molecular weight is almost twice higher than the one which was determined for the Pal14C19ox-Pt mixture. Importantly, the
largest signal in this MS spectrum still belongs to the MW of apopeptide which almost 
disappears in the SDS-PAGE experiment. This result shows the decomposition of the platinum-
peptide complexes under the ionizing conditions of MALDI-MS, even for the more stable 
peptide.

Figure IV.26. MALDI-MS experiment for reaction mix of Pal14C19nox with 
ethylenediamine platinum (II).
IV.3.6. Size exclusion high performance chromatography

As it was shown in the platinum-Pal14C19ox reaction, HPSEC allows separation the metal-peptide assemblies which are not seen by SDS-PAGE or MS methods. (Chapter III.2) Therefore the platinum-Pal14C19nox assemblies with MW > 35 kDa were expected to be registered by these techniques. In order to get a better resolution in the HPSEC chromatograms for the Pl14C19nox-palatinum complexes, a size exclusion column with a molecular weight determination range of 100-300kDa was used. Monitoring the reaction of the ethylenediamine platinum complexes with Pal14C19nox by HPSEC is shown in figure IV.27:

Figure IV.27. Formation of the ethylenediamine platinum complexes with Pal14C19nox by HPSEC. Phosphate buffer pH7 Superdex 200
As expected, with the propagation of the platinum-Pal14C19nox reaction the HPSEC chromatogram shows the presence of many large molecular structures. These structures are capped by unreacted apopeptide molecules in a mixture of assemblies which have different sizes. Similar to the reaction with Pal14new (Chapter III.3.), the different sizes of these assemblies are responsible for the jagged shape of the HPSEC diagram. The assemblies of a low molecular weight 7 kDa and 14 kDa \((n = 1, n = 2)\) formed the smallest fractions on the SE chromatogram. In contrast, high molecular weight species \((n > 6)\) dominate the HPSEC chromatogram on the 8th day of reaction.

The results of HPSEC experiments suggest the formation of large platinum-peptide assemblies through the metalation of the peptide. These assemblies are different in size and most likely have a linear structure with platinum-peptide complexes forming the body. The domination of the large molecular assemblies in the HPSEC for platinum-Pal14C19nox reaction mixture suggests almost complete reaction and polymer formation. The observation of a small precipitate at the end of the reaction suggests that the high molecular weight polymers of platinum-Pal14C19nox have a low solubility, which is expected of “infinitive” polymer lines.

**IV.3.7. Atomic force microscopy**

Atomic force microscopy (AFM) was used to visualize the formed platinum-peptide polymers (See Chapter III.3.). To prevent formation of artifacts during solvent evaporation, the staining-rise technique was used to prepare the sample for AFM imagining. The image of the aqueous solution with the concentration of 1mg/L reveals the presence of many worm-like structures (Figure IV.28.). The length of these assemblies varies from 15 to 100 nm. The
The dimensions of these assemblies are 15-25Å high and 2-10nm wide. These dimensions are similar to the size of a single apopeptide molecule (Figure IV.29) lying on the surface of mica. Therefore, these structures are metal-peptide polymers in which peptides of coiled-coil structure are coordinated by Pt(en)(NO₃)₂ complexes into linear chains. The formation of similar structures have been observed in the AFM experiment of Pal 14new with Pt(en)(NO₃)₂ (Chapter III.3).

Figure IV.28. Left: AFM images of diluted solution of complex of Pal14C19nox with Pt(en)(NO₃)₂ (incubation-rinse technique, water solution of 1 microgram/ml). Right: Criss-cross profile of the formed assemblies.

Increasing in the concentration of the metal-peptide samples to 10mg/l led to the formation of globular metal-peptide assemblies on the surface of mica (Figure IV.3.7.2.). The dimensions of these globular structures are 25-50 nm in diameter and 1-3 nm high. These dimensions would fit the observed linear assemblies if they would form circular structures. The
criss-cross profile some of these globules revealed the presence of a gap in the center of the formed globules, indicative of a doughnut-like structure, formed from the linear structures. This shows that the formed globular assemblies are indeed linear metal-peptide assemblies which are forced by steric repulsion to form circles on the surface of mica. Importantly, this doughnut-like morphology of the platinum-Pal14C19ox was not observed in the case of covalent platinum-peptide assemblies which suggests a difference in the mechanism of their formation.

Nevertheless, the change of the linear-globular morphologies with concentration is similar for both non-covalent and covalent crosslinked metal-peptide assemblies. These results suggest some similarity in the structure and the formation of the platinum-peptide assemblies.

Figure IV.29. Left: AFM images of Pal14C19nox complex with Pt(en)(NO₃)₂. The images were obtained by incubation-rinse technique from a solution with concentration 10 micrograms/ml. Right: Criss-cross profile of the formed assemblies.
IV.3.8. Effect of different metals on the formation of the metal-peptide complex

Several different metal complexes were examined for the ability to form a stable complex with Pal14C19ox and Pal14C19nox. Similarly to the reaction with non-covalent peptides, the Ni-Pd-Pt and Ru complexes was examined by mixing the equal molar amount of peptides with metals.

A “naked” ion of Ni$^{2+}$ did not show any stable products with any of the peptide ligands. Ru(bpy)$_2^{2+}$ was reacted with Pal14C19nox. After one week of monitoring the reaction mixture by SDS-PAGE, the experiment revealed the formation of several Ru-peptide complexes (Figure IV.30.) with molecular weight about 14, 21 and 28 kDa which corresponds to the two, three and four peptide molecules bound together by Ru(bpy)$_2^{2+}$. Under similar conditions during two weeks, the reaction of Pd(en)$^{2+}$ with Pal14c19nox did not show any results.

![SDS-PAGE image](image)

**Figure IV.30.** The reaction Ru(bpy)$_2^{2+}$ with Pal14C19n in D$_2$O by SDS-PAGE
IV.3.9. Summary

Ethylenediamine platinum (II) reacts with Pal14C19ox with the formation of multiple metal-peptide assemblies. The results of MALDI-MS, SDS-PAGE and HPSEC show that the platinum-peptide complex formation results in longer metal-peptide chains in comparison to the reaction of the “original” peptide. The peptide ligands in these assemblies are coordinated by Pt(en)(NO3)2 complex into linear chains. The main reason in the stability of the formed high molecular weight species is most likely the higher stability of Pt-pyridylalanine bond of the peptide with IAALEQK repeat sequence.

Similar to the results of the Pal14new (See Chapter III.3.), the assemblies of Pal14C19nox have linear or globular morphology on the surface of mica. The observation of doughnut-like structures (Figure IV.30.) suggests that the formed globular structures are indeed linear metal-peptide assemblies which are forced by sterical repulsion to form circles on the surface of mica.

Figure IV.31. Doughnut-like structures of Pal14C19nox complex with Pt(en)(NO3)2 on the surface of mica
CHAPTER IV REFERENCES:

CHAPTER V: METAL COORDINATION OF PEPTIDE LIGANDS HAVING MULTIPLE COORDINATION SITES

V.1. STRUCTURAL DESIGN

V.1.1. Introduction

The following chapter describes the effect of metal coordination on the oligomerization state of peptides with multiple coordination sites. The presented work explores the use of two metal binding sites in the hydrophilic exterior of the two-stranded coiled-coil to manipulate the spatial organization of the peptide assemblies. It was anticipated that the incorporation of multiple metal binding sites might decrease the degree of freedom of the formed metal-peptide complexes, and force the formation of a cyclic, enthalpy driven product rather then a polymeric, entropy driven product. Similar to the formation of non-covalent and covalently crosslinked metal-peptide assemblies, Pt(en)(NO$_3$)$_2$ was expected to bind the peptide ligand. The schematic view of this strategy is shown in figure V.1:

![Figure V.1. Strategy of the formation of metal-peptide assemblies](image-url)
V.1.2. Design of peptides ligands

The peptide used in this work was designed to form a non-covalent two-stranded $\alpha$-helical coiled-coil$^{4-9}$. As previously stated, the peptides with new sequence (IAALEQK) form the stabilize bonds with Pt(en)(NO$_3$)$_2$ (Chapter III.3.; Chapter IV.3). Therefore, the design of the peptide in this work was also based on the IAALEQK heptad where amino acid residues at positions a, b, c, and d (IAAL) are responsible for the formation of a very stable two-stranded $\alpha$-helical coiled-coil peptide$^{10,11}$. To create the metal-binding ability of the peptide, a strong metal-binding site, non-natural amino acid 4-pyridylalanine$^{12}$, was placed at positions 14 and 21 of the sequence, which occupy the highly exposed “f” positions of the second and third heptads. This peptide was named Pal14Pal21n. Substitutions in the hydrophilic reign of coiled coil peptides in general, and position “f” in particular, have the smallest effect on the conformation and oligomerization state of the $\alpha$-helical coiled coil peptides$^{11}$. Another feature of our design is the positioning of the metal-binding sites i, i+7, which are two $\alpha$-helical turns apart, makes intramolecular metal-peptide binding highly unfavorable due to the distance between the sites. Therefore, the products of the reaction were expected to be intermolecular platinum-peptide complexes which will keep the original $\alpha$-helical coiled-coil conformation of the peptide. The sequences and computer generated model of the peptides used in this work are shown in Figure V.2
V.1.3. Synthesis of the peptide ligand

Solid phase method was used to prepare the 30-residue polypeptide Pal14Pal21new (Pal14Pal21n) peptide. The purity of the peptide was confirmed by analytical high performance size exclusion chromatography (HPLC), ESI-MS and MALDI-MS (Figure V.3.). ESI-MS shows signals m/z = 1101, 826, 661, and 551 which corresponds to the +3, +4, +5, and +6 ions of a species with a molecular weight 3300 (calc’d: 3299.89). MALDI-mass spectroscopy reveals the single peak of the molecular ion (calc’d: 3299.89; obs’d: 3300.15).
Figure V.3. Characterization of apopeptide Pal14Pal21: The purity of the peptide was confirmed by analytical HPLC (A) and mass spectroscopy experiments MALDI-MS (B), ESI-MS (C).

V.1.4. Conformational stability of Pal14Pal21n

Circular dichroism (CD) spectroscopy is used to evaluate the secondary structure of the apopeptide. The circular dichroism (CD) spectrum of the Pal14Pal21n apopeptide consists of
minima at 208 and 222 nm which indicates that the peptide exists within the α-helical conformation\textsuperscript{13, 14} (Figure V.4.(left)). The ratio between the signals at 208 and 222 nm, which is $\theta_{222}/\theta_{208} = 1.04$, corresponds to the generally accepted range ($\theta_{222}/\theta_{208} > 1$) indicating the formation of the α-helical coiled-coil\textsuperscript{13}. The observed value of $\theta_{222} = -26,538$ deg cm\textsuperscript{2} dmol\textsuperscript{-1} indicates that the apopeptide is 76% helical in comparison to that predicted for a pure 30-residue α-helix\textsuperscript{15, 16}. These values are comparable to the values for Pal14 and Pal14n (See Chapter III.). The helicity of Pal14Pal21n is in between of the helicity of Pal14 (57%), which does not form stable metal-peptide products, and Pal14new (91%), which forms highly stable metal-peptide complexes.

\textbf{Figure V.4.} Left: CD spectrum of Pal14Pal21n; Concentration of the apopeptide Pal14Pal21n is $2.5 \times 10^{-4}$ mole/l Right: temperature diagram of the CD signal at 222 nm. Measurement was made at 25\textdegree C in 20mM phosphate buffer with 50mM KCl.

Pal14Pal21n does not drastically denature with increasing temperature (Figure V.4.(right)). The apopeptide still exists as 50% of α-helical structure at 80\textdegree C. Similarly to the
data for Pal14new, the beginning of decomposition curve for Pal14Pal21n appears only at temperatures higher then 85°C.

The conformational stability of the Pal14Pal21 coiled-coil was studied by guanidinium chloride (GdnHCl) titrations\textsuperscript{17-19} Figure V.5 shows the denaturation plot of Pal14Pal21n in comparison to Pal14 and Pal14new. The denaturation curve of Pal14Pal21n lies closer to the curve of Pal14new showing a high conformational stability of the apopeptide.

![Figure V.5](image)

**Figure V.5.** The plot of denaturation of Pal 14 (blue), Pal14new (red) and Pal14Pal21n (green) with increasing concentration of guanidinium chloride. The concentrations of peptide were within of 40-80 μM.

The free energy of the folding was determined by extrapolating the linear factor in the change of the free energy with increasing concentration of guanidinium chloride to y axial (Figure V.6.). The full description of the procedure is given in the material and methods chapter. A mid-point concentration of \([\text{GdnHCl]}_{1/2} \approx 6\) M shows that Pal14Pal21n peptide is indeed very
stable, having a $\Delta G^{H2O} = 12.1$ kcal/mol. Pal14new has a midpoint around 7.5 M GdnHCl and $\Delta G^{H2O} = 13.8$ kcal/mole, which makes the difference between the stability of the apopeptides only $\Delta G^{H2O} = 1.7$ kcal/mole. For comparison, the denaturation midpoint for Pal14 is 2.5 M GdnHCl and $\Delta G^{H2O} = 7.3$ kcal/mole. Together the results show high conformational stability of Pal12Pal21n which is comparable with that of Pal14new.

Figure V.6. The free energy of Pal14Pal21n coiled-coil folding with increasing concentration of guanidinium chloride.
V.2. PURIFICATION AND CHARACTERIZATION OF THE PT-PAL14PAL21 REACTION PRODUCTS

V.2.1. Characterization of the reaction Pal14Pal21 with ethylenediamine platinum (II)

The Pt-peptide complex was prepared by reaction of Pt(en)(NO$_3$)$_2$ with Pal14Pal21n for several days at 60°C in water. The reaction of the product was observed by a characteristic change in the UV spectrum which appears as an absorption band centered at 250 nm with a shoulder appearing at 270 nm (See Chapter III.1.4.). The crude reaction mixture was characterized by SDS-PAGE and HPLC techniques.

The SDS-PAGE experiment (Figure V.7.) revealed the presence of only a single reaction product having a molecular weight of approximately 14 kDa. The molecular weight of the product is consistent with the formation of four peptide molecules linked together by coordination of the *cis* positions of the square planar platinum complexes. The species appearing at ca. 3 kDa indicates the presence of unreacted apopeptide which is expected to travel as a monomer under the denaturing gel conditions.

![SDS-PAGE experiment](image)

**Figure V.7.** Analysis of the reaction mixture by SDS-PAGE experiments.
Because Pal14Pal21n has two metal-bonding sites can lead to different orientations of the peptide molecules within the formed platinum-peptide complex and therefore, the presence of several products in the reaction mixture is possible. Therefore, reverse-phase C$_{18}$ HPLC was used to characterize the reaction mixture (Figure V.8). To prevent decomposition of the formed metal-peptide complexes in the acetonitrile/water media of the HPLC experiment, an alternative mobile phase gradient of methanol/water was used (See Chapter II). The HPLC chromatogram shows the presence of three products (peaks 1, 3 and 4) with retention times different from the retention time of the apopeptide (48 min, peak 2).

![HPLC Chromatogram](image)

**Figure V.8.** Analysis of the reaction mixture HPLC experiments.

The different retention times in the HPLC experiment suggests a difference in the hydrophobicity of different products of the reaction. The results of the SDS-PAGE experiment showed that all products formed in the reaction of Pal14Pal21n with ethylenediamine platinum (II) have similar molecular weight of about 14 kDa. To clarify this observation, further studies
were performed to separate and characterize these peaks. The amount of fraction 3 is low in comparison with fraction 1 and 4. Therefore, we have chosen to focus on characterization of only the main products in this reaction (Fraction 1 and 4). These products were successfully separated from the reaction mixture by preparative HPLC. The analytical HPLC chromatograms of the collected fraction 1 (FR1) and 4 (FR4) show that the samples keep their retention time (Figure V.9.) and survive the HPLC separation. Longer elution time of the FR4 suggests that it has a higher hydrophobicity in comparison to FR1, the elution time of which is 5 minutes less.

![Figure V.9. Analytical HPLC chromatogram of purified fraction 1 (left) and fraction 4 (right).](image)

**Figure V.9.** Analytical HPLC chromatogram of purified fraction 1 (left) and fraction 4 (right).

### V.2.2. SDS-PAGE experiment of purified products of the platinum-peptide reaction

All collected HPLC fractions characterized by SDS-PAGE (Figure V.10.). SDS-PAGE shows that HPLC fractions 1-2 contained a mixture of 3 and 14 kDa species whereas fraction 3 and 4 contained only the 14 kDa. The presence of low molecular weight species in the first fraction is due to poor purification. The HPLC peak of the first fraction overlaps with the peak of unreacted apopeptide which further appears as an impurity in the SDS-PAGE experiment.
Interestingly, the expected products of the reaction such as platinum-peptide dimer (MW ~ 6.6 kDa), trimer (MW ~ 9.9 kDa and complexes with a molecular weight higher than tetramer (MW > 14 kDa) almost do not form. Together, these results show that the four peptide molecules are crosslinked together by the four platinum complexes.

![Image](image.png)

**Figure V.10.** Analyze of the reaction mixture HPLC experiments.

V.2.3. **Mass spectroscopy of purified products of the platinum-peptide reaction**

The SDS-PAGE experiment gives only the approximate molecular weights of the samples which is useful for determination of oligomerization state of peptides. Thus MALDI-MS and ESI-MS were carried out for each collected peak in the HPLC experiment to obtain complete information about their molecular weights.

Among the variety of MS techniques MALDI has the softest ionization conditions and allow visualization of mono charged molecular ions\(^ {20}\). The MALDI experiment for FR1 and FR4 similarly shows the presence of a series of signals which are divisible by the molecular weight of the apopeptide bound to one platinum ethylenediamine complex and has the highest ratio m/z
around 14.3 kDa (Figure V.11 - Figure V.12.). The presence of species with molecular weights about of 7 and 11 kDa in the MALDI experiment, but their absence in the SDS-PAGE experiment suggests a low stability of the formed metal-peptide complexes which mostly decompose even under the ionizing conditions of MALDI-MS. However, the highest molecular weight (~ 14.4 kDa) in the MALDI experiment, in agreement to the gel electrophoresis data, corresponds to the molecular weight of a metal-peptide tetramer. This behavior of similar platinum-peptide complexes has been observed in Chapter IV. Another example of this behavior is the complex of platinum ethylenediamine with Pal14new which completely decomposes under ionization conditions of MALDI-MS but shows high stability in SDS-PAGE (See Chapter III). Therefore, the results of MALDI-MS of FR1 and FR4 suggests the of platinum-peptide complex in which the four peptide molecules are crosslinked together by four metal complexes.

Figure V.11. MALDI mass spectrum for the fraction 4.
Electro spray ionization mass spectroscopy is an alternative for MALDI-MS\textsuperscript{21,22}. ESI-MS spectra of FR1 and FR4 were identical and showed only peaks at m/z = 1425, 1188, 1018, 891, and 792 which could correspond to the +10, +12, +14, +16 and +18 ions of a species with an apparent mass of 14.24 kDa (Figure V.13.). However, the presence of only one evenly charged molecular ions in the mass spectrum is unusual and therefore these peaks can be interpreted to correspond to the +5, +6, +7, +8 and +9 ions of a species with a parent mass of 7.12 kDa which is a metal-peptide dimer. This result is inconsistent with the results of the SDS-PAGE and MALDI-MS experiments in which the formation of a metal-peptide tetramer complex
is clearly seen. Therefore, the dimer species in ESI mass spectrum is thought to be a product of the metal-peptide tetramer complex decomposition.

**Figure V.13.** Top: ESI mass spectrum of the fraction 1 and 4. Bottom: ESI mass spectrum of Pal14Pal21

**V.2.4. Circular dichroism spectroscopy of purified products of the platinum-peptide reaction**

FR1 and FR4 have a similar molecular weight, but deposit different retention times in the HPLC experiment. The identical molecular weight of the metal-peptide complex points out the high similarity of the products which are most likely structural isomers. The results of the SDS-PAGE, MALDI-MS and HPLC experiments showed that the metal-peptide exists as a platinum-
peptide tetramer (PtPal14Pal21n)_4 in which the peptide molecules which could possibly exist as four stranded α-helical coiled-coil structures.

The information about the conformation of the metal-peptide complexes in solution can be obtained by circular dichroism CD spectroscopy. The (CD) spectrum of both the apopeptide and the products of the metal-peptide reaction in water consist of minima at 208 and 222 nm showing that the peptides exist within the α-helical conformation^{11, 13} (Figure V.14.). Similarly with the apopeptide the metallopeptide complexes have the ratio \( \theta_{222}/\theta_{208} > 1 \) (\( \theta_{222}/\theta_{208} \approx 1.04 \) for FR1 and \( \theta_{222}/\theta_{208} \approx 1.02 \) for FR4 which is generally considered indicative of the α-helical coiled-coil conformation^{14}. However, the helical content, which is determined by intensity at 222nm,^{15, 16} is 52% for FR1 (\( \theta_{222} = -15,700 \) deg cm\(^2\) dmol\(^{-1}\)) and 50% for FR4 (\( \theta_{222} = -15,200 \) deg cm\(^2\) dmol\(^{-1}\)), is lower then the apopeptide (\( \theta_{222} = -22,500 \) deg cm\(^2\) dmol\(^{-1}\) 71%) and can be explained by distortion of the peptide conformation after metal binding. These results suggest that the metal-peptide complexes keep the original α-helical coiled-coil conformation in the formed metal-peptide complex, but their helicity is lower then the apopeptide.

Figure V.14. CD spectrum of Pal14Pal21n (black) fraction 1(red) and fraction 4(green); Concentration of the samples is around 1 \( \times 10^{-5} \) mole/l (25C°, water).
Increasing the ionic strength of aqueous solvent enhances the free energy formation of hydrophobic interaction. For coiled-coil peptides an increase in ionic strength of the solvent results in the formation of stronger coiled-coil structures. This can be observed in CD spectrum of a coiled-coil peptide by higher value of helicity (θ_{222}) in a buffered solution in comparison with water. This observation is true for all apopeptides used in this PhD project. This increase in helicity is usually about 10 ± 5% for 50mM monobasic phosphate buffer in the presence of 100mM KCl (ionic strength is 150mM). Pal14Pal21n has an increase in helicity about 12% if the solvents changes from water 76% (θ_{222} = -27,000 +/- 2,000 deg cm^2 dmol^{-1}) to the buffer 89.8% (θ_{222} = -31,000 +/- 2,000 deg cm^2 dmol^{-1}) buffer as shown in figure V.15:

Figure V.15. CD spectrum Pal14Pal21n 1 in water (black) and phosphate buffer (red); Concentration of the samples is around 6x10^{-4} mole/l (25°C, water).

In contrast to the apopeptide which shows the increase of the helicity, the helicity of both FR1 and FR4 drops with an increase of ionic strength. Moreover, the formation of a precipitate was observed if a concentrated solution of the fraction was mixed with the phosphate buffer. In
order to understand the nature of this precipitate, fractions 1 and 4 were titrated with increasing concentrations of buffer.

Both fractions 1 and 4 show a decrease of helicity (CD signal at 222nm) with an increase in the concentration of buffer solution (Figure V.16.). FR1 keeps its shape in the CD spectrum and the ratio $\theta_{222}/\theta_{208} = 1$ suggests it is still in a coiled-coil conformation. In contrast, the shape of CD signals changes dramatically for FR4 in which the ratio $\theta_{222}/\theta_{208}$ becomes higher than 1.5. This behavior of CD spectrum was observed before for peptides and proteins which assemble into linear polymers through the formation of coiled coils. It was suggested that this behavior of CD spectrum is indicative of the formation of large molecular assemblies. These results suggest that the precipitation of the FR1 and FR4 in the phosphate buffer could be due to the aggregation through the formation of hydrophobic interactions. To characterize the formation of the assemblies, a HPSEC-LS experiment was carried out.

![Figure V.16. CD spectrum](image)

**Figure V.16.** CD spectrum Left: fraction 1 in water (black) and phosphate buffer (red) Right: fraction 4 in water (black) and phosphate buffer (red). Concentration of the samples is around $1 \times 10^{-4}$ mole/l; $25^\circ\text{C}$. 
V.2.5. HPSEC experiment in non-denaturing conditions

HPSEC allows separation of non-covalent peptide assemblies\textsuperscript{25,26}. The conditions of HPSEC experiment are phosphate buffer (pH7) which induces the formation of molecular aggregates of fraction 1 (FR1) and fraction 4 (FR4). Therefore, the formed aggregates which would come out from the SEC column were expected to be characterized by in-line light scattering. The HPSEC diagram for fraction 1 and 4 are shown in figure V.17:

![HPSEC experiments for Fraction 1(blue) Fraction 4 (green) and Pal14Pal21n (red). Phosphate buffer pH7 Superdex 75](image)

**Figure V.17.** HPSEC experiments for Fraction 1 (blue) Fraction 4 (green) and Pal14Pal21n (red). Phosphate buffer pH7 Superdex 75

The main fraction in the HPSEC chromatogram for FR1 and FR4 is coming with the elution time of 20 minutes, which corresponds to the void volume of the packing, and therefore can not be characterized by the column. This behavior is characteristic for the large non-covalent molecular assemblies (Mw > 70 kDa) (See Chapter III). The HPSEC chromatogram for FR1, additionally to the peak at 20 min, shows the peak, elution time of which is slightly higher of the apopeptide. This peak can be assigned to the single metal-peptide tetramer.
Samples FR1 and FR4 registered at the UV wavelength of 250nm which is characteristic of the platinum-pyridine bond ($\varepsilon = \log 3.5$) in the HPSEC experiment (Chapter III.1.). Therefore, the intensity of the signals in this chromatogram should correspond to the concentration of the sample. However, the observed intensity of the signals for FR1 and FR4 registered by UV and LS was much lower than the starting concentration of the injected samples (injected concentration is 5 mg/ml and registered about 0.1 mg/ml). This suggests the formation of a precipitate in the SEC column which can not be registered by nor UV/Vis nether LS detectors. The low signal in the HPSEC experiment usually prevents the application of LS techniques for the characterization of molecular weight and size for the formed aggregates. Therefore, use of denaturizing conditions is necessary for preventing the non-covalent aggregation and the characterization of a single metal peptide complex. The denaturation conditions of 6M guanidinium chloride (GdnHCl) in phosphate buffer pH7 to avoid the formation of non-covalent aggregates.

V.2.6. HPSEC-LS experiment under denaturing conditions

High performance size exclusion chromatography followed by light scattering experiment (HPSEC-LS) was carried out to get more accurate information about the molecular weight of the metal-peptide complexes in aqueous solutions\(^{27-30}\). Static light scattering experiment for the products of the platinum-peptide reaction show the molecular weight of the assemblies is 13.3 +/- 0.9 kDa for the fraction 1 and 14.3 +/- 0.6 kDa for the fraction 4 (Figure V.18. (left)). The dn/dc value for the samples was calculated form the change in UV absorption signal at 250 nm by using the extinction coefficient of apopeptide before ($\varepsilon = \log 3.3$) and after ($\varepsilon = \log 3.5$) of the reaction correspondently.
Concurrently, the results of HPSEC experiment of the fraction 1, 4 and peptides of known molecular weight were plotted on the calibration graph of log(Mw) against Kd, which can in general show the extent of size separation (see Chapter II). The samples of similar shape morphology but different molecular weight can be fitted in a line in this graph. The calibration graph of the purified platinum-peptide complexes revealed the platinum-peptide complexes have similar molecular weight about 15.0 +/- 20% kDa with the ratio of size-log(Mw) in good agreement with standard peptides of known molecular weight (Figure V.18. (right)).

![Figure V.18](image)

**Figure V.18.** Left: Molar mass distribution plot by multangle light scattering of purified samples of fractions 1 (red) and 4 (blue). The solid lines indicate the HPSEC traces using absorption detection at 250 nm. The hashed-lines are the weight-averaged molecular masses for the two compounds. Right: Calibration graph for SE Superdex 75 in 6M GdnHCl, phosphate buffer pH7.

The results of LS – measurements and the retention time from HPSEC it was shown that the molecular weight of the apopeptide is about 3.5 kDa under denaturation conditions of 6M guanidinium chloride (GdnHCl). The calculation of the molecular weight of platinum-peptide
complexes of the fraction 1 and 4 has shown that the complexes have size and molecular weight of a peptide tetramer. The absence of high molecular weight assemblies in the SE experiment suggests that the FR1 and FR4 complexes exist as cyclic platinum-peptide tetramers. One of the reasons for aggregation of the platinum-peptide complexes could be a hydrophobic part of the peptide molecules exposed to exterior. This hydrophobic part would force the FR1 and FR4 assemblies to aggregate in the presence of phosphate buffer.

**V.2.7. Hydrophobic probe**

The results of HPSEC-LS experiments in the presence and absence of denaturizing agents suggest that the molecular structures of Fraction 1 and 4 could potently aggregate through the formation of hydrophilic interaction. Hydrophobic probe was used to show the presence of exposed to the exterior hydrophobic part of the platinum-peptide molecules.

ANS dye (1-amlinonaphtalene-8-sulfonic acid) has a very low fluorescence quantum yield in aqueous solution, but enhances its fluorescent intensity when it is in hydrophobic inviroment. Importantly, this dye does not bind peptide in their random coil conformation. It was shown that ANS spontaneously binds itself to the hydrophobic exterior of peptide oligomers. However, a control experiment with ANS alone and apopeptide is necessary for the examination of the hydrophobic interior of Fractions 1 and 4. Figure V.19 shows the emission spectrum and maximum fluorescent enhancement of the samples:
The intensity of FR1 and FR4 are only slightly (27-30%) higher than the intensity of the apopeptide. Nevertheless, the observed increase in fluorescence suggests that the metal-peptide structures of FR1 and FR4 have a hydrophilic part exposed to water and therefore can potentially participate in the formation of molecular aggregates. Interestingly, the apopeptide showed negative value of maximum ANS fluorescence enhancement, the explanation of which was not found.

An explanation for the low intensity in the hydrophobic probe experiment can be the aggregation of FR1 and FR4 which was shown by HPSEC and CD spectroscopy. This aggregation leads to the formation of soluble aggregates which partially keep their hydrophobic part inside of the formed structures and prevents binding of the hydrophobic probe. Therefore, only a small portion of the hydrophobic part of these molecules, which is located on the surface
of the formed aggregates, can participate in binding with the hydrophobic probe. This suggests that FR1 and FR4 exist as peptide tetramers only under denaturation conditions. In aqueous solutions FR1 and FR4 form aggregates which are soluble in water, but precipitate in phosphate buffer.

V.2.8. Competition experiments

It was suggested that FR1 and FR4 can aggregate through the formation of hydrophobic interactions. A competition experiment, in which excess of the apopeptide is mixed with FR1 or FR4, is used to examine this suggestion.

In this competition experiment, excess of apopeptide were expecting to bind to the hydrophobic part of the platinum-peptide complexes of FR1 And FR4 and therefore prevent them from forming high molecular weight aggregates. In other words, the molecules of the apopeptide will compete with the platinum-peptide complex itself in order to bind the hydrophilic part of these platinum-peptide complexes. The formation of heteromolecular assemblies is proportional to the binding constant and the concentration of the samples. Therefore, the presence of several hetero-molecular assemblies containing one, two, three or more platinum-peptide complexes will be capped by bonding with apopeptides. The presence of these molecules was observed by HPSEC experiment, as shown in Figure V.20.

The HPSEC chromatogram shows the formation of low molecular weight assemblies which appear in the region of 30-40 min of elution time (shown by arrows). The apopeptide itself can not form these assemblies and exist as a coiled-coil dimer with the elution of 44 min. On the other hand, the fraction1 and fraction 4 alone did not exhibit such assemblies in HPSEC experiment alone (Figure V.17.). Therefore these low molecular weight assemblies are the non-
covalent complexes of the apopeptide and the platinum-peptide structures of FR1 and FR4. The use of the apopeptide as a template agent suggests that the metal-peptide complexes of FR1 and FR4 can bind peptides possibly through the formation of the coiled-coil.

Figure V.20. HPSEC chromatogram of the competition experiment for the fractions 1 (right) and 4 (left). Phosphate buffer pH7 Superdex 200.
V.2.9. Atomic force microscopy

HPLC fractions 1 and 4 isolated from the reaction mixture were analyzed by HPLC, SDS-PAGE, MALDI-MS and HPSEC-LS which showed that these species were peptide tetramers under denaturation conditions. The fractions appeared identical except their HPLC properties. The longer elution time of FR4 suggests a higher hydrophobicity compared to FR1, the elution time of which is 5 minutes less. The identical molecular weight of the products points out the high similarity of the metal-peptide complexes which are most likely structural isomers. It was suggested that the difference in the structure of these isomers can appear in the difference morphology of aggregates which they form.

Atomic force microscopy (AFM) was used to visualize the aggregates of FR1 and FR4. Due to its reliability, and to prevent the formation of artifacts during solvent evaporation, the staining-rise technique was used to prepare the sample for AFM imaging. All images were made from a water solution with a peptide’s concentration of 1mg/l.

Figure V.21 shows that the aggregates of FR1 on the surface of mica exists as wide (50 - 500 nm) linear structures with height of 0.5-1.5 nm (Images A and B). In some cases, these linear structures are accompanied by round, flat aggregates (Images C and D). The height of these aggregates structures varies, but the height is constant within 0.5-1.5 nm. The height of these structures is comparable to the dimension of a single molecule of the metal-peptide tetramer (which is estimated in the range of 1 - 2 nm wide and 4 - 5 nm high) lying on surface of mica. Therefore, these linear aggregates are most likely assemblies of the metal-peptide complexes that form a single layer on the surface of mica.
Figure V.21. AFM Images of fraction 1 sample (incubation-rinse technique, water solution of 1 microgram/ml).
An increase in magnification reveals that these linear and round fraction one aggregates consist of smaller structures, most of which exist as round, flat objects (image B and D). The height of these structures is uniform to 0.5-1.5 nm, which is similar to the height of the larger, linear aggregates. Together, these results show that the platinum-peptide complexes of FR1 forms supramolecular structures which aggregate on the surface of mica in flat (“two dimensional”) structures.

Figure V.22 shows that the assembly of FR4 exists on the surface of mica as large globular structures of various sizes. The diameter of these globular assemblies varies from 20 to 150 nm and the height is the range of 1- 100 nm. The increase in magnification shows the presence of smaller globular structures of different sizes on the surface with a shape similar to that of the large ones (images B and D).

AFM images of the FR1 and FR4 shows the difference in the morphology of formed aggregates. In contrast to fraction 1, which forms flat aggregates, fraction 4 aggregates into globular structures. Increased magnification of AFM images of FR1 and FR 4 allows visualization of structures that are about 20 nm and corresponds to several metal-peptide complexes on the surface of the globular structure. (Figure V.23) Theses assemblies are looking highly similar for both fractions one and four.
Figure V.22. AFM Images of fraction 4 sample (incubation-rinse technique, water solution of 1 microgram/ml).
Figure V.23. High magnitude AFM Images of fraction 1 (images A and B) and fraction 4 (images C and D) (incubation-rinse technique, water solution of 1 microgram/ml).

The AFM results show that FR1 and FR4 have different morphology of their aggregates.

The FR1 and FR4 form aggregate of different shape (flat and spherical) which suggests structural difference (geometry) of these platinum-peptide complexes.
V.2.10. Covalently crosslinked Pal14Pal21n

The characterization of FR1 and FR4 suggests a difference in the geometry of these platinum-peptide complexes. To examine this difference a covalent crosslink was engineered in the hydrophobic part of Pal14Pal21n (Figure V.24). This modification was made to reinforce the two stranded coiled-coil conformation of the Pal14Pal21n peptide and prevent the exposure of the hydrophobic portion to the solvent. This peptide (Pal14C19Pal21n) was synthesised and examined on the ability to be coordinated by Pt(en)(NO$_3$)$_2$\textsuperscript{1-3}. It was expected that the “secured” coiled-coil structure of Pal14Pal21n would prevent the formation of platinum-peptide complexes FR1 and FR4.

Figure V.24. Top: Schematic views helical wheel diagram of the coiled coil; view down helical axes of the coiled coil with pyridine rings at f positions. Bottom: amino acid sequence of Pal14C19Pal21n.
The peptide Pal14C19Pal21n was synthesized by solid-phase techniques and purified by reverse-phase HPLC (Figure.V.25. (left)). The molecular weight was proven by MALDI mass spectroscopy (Figure.V.30.4). MALDI-MS reveals the peak of the molecular ion (calc’d: 3289.89; obs’d: 3288.62) and a smaller peak with the molecular weight of the apoprotein bound to one atom of sodium (calc’d: 3312.89; obs’d: 3310.61), which is a common byproduct of this technique (Figure.V.26. (left)). The disulfide crosslinked peptide, Pal14C19ox, was prepared by oxidation of the cysteine residues with I₂ and also purified by HPLC (Figure.V.25. (left)).

Similar to the oxidation of Pal1419n (Chapter IV), the retention time of Pal14C19Pal21nox in the HPLC chromatogram decreased in comparison with the non-oxidized form of the peptide. Analysis of Pal14C19Pal21nox by MALDI-MS shows the presence of the oxidized peptide (calculated: 6577.78; observed: 6576.67) in addition to a smaller peak of the reduced peptide monomer (calculated: 3289.89; observed: 3221.76), which may be due to the thermal decomposition of the disulfide bond of cysteine crosslinking (Figure.V.26.).

Figure.V.25. Analytical HPLC experiments for Pal14C19Pal21n before (left) and after (right) oxidation. H₂O/AcN linear gradient 0.1% TFA
Figure V.26. MALDI-MS spectra of Pal14C19Pal21n before (top) and after (bottom) oxidation.

The platinum-Pal14C9Pal21n complex was prepared by reaction of Pt(en)(NO$_3$)$_2$ with Pal14C19Pal21nox for several days at 60 °C in water. The formation of the platinum-peptide complexes was monitored by SDS-PAGE and compared to the results of the reaction of Pal14Pal21n with Pt(en)(NO$_3$)$_2$. 
The SDS-PAGE (Figure V.27.) revealed the presence series of bands which correspond to two (MW ~ 14 kDa), three (MW ~ 21 kDa), and four (MW ~ 28 kDa), peptide molecules bound together by platinum ethylenediamine. This observation is very similar with the results of rhenium-Pal14C19ox reaction which were discussed in Chapter IV. In contrast to this reaction of Pal14Pal21n revealed the presence of only a single product having a molecular weight of approximately 14 kDa.

![Image](image.png)

**Figure V.27.** Left: Monitoring of the formation of ethylenediamine platinum (II) with Pal14Pal21n by SDS-PAGE experiment. Right: Monitoring of the formation of the platinum complex with Pal14C19Pal21nox by SDS-PAGE experiment.

The difference between these two reaction shows that the covalent crosslinking does prevents the formation of a single product in the reaction of Pt(en)(NO$_3$)$_2$. Importantly, the difference between Pal14C9Pal21nox and Pal14Pal21 is only one amino acid which on the examples of Pal14C19ox and Pal14C19nox has been shown do not change the coiled-coil conformation of the peptides, but prevents its dissociation.
Both Pal14Pal21n and Pal14C19Pal21n exist as two stranded $\alpha$-helical coiled-coil in the absence of metal ions. However, the “secured” coiled-coil structure of Pal14C19Pal21nox restrict the conformation changes within the molecule which does not allow the platinum complex to orient the peptide ligand in order to form structures such as FR1 and FR4. The results of this experiment show that strong conformational changes of the peptide molecule are necessary for the formation of FR4 and FR4.

V.2.11. Reaction of Ru(bpy)$_2^{2+}$ with Pal14Pal21

Cis-dichlorobis(2,2-bipyridine)ruthenium (II) was examined for the ability to form a stable complex with Pal14Pal21. The reaction of Ru(bpy)$_2^{2+}$ with Pal14n and Pal14C19nox showed the ability of this complex to coordinate the peptide ligands (See Chapter III and Chapter IV). Therefore, it was expected that a ruthenium-peptide tetramer could be formed in the reaction of Ru(bpy)$_2^{2+}$ with Pal14Pal21.

Similarly to the reaction of ethylenediamine platinum (II) with Pal14Pal21n, the ruthenium-peptide complex was prepared by mixing the equal molar amount of the apopeptide with Ru(bpy)$_2^{2+}$. After several days the reaction mixture was examined by SDS-PAGE which revealed that the reaction of Ru(bpy)$_2^{2+}$ with the apopeptide is slow and the main product of the reaction is unreacted apopeptide. Nevertheless, the formation of two ruthenium-peptide complexes (Figure V.28.) with molecular weight about 6.5 and 14 kDa is seen on the gel. The molecular weight of these complexes corresponds to the two and four peptide molecules bound together by Ru(bpy)$_2^{2+}$. 
Figure V.28. The reaction $\text{Ru(bpy)}_2^{2+}$ with Pal14Pal21 in MeOH by SDS-PAGE

Concurrently, the reaction mixture was characterized by Uv-Vis spectroscopy. The spectrum of $\text{Ru(bpy)}_2^{2+}$ consists of characteristic absorption bands centered at 378 nm and 548 nm. After the reaction with Pal14Pal21n, the spectrum of the reaction mixture shows blue shift to 350 nm and 490 nm respectively. These changes in the UV-Vis spectrum is characteristic for the formation of $\text{Ru(bpy)}_2^{2+}$-pyridine bond$^{34,35}$. Moreover, the reaction mixture shows broad emission centered at 700nm (excitations 450nm) which reveal the presence of di-substituted $\text{Ru(bpy)}_2^{2+}$ in which both of the ligands could be pyridylalanine residues of Pal14Pal21. The emission spectrum of the reaction mixture is similar to the emission spectra of $\text{Ru(bpy)}_2\text{Py}_2^{36}$ but have 50 nm red shift; this additionally support the presence of di-substituted ruthenium-Pal14Pal21n complex.
V.2.11. Summary

This work shows how α-helical polypeptides can form organized assemblies when they bind square-planar transition metal ions. In particular, coordination of ethylenediamine platinum (II) to the pyridylalanine residues of Pal14Pal21n changes the peptide conformation from a highly stable two-stranded coiled-coil to a metal-stabilized, four-helix bundle. The reverse-phase HPLC experiment shows that at least two different forms of the platinum-peptide tetramer exist. Such behavior suggests that the different tetrameric assemblies are different in their hydrophobic properties. The results of SDS-PAGE, MALDI-MS and HPSEC-LS experiments all showed no evidence for the presence of any other differences in the structure of these isomers. The identical molecular weight of the products points out the high similarity of the metal-peptide complexes which are most likely structural isomers. Many isomers must be considered because
of the parallel and anti-parallel orientation of the peptides into the metal-peptide complex. The
direction of the metal-peptide crosslinking adds more variety for possible structures of the
complex, but stoichiometry of the metal and peptide at 1:1 suggests a cyclic structure of the
metal-peptide complexes. In other words, the metal-peptide tetramer exists as a cyclic array of
$\alpha$-helices where the two most likely directions of metal-peptide bonding are shown in Figure
V.28:

![Figure V.28. ESI-MS (top) data for purified HPLC fractions 1 and 4. Scheme of possible crosslinking isomers (bottom)](image)

The results of CD spectroscopy suggest that the peptide molecules keep their $\alpha$-helical
conformation in the formed metal-peptide complexes. In the same time the results of the
hydrophobic probe shows that the formed platinum-peptide aggregates have exposed to solvent
hydrophilic part of peptide molecules. Moreover, the competition experiment showed that the
exposed hydrophobic part of FR1 and FR4 can bind apopeptide possibly through the formation
of coiled-coil structures. The reaction of Pal14C19Pal21nox with Pt(en)(NO$_3$)$_2$ showed that that
strong conformational changes of the peptide molecule are necessary for the formation of FR4
and FR4.
CHAPTER V REFERENCES:


VI: CONCLUSION

The work presented in this dissertation demonstrates how de-novo designed peptides having the double stranded α-helical coiled-coil conformation can be organized into molecular assemblies by metal coordination. The design of these peptides explores the positioning of a strong metal binding site, 4-pyridylalanine, within the hydrophilic portion of the peptides in order to coordinate the metal complexes into well-defined geometries. Six peptides of different design were examined for the ability to form metal-peptide complexes, the results of this which are outlined in Table VI:

Table VI.1. Structure of metal-peptide assemblies

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Pt(en)(NO₃)₂</th>
<th>[NE₄]₂[ReBr₃(CO)₃]</th>
<th>Ru(bpy)₂Cl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pal14</td>
<td>Di-substituted complex</td>
<td>Di-substituted complex</td>
<td>Does not react</td>
</tr>
<tr>
<td></td>
<td>(not stable)</td>
<td>(not stable)</td>
<td></td>
</tr>
<tr>
<td>Pal14n</td>
<td>Di-substituted complex,</td>
<td>Di-substituted complex</td>
<td>Di-substituted complex</td>
</tr>
<tr>
<td></td>
<td>forms globular structures in solution</td>
<td>(not characterized)</td>
<td>(very slow reaction)</td>
</tr>
<tr>
<td></td>
<td>Exist as linear and globular assemblies on the surface</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pal14C19ox</td>
<td>Linear metal-peptide assemblies n = 1-7</td>
<td>Linear metal-peptide assemblies n = 1-5</td>
<td>Does not react</td>
</tr>
<tr>
<td></td>
<td>(stable)</td>
<td>Forms hydrogel</td>
<td></td>
</tr>
<tr>
<td>Pal14C19nox</td>
<td>Linear metal-peptide assemblies n &gt; 7</td>
<td>Linear n &gt; 7 (not characterized)</td>
<td>Linear metal-peptide assemblies n = 1-5 (slow reaction)</td>
</tr>
<tr>
<td></td>
<td>Exist as linear and globular assemblies on the surface</td>
<td></td>
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<tr>
<td>Pal14Pal21n</td>
<td>Tetramer (inverted structure)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pal14C19Pal21nox</td>
<td>Linear metal-peptide assemblies n = 1-4</td>
<td></td>
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</tbody>
</table>
This work demonstrates how peptide design can be used to manipulate the stability of metal-peptide complexes. The effect of the peptide’s sequence on the stability of the formed metal-peptide complex has been shown through the reaction of Pal14 and Pal14n with ethylenediamine platinum (II). In particular, it was shown that peptides based on an IEALEGK repeat do not form stable metal complexes. In contrast, peptides based on an IAALEQK repeat do form stable platinum complexes which in water assemble into globular structures that are 40 nm in diameter. Incorporation of a covalent crosslink into the Pal14n coiled-coil structure (Pal14C19nox) forces this peptide to form linear assemblies after the reaction with the platinum complex.

The last chapter of this dissertation reveals that multiple metal binding sites on the hydrophilic exterior of the two-stranded coiled-coil Pal14Pal21n forces the formation of cyclic tetramers, rather than a polymeric product. It was also shown that the conformational “plasticity” of the non-covalent coiled coil Pal14Pal21n is necessary for the formation of the platinum-peptide tetramer. In contrast, the covalently “secured” coiled-coil structure of Pal14C19Pal21nox restricts conformation changes within the molecule, which inhibits the platinum complex from orienting the peptide ligand in order to form the tetramer structure.