



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

SYNTHESIS OF CARBON NANODOTS-PEPTIDE CONJUGATES DECORATED WITH GERMANIUM FOR BIOIMAGING

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Declaration of originality

I, at this moment, state that I conducted the work recorded in this document. This thesis was wholly conducted and written by me under the supervision of Dr Maya Makatini and Dr Manoko Maubane-Nkadimeng. I have rightfully credited the sources used in the thesis. This thesis has not been submitted before for any degree.



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Abstract

The World Health Organization Global Cancer Observatory estimates that cancer caused 9.96 million deaths worldwide in 2020, making early detection crucial for diagnosis and treatment. Accurate identification of cancer plays a crucial role in the diagnosis and treatment process. It allows for customized and efficient therapies, minimizes unnecessary procedures and adverse effects, and improves the prognostic insights for patients and healthcare providers alike. The challenges in diagnosis include overdiagnosis, false positives/negative outcomes, and limited sensitivity. Advanced technologies are needed for better imaging accuracy and minimizing harm. This study aims to fabricate carbon dot-peptide conjugates to enhance bio-imaging capacity and selectivity. The peptides used are derived from the GKPIILFF cell-penetrating peptide sequence and the RLRLRIGRR peptide, which is selective to cancerous cells. The Carbon dots were used to provide the photoluminescent properties required for bio-imaging of cancerous cells.

Carbon dots (CDs) were synthesized using iso-ascorbic acid as the source of carbon using a microwave-assisted method. The nitrogen and germanium-modified carbon dots (*Iso-N-Ge-CDs*) demonstrated the highest photoluminescent properties compared to the unmodified CDs (*Iso-CDs*) and those with either N (*Iso-N-CDs*) or Ge (*Iso-Ge-CDs*). Photoluminescence emissions of longer wavelengths suitable for cell imaging were observed for the CDs, and the *Iso-N-Ge-CDs* demonstrated excitation-dependent emission wavelength behavior, pH sensitivity, and Fe^{3+} sensitivity.

The 13 peptides derived from the peptide accelerating sequence GKPIILFF and the cancer-selective peptide RLRLRIGRR were successfully synthesized. The peptides were characterized using Liquid Chromatography Mass Spectrometry (LCMS) and purified using preparative High-Pressure Liquid Chromatography (prep-HPLC). The secondary structure of the L-GKPIILFF penetration acceleration peptide sequence (Pas) adopted a helical secondary structure. The D-GKPIILFF derivative was found to adopt a random coil structure. These were confirmed using Nuclear Magnetic Resonance (NMR) techniques such as Total Correlation Spectroscopy (TOCSY) and Rotating Frame Overhauser Enhancement Spectroscopy (ROESY) NMR.

The CDs-peptide conjugates were successfully synthesized, and the confirmation of conjugation involved multiple methods, including UV-Vis and PL techniques. To the best of

our knowledge, the thesis incorporates the first study to demonstrate long-range interactions through ROESY NMR. The NMR analysis indicated that the helical structure of the peptide could be affected after conjugation, leading to notable peak shifts. Since the helical structure is crucial for the peptide's bioactivity and stability enhancement, NMR spectra with fewer structural changes in the peptide region may improve its biological properties. The research contained valuable information for scientists aiming to design and characterize Carbon dot-peptide conjugates with enhanced permeability and selectivity that can effectively deliver materials into cytosolic space.

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List of abbreviations

AA	Amino Acid
Ada	adamantane carboxylic acid
ACN	Acetonitrile
CDs	Carbon dots
CD	circular dichroism
CPPs	cell-penetrating peptides
AlCl ₃	Aluminium chloride
Boc	<i>tert</i> -butoxycarbonyl
CDCl ₃	deuterated chloroform
COSY	correlated spectroscopy
(CD ₃) ₂ SO ₂	Deuterated Dimethylsulfoxide
DCM	Dichloromethane
DIC	1, 3-diisopropyl carbodiimide
DIPEA	<i>N, N</i> -diisopropylethylamine
DMF	<i>N, N</i> -dimethylformamide
DMSO	Dimethylsulfoxide
EDT	1,2-Ethanedithiol
EtOAc	Ethyl acetate
FDA	Food and Drug Administration
Fmoc	9-Fluorenylmethoxycarbonyl
FWHM	Full width at half maxima

HATU	Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium
HBTU	O-Benzotriazole- <i>N,N,N',N'</i> -tetramethyl-uronium hexafluorophosphate
HOBt	1-Hydroxybenzotriazole
Hz	Hertz
UHPLC	Ultra High-Performance Liquid Chromatography
Prep-HPLC	Preparative High-Performance Liquid Chromatography
Ppm	parts per million
HRMS	High-resolution mass spectroscopy
Mg	Milligram
MIC	Minimum Inhibitory Concentration
Min	Minute
ml	Milliliter
mM	Millimolar
MS	Mass spectrometry
m.p	Melting point
m	Multiplet
m/z	mass to charge ratio
nm	Nanometres
NMR	Nuclear Magnetic Resonance
NMM	N-Methylmorpholine
NOESY	Nuclear Overhauser Effect Spectroscopy
PDCs	Peptide–drug conjugates

ROESY	Rotating Frame Overhauser Enhancement Spectroscopy
SPPS	Solid phase peptide synthesis
SPR	Surface plasmon resonance
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TIPS	Triisopropyl silane
TIC	Total ion chromatograms
TOCSY	total related spectroscopy
μg	Microgram
μL	Microliter
TLC	Thin Layer Chromatography
UV-VIS	Ultraviolet-visible spectroscopy

1. Introduction

1.1 Background and motivation

Carbon dots (CDs) are non-toxic and extremely biocompatible nanoparticles, making them suitable for use in biological applications.¹ They are highly photostable, which means they may be exposed to light repeatedly without deteriorating or losing their luminous qualities.² CDs are also known to have a high fluorescence quantum yield, which indicates that when activated by light, they create intense fluorescence signals.³ These properties of CDs make them extremely sensitive to bioimaging and suitable for long-term bioimaging research.

CDs may be targeted to cancer cells with remarkable selectivity by conjugating them with certain peptides.^{4,5} This allows for the identification of cancer cells while ignoring healthy ones. Therefore, carbon dot-peptide conjugates have the potential to increase bioimaging sensitivity and specificity for cancer detection and surveillance, as well as to enable multimodal imaging for more thorough cancer diagnosis.

1.2 Problem statement

According to The World Health Organization, cancer is the leading cause of death globally and was responsible for almost 10 million deaths and 19.3 million new cancer cases in 2020.⁵ Current technologies for the treatment of cancer include surgery, radiation therapy, and chemotherapy which are toxic to the body.⁶ Remission of cancer might last months, years, or one's entire life with current treatment. However, it does not always imply that one is cancer-free or cured.⁷ Additionally, overdiagnosis, false positive/negative results, and low sensitivity of instruments are one of the challenges faced when diagnosing cancer. Patients labeled as 'cancer-free' sometime develop cancer again even after years.⁸ This calls for new technologies for diagnosing cancer that have high imaging efficiency and is less harmful to the body. This work proposes the use of nanotechnology for bio-imaging and monitoring methods using nitrogen-doped carbon dots (N-CDs) with germanium to be conjugated to tumour-homing peptides. This conjugate should be able to target cancer for imaging. Germanium, which is known to have anticancer properties, was successfully incorporated into the as-synthesized carbon dots. Optical and photoluminescence properties of GeO₂ have

been well documented and which was incorporated into the carbon dots. However, Germanium, which is known to have anticancer properties, was successfully incorporated into the as-synthesized carbon dots.⁹ Spiro-germanium Compounds, 2-Carboxyethyl Germanium Sesquioxide (Ge-132), Amino Acid Germanium, and Lactic Acid-Citrate Germanium are organogermanium compounds that have been proven to have activity against tumors and have been incorporated into drugs. A study conducted by Mark G. Mainwaring and colleagues at the University of Florida College of Medicine and Veterans Affairs Medical Center, Gainesville, demonstrated that treatment of Spindle cell carcinoma with orally administered germanium sesquioxide could cause complete remission.¹⁰ Herein, cancer-selective bio-imaging carbon dot-peptide conjugate was explored. The incorporation of germanium to the N-doped carbon dots is an area that has scarce information and we hypothesized that it may exhibit superior fluorescence. Additionally, the peptides used in this study are known for their enhanced permeability and selectivity which are crucial properties for conjugates.

1.3 Carbon Dots as Potential Cancer Diagnostic and Therapeutic Agents

The increasing demand for low-cost and highly efficient diagnostic and therapeutic agents is increasing exponentially globally, which requires research and development for creating new and innovative drugs and diagnostics. Numerous well-known and effective drugs should be reformulated to provide enhanced efficiency or more beneficial therapy in efforts to meet this demand.

A nanotherapeutic delivery and diagnostic system are drug delivery concepts in nanotechnology, which generally refers to a technique that develops nanoscale devices (1 to 100 nm) for enhanced delivery of small drug molecules to the cells of interest for treatment or detection.^{11, 12}

In 2005, the Food and Drug Administration (FDA) approved Abraxane as the first commercially available nanomedicine for metastatic breast cancer, which encapsulates the anticancer drug paclitaxel within protein (albumin) nanoparticles.¹³ Other nanomedicines encapsulated with albumin include 5-fluorouracil, doxorubicin, 10-hydroxy camptothecin, and methotrexate, where chemotherapy drugs were utilized.¹⁴ This shows that conjugates

have great prospects in the medical field, and selective bio-imaging conjugates may address the limitations of current technologies for bioimaging.

1.4 Limitations of current technologies

Imaging tools such as Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET), and Computed Tomography (CT) scans can assist to diagnose cancer and identify the amount of its spread, a process known as staging.¹⁵ This information is essential for selecting the best treatment and predicting prognosis.¹⁶ Despite developments in imaging technology, visualizing malignancies or tumours still poses various problems. Among the current challenges are sensitivity, selectivity towards cancer cells and deep penetration of the cells.^{17,18} This means that emerging imaging technology should be able to identify even tiny tumours while also discriminating between diseased and non-cancerous cells with accuracy.

Although imaging is crucial in cancer diagnosis and therapy, more research and innovation are still needed to overcome these problems and enhance accuracy and sensitivity. In this case, cancer-selective carbon-peptide conjugates will be used to overcome these challenges.

1.5 Conjugation of carbon dots to peptides for bio-imaging

There are several conjugate systems with promising results such as antibody-dye conjugates which are agents that can detect and bind to particular targets, such as proteins or cells by labelling antibodies with fluorescent but used dyes with dose-limiting toxicities.¹⁹ Other bio-imaging conjugates include quantum dot-protein conjugates, green fluorescent protein (GFP) and magnetic nanoparticle-antibody conjugates.^{20, 21, 22} The drawback with these conjugates is that they often make use of chemical dyes which can be toxic if used inappropriately.²³ Although they can be highly selective, the cost of producing synthetic antibodies or proteins can be high depending on several factors, including the complexity of the molecule, the type and scale of production method used, and the purification and characterization requirements.²⁴ Carbon dots are metal free, biocompatible and cheap to synthesize because waste can be used as a carbon source. Additionally, short peptides significantly reduce the cost of synthesis because fewer reagents are used and purification is more facile.²⁵

1.6 Aim and objectives

Aim

The purpose of this research work is to synthesize carbon dot-peptide conjugates to enhance the bio-imaging capacity and selectivity of the conjugates. Factors that affect permeability include bioavailability, potency, stability, and half-life of the drug in the body. Theragnostic is a rising area of study which is a combination of therapeutics and diagnostics.

To achieve the aims of this thesis, the following objectives were outlined:

- Synthesize **N-doped carbon** dots to enhance fluorescence, using a microwave assisted synthesis method.
- Synthesize **Ge-modified carbon** dots to add anticancer properties and investigate other defects that may introduce enhanced fluorescent properties to the carbon dots.
- Synthesize **L and D amino acid derivatives** of cell-penetrating and/or cancer-homing peptides for structure-activity studies.
- Synthesize peptides with **adamantane and palmitoyl moieties** to enhance cell permeability.
- To **fully elucidate the synthesized peptide** analogues using **NMR** for insight into the structure vs. activity study.
- To determine the **optical properties** of the nanoparticles and the conjugates by using ultraviolet-visible spectroscopy and photoluminescence spectroscopy.
- To investigate the **bio-imaging capacity** of the nanoparticles and the conjugates by using optical fluorescent microscopy

1.7 The study's novelty and prospective applications in the context of South Africa

The preparation of carbon dots (CDs) and nitrogen-doped carbon dots (NCDs) with germanium oxide (GeO_2) using a facile one-step microwave-assisted synthesis procedure was explored. Studies have shown that the CDs, NCDs, and GeO_2 nanoparticles individually have excellent qualities for bio-imaging applications, including photoluminescence, biocompatibility, good stability, and low toxicity.^{26,27} While CDs have been thoroughly explored in literature, CDs with germanium oxide on their surface are an avenue that has scarce information.

To enlarge the scope of these CDs-Ge/ GeO_2 composites in practical applications, particularly bioimaging applications, this study reports on a simple microwave reaction for the synthesis of germanium oxide-modified NCDs. Iso-ascorbic acid and urea were utilized to evaluate the response of Ge-132. Ge-NCDs produced were fluorescent, water-soluble, and emitted different colors due to the different sizes. To the best of our knowledge, the effect of both germanium and nitrogen on the photoluminescent traits of CDs has not been reported. We found that the CDs modified with germanium oxide have excellent photoluminescent traits, which are crucial for bio-imaging.

The short penetration accelerating sequence (PAS) has gained increasing attention from researchers due to its ability to enhance cellular uptake.²⁸ The seven-amino acid sequence recognized by lysosomal protease cathepsin D promotes the endosomal escape of cell-penetrating peptides (CPPs) and improves the cytosolic release of the delivered cargo.²⁹ To date, the peptide's mechanism is still unknown, and there is no available NMR data or other structural information. This is a major limitation in understanding the mechanism of penetration and how the peptide interacts with the target site.³⁰

In short, carbon dot-peptide conjugates offer a lot of potential in bioimaging.³¹ It is feasible to generate fluorescent probes that may selectively target certain cells or tissues by conjugating peptides to carbon dots, giving a potent tool for observing biological processes. Carbon dots' strong photostability makes them useful for long-term imaging research. Many studies have shown that carbon dot-peptide conjugates may be used for bioimaging applications such as *in*

vivo imaging of malignancies, imaging of cellular uptake, and imaging of protein-protein interactions and this study expands the scope of carbon dot-peptide conjugates.

1.8 Outline of the thesis

Chapter 1

This chapter serves as a layout of the context and rationale for drug delivery for anticancer drugs and emphasizes the need for a new class of drugs that are multifaceted to overcome the current limitations and enhance pharmacokinetic properties. Moreover, the main aim and objectives of the study are also discussed.

Chapter 2

First, the impact of tumours and cancerous diseases were reviewed. Subsequently, carbon dots and peptides are thoroughly explored to fully understand the synergetic effects of the Peptide-Carbon dot conjugates for treatment and drug delivery. Furthermore, the target sites and receptors involved in enhanced drug delivery were examined.

Chapter 3

The synthesis and characterization of carbon dots were investigated, where we evaluated the effects of introducing germanium into the CDs as well as doping with nitrogen. The nitrogen doped CDs containing germanium were found to have the highest photoluminescent properties.

Chapter 4

Herein, the synthesis and characterization of peptide derivatives were analysed following the conjugation of the peptides to the carbon dots. There were 13 peptides which were successfully synthesized, and the different retention times were indicative of different structures for each peptide derivative. These peptides were variations of the penetration accelerating peptide (sequence: GKPIILFF) and cancer targeting peptide (sequence: RLRLRLIGRR).

Chapter 5

In this section, the full elucidation of the penetration accelerating GKPIILFF peptide derivatives was examined to provide further insight into drug delivery and bioactivity. The peptide was found to have a helical structure known to enhance stability in bioactive peptides.

Chapter 6

In this part, the full elucidation of the conjugates was examined to provide further insight into drug delivery and bioactivity. Long-range interactions between the peptide and the CDs were confirmed in the ROESY NMR spectra of the conjugates.

Chapter 7

Finally, this chapter will supply overall conclusions and recommendations for possible future work on the synthesis of carbon dot Peptide conjugates for drug delivery and treatment of cancer. The NMR results of the conjugates indicate that the helical structure may be impacted due to noticeable peak shifts. Considering the potential influence of the helical structure on its activity, conjugates with NMR spectra showing less structural deviation from the peptide may possess more favourable biological properties. These promising options include GKP-*Iso-N-Ge*, GKP-*ada-Iso-N-Ge*, BP-*pal-Iso-N-Ge*, and DBP-*ada-Iso-N-Ge*, which still exhibit distinguishable peaks compared to the original peptide.

Chapter 8

The detailed procedures for synthesizing the carbon dots and peptides are fully explained in this chapter. Additionally, the instruments used for characterization and calculations used was presented.

1.9 References

1. Wang, Y., Zhu, Y., Yu, S. & Jiang, C. Fluorescent carbon dots: rational synthesis, tunable optical properties and analytical applications. *RSC Adv.* **7**, 40973–40989 (2017).
2. Peng, Z., Miyanji, E. H., Zhou, Y., Pardo, J., Hettiarachchi, S. D., Li, S., Blackwelder, P. L., Skromne, I. & Leblanc, R. M. Carbon dots: promising biomaterials for bone-specific imaging and drug delivery. *Nanoscale* **9**, 17533–17543 (2017).
3. Zhang, Y., Liu, X., Fan, Y., Guo, X., Zhou, L., Lv, Y. & Lin, J. One-step microwave synthesis of N-doped hydroxyl-functionalized carbon dots with ultra-high fluorescence quantum yields. *Nanoscale* **8**, 15281–15287 (2016).
4. Zhang, Q., Zhang, Y., Li, K., Wang, H., Li, H. & Zheng, J. A Novel Strategy to Improve the Therapeutic Efficacy of Gemcitabine for Non-Small Cell Lung Cancer by the Tumor-Penetrating Peptide iRGD. *PLoS ONE* **10**, e0129865 (2015).
5. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.* **71**, 209–249 (2021).
6. Abola, M. V., Prasad, V. & Jena, A. B. Association between treatment toxicity and outcomes in oncology clinical trials. *Ann. Oncol.* **25**, 2284–2289 (2014).
7. Imura, M., Katada, J. & Shiga, T. Epidemiological Study Regarding the Incidence of Venous Thromboembolism in Patients After Cancer Remission. *Cardiol. Ther.* **11**, 611–623 (2022).
8. Understanding Cancer Prognosis - NCI. (2014). at <<https://www.cancer.gov/about-cancer/diagnosis-staging/prognosis>> (Last accessed: 21 Oct. 2023)
9. Schauss, A. G. Nephrotoxicity and neurotoxicity in humans from organogermanium compounds and germanium dioxide. *Biol. Trace Elem. Res.* **29**, 267–280 (1991).
10. Li, L., Ruan, T., Lyu, Y. & Wu, B. Advances in Effect of Germanium or Germanium Compounds on Animals—A Review. *J. Biosci. Med.* **5**, 56–73 (2017).
11. Sinha, R., Kim, G. J., Nie, S. & Shin, D. M. Nanotechnology in cancer therapeutics: bioconjugated nanoparticles for drug delivery. *Mol. Cancer Ther.* **5**, 1909–1917 (2006).
12. Werengowska-Ciećwierz, K., Wiśniewski, M., Terzyk, A. P. & Furmaniak, S. The Chemistry of Bioconjugation in Nanoparticles-Based Drug Delivery System. *Adv. Condens. Matter Phys.* **2015**, e198175 (2015).

13. Zhong, H., Chan, G., Hu, Y., Hu, H. & Ouyang, D. A Comprehensive Map of FDA-Approved Pharmaceutical Products. *Pharmaceutics* **10**, 263 (2018).
14. Lee, S., Trinh, T. H. T., Yoo, M., Shin, J., Lee, H., Kim, J., Hwang, E., Lim, Y. & Ryou, C. Self-Assembling Peptides and Their Application in the Treatment of Diseases. *Int. J. Mol. Sci.* **20**, 5850 (2019).
15. Bagci, U., Udupa, J. K., Mendhiratta, N., Foster, B., Xu, Z., Yao, J., Chen, X. & Mollura, D. J. Joint segmentation of anatomical and functional images: Applications in quantification of lesions from PET, PET-CT, MRI-PET, and MRI-PET-CT images. *Med. Image Anal.* **17**, 929–945 (2013).
16. Wang, Z. J., Reddy, G. P., Gotway, M. B., Higgins, C. B., Jablons, D. M., Ramaswamy, M., Hawkins, R. A. & Webb, W. R. Malignant Pleural Mesothelioma: Evaluation with CT, MR Imaging, and PET. *RadioGraphics* **24**, 105–119 (2004).
17. O’Farrell, A., Shnyder, S., Marston, G., Coletta, P. & Gill, J. Non-invasive molecular imaging for preclinical cancer therapeutic development. *Br. J. Pharmacol.* **169**, 719–735 (2013).
18. Heidenreich - 2011 - Consensus Criteria for the Use of Magnetic Resonance.pdf. at <https://citeseerx.ist.psu.edu/document?repid=rep1&type=pdf&doi=efa8046bca2688acb5ae3f59df20e453bcef3705> (Last accessed: 21 Oct. 2023)
19. Lu, G., Nishio, N., van den Berg, N. S., Martin, B. A., Fakurnejad, S., van Keulen, S., Colevas, A. D., Thurber, G. M. & Rosenthal, E. L. Co-administered antibody improves penetration of antibody–dye conjugate into human cancers with implications for antibody–drug conjugates. *Nat. Commun.* **11**, 5667 (2020).
20. Bilan, R., Nabiev, I. & Sukhanova, A. Quantum Dot-Based Nanotools for Bioimaging, Diagnostics, and Drug Delivery. *ChemBioChem* **17**, 2103–2114 (2016).
21. Miyawaki, A. & Niino, Y. Molecular Spies for Bioimaging—Fluorescent Protein-Based Probes. *Mol. Cell* **58**, 632–643 (2015).
22. Kale, A., Kale, S., Yadav, P., Gholap, H., Pasricha, R., Jog, J. P., Lefez, B., Hannoyer, B., Shastry, P. & Ogale, S. Magnetite/CdTe magnetic–fluorescent composite nanosystem for magnetic separation and bio-imaging. *Nanotechnology* **22**, 225101 (2011).
23. Wang, Y., Hu, R., Lin, G., Roy, I. & Yong, K.-T. Functionalized Quantum Dots for Biosensing and Bioimaging and Concerns on Toxicity. *ACS Appl. Mater. Interfaces* **5**, 2786–2799 (2013).

24. Roque, A. C. A., Lowe, C. R. & Taipa, M. Â. Antibodies and Genetically Engineered Related Molecules: Production and Purification. *Biotechnol. Prog.* **20**, 639–654 (2004).
25. B. Essner, J., H. Laber, C., Ravula, S., Polo-Parada, L. & A. Baker, G. Pee-dots: biocompatible fluorescent carbon dots derived from the upcycling of urine. *Green Chem.* **18**, 243–250 (2016).
26. Edison, T. N. J. I., Atchudan, R., Sethuraman, M. G., Shim, J.-J. & Lee, Y. R. Microwave assisted green synthesis of fluorescent N-doped carbon dots: Cytotoxicity and bio-imaging applications. *J. Photochem. Photobiol. B* **161**, 154–161 (2016).
27. Karatutlu, A., Song, M., P. Wheeler, A., Ersoy, O., R. Little, W., Zhang, Y., Puech, P., S. Boi, F., Luklinska, Z. & V. Sapelkin, A. Synthesis and structure of free-standing germanium quantum dots and their application in live cell imaging. *RSC Adv.* **5**, 20566–20573 (2015).
28. Takayama, K., Hirose, H., Tanaka, G., Pujals, S., Katayama, S., Nakase, I. & Futaki, S. Effect of the Attachment of a Penetration Accelerating Sequence and the Influence of Hydrophobicity on Octaarginine-Mediated Intracellular Delivery. *Mol. Pharm.* **9**, 1222–1230 (2012).
29. Woldetsadik, A. D., Vogel, M. C., Rabeh, W. M. & Magzoub, M. Hexokinase II–derived cell-penetrating peptide targets mitochondria and triggers apoptosis in cancer cells. *FASEB J.* **31**, 2168–2184 (2017).
30. Majumdar, S. and Siahaan, T.J. Peptide-mediated targeted drug delivery. *Medicinal research reviews*, **32**, 637-658 (2012).
31. Li, J., Wu, D., Miao, Z. and Zhang, Y. Preparation of quantum dot bioconjugates and their applications in bio-imaging. *Current Pharmaceutical Biotechnology*, **11**, pp.662-671 (2010).

2. Literature review

2.1 Introduction

This Chapter investigates the prevalence of cancer-related mortality internationally and nationally. The imaging of tumors is important for prognosis and treatment; this is an area where the development of nanotechnology could assist. Carbon dots conjugated to peptides have been studied for their potential use in the bio-imaging of cancer cells, and this is explored in this chapter. Furthermore, by conjugating peptides to the surface of carbon dots, they can selectively target cancer cells or biomolecules that are overexpressed on cancer cells, allowing for more precise imaging of the tumor. The effects of doping carbon dots and manipulating peptides were discussed to further understand the structure-activity relationship.

2.2 Global and local impact of diseases caused by cancer and tumors

At the end of 2020, the Global Cancer Observatory (GLOBOCAN) 2020 supplied an updated approximation of cancer-related mortality and prevalence which was estimated at 19.3 million new cancer cases globally and nearly 10.0 million people deaths in the year 2020.¹ It is caused by damaged cells due to malfunctions during cell division induced by harmful substances or inherited DNA genes.² When a patient develops a tumor that leads to cancer, it can be due to the environment that the patient was exposed to or their lifestyle habits such as smoking, which accounts for 90-95% of reported tumor cases; moreover, only 5% and less of the cases are due to inheritable defected genes from other family members.³ In South Africa, 56 802 people died of cancer in 2021, as seen in **Figure 2.1**, according to the American Society of Clinical Oncology (ASCO) Post, and the deadliest forms of cancer were found to be lung cancer, oesophageal cancer, and cervical cancer.⁴

Mine workers are often exposed to excessive chemicals underground, which cause them to develop cancer. A clinical study of South African mine workers by Ndlovu and colleagues at the School of Public Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, assessed the asbestos-related diseases in mine workers and demonstrated that the chemical exposure underground caused asbestosis, mesothelioma, and lung cancer.^{5,6} Exposure to UV rays from the sun and strong air pollution also cause cancer. Studies show

that an unhealthy lifestyle can also induce cancer, such as smoking, passive smoking, drinking alcohol, and obesity.⁷

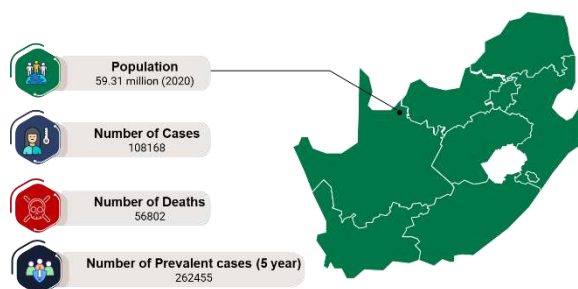


Figure 2.1 Stats as per The ASCO Post, in partnership with the American Society of Clinical Oncology 2021.⁸

Any kind of swelling caused by abnormal growth of tissue is regarded as a tumor, but not all tumors are cancerous or malignant.⁹ Normally, dead cells that have gone through apoptosis are replaced by new cells, but tumors form when the dead cells remain to form a growth. Life-threatening tumors are classified as malignant tumors because they can spread throughout the body and also limit the function of other organs. Malignant tumors can be surgically removed, but regrowth is possible.¹⁰ On the other hand, benign tumors pose a lower health risk because they do not spread in the body and can often be left untreated.¹¹

2.2.1 Treatment and monitoring

Imaging procedures play a crucial role in determining the appropriate treatment and location of malignant tumors, as well as determining the stage of development.¹² More research has gone into diagnosing and treating malignant tumors because they are fatal and aggressive.¹³ One of the most important aspects of treating malignant tumors is monitoring their size and growth. This can assist in determining the correct treatment and help monitor the response of the tumor toward certain treatments.

One way to image and monitor the size and growth of the tumor is through the use of nanotechnology. Nanomaterials such as gold nanoparticles, iron oxide nanoparticles, and quantum dots have been explored extensively for tumor monitoring due to their unique optical properties and biocompatibility.¹⁴ These nanomaterials can be functionalized with targeting molecules such as antibodies or peptides to specifically bind to cancer cells, making them useful for early cancer diagnosis and targeted therapy.¹⁵

Among the carbon nanomaterial family, carbon dots (CDs) have the potential to be used in tumor monitoring due to their unique optical properties and biocompatibility.¹⁶ CDs are small carbon-based nanoparticles (diameters less than 10 nm) that exhibit strong fluorescence, making them ideal for use in biological imaging.¹⁷ In addition, CDs have low toxicity and can be easily modified to target specific cells or tissues. Moreover, the competitive advantage CDs possess over other nanomaterials is that they can be made from waste materials such as agricultural waste, food waste, and industrial waste, which makes their production cheap and sustainable.¹⁸

Several studies have demonstrated the potential of CDs for tumor imaging and monitoring.¹⁹ For example, CDs have been used to label cancer cells and track their migration and proliferation *in vivo*.²⁰ Additionally, they have been functionalized with targeting molecules such as antibodies or peptides to specifically bind to cancer cells, making them useful for early cancer diagnosis and targeted therapy.²¹

2.3 Carbon dots

2.3.1 Carbon dots – what are they?

CDs are nanoparticles that are less than 10 nm in sizes and predominantly contain carbon atoms, as seen in **Figure 2.2**.²² These nanoparticles are biocompatible, which means that they are not harmful to living tissue and maintain their efficiency *in vitro* and *in vivo*.^{23,24} These nanoparticles have strong luminescent properties and give off bright colors under UV light, which allows for many bio-applications to emerge, such as fluorescence bio-imaging, bio-analysis, and micro-arrays. CDs have both excellent antimicrobial properties and anti-cancer properties.²⁵

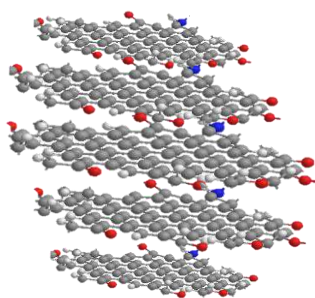


Figure 2.2 This is an illustration of a carbon dot with functional groups.

2.3.2 The potential of carbon dots for bio-application

Several studies have been conducted with CDs which explore the potential of using them in the medical industry, especially for cancer.²⁶ Below is a list of some of the fluorescent bio-applications of carbon nanomaterials that have been used in biology or medicine:

- Fluorescent biological labels

CDs have been employed in a variety of biological applications, including cellular imaging, bioimaging, and biosensing.²¹ They are useful in both *in vitro* and *in vivo* labelling of diverse biological substances such as proteins, nucleic acids, and lipids.²²

One advantage of utilizing CDs as fluorescent biological identifiers is their high photostability, which means they do not easily bleach or fade, which is useful for long-term imaging.²⁹ They are also less susceptible to photobleaching and photodamage than standard fluorescent dyes, making them excellent for live cell imaging.³⁰

- Drug and gene delivery

The fluorescence of CDs can be used to trace the distribution of medicines and genes.³¹ CDs, for example, can be functionalized with specific targeting ligands like antibodies or peptides, allowing them to bind to specific cells or tissues.³² The fluorescence of the CDs can be monitored once they have been picked up by the target cells to follow the intracellular delivery of the medication or gene.³³⁻³⁵

- Bio-detection of pathogens

The fluorescence features of the CDs can be used to detect the presence of the pathogen or biomarker once they have been functionalized and exposed to the target pathogen or biomarker.³⁶ This can be accomplished through the use of several detection techniques like fluorescence microscopy, flow cytometry, and spectroscopy.

- Magnetic resonance imaging (MRI) contrast enhancement

The fluorescence and magnetic resonance imaging (MRI) are separate imaging modalities, therefore, the fluorescence properties of CDs are not directly applicable to MRI contrast enhancement.³² However, CDs can be functionalized with magnetic materials such as iron oxide nanoparticles to improve their magnetic characteristics and allow them to be used as MRI contrast agents.³³

Magnetic CDs can also be functionalized with specific targeting ligands like antibodies or peptides to allow them to bind to certain cells or tissues.³⁹ This can improve the sensitivity and specificity of MRI imaging and allow for earlier disease identification. Moreover, the fluorescence of CDs is used for probing DNA structures and bio-sensing, which demonstrates the prospects of fluorescent CDs in the medical field.^{40, 41}

2.3.3 *In vivo* and *in vitro* studies using fluorescent carbon dots

Carbon nanoparticles for bio-labelling studies are gaining popularity because of their low toxicity and efficiency. One of the advantages of CDs is that they can be manipulated to exhibit bright luminescent properties for bio-imaging experiments.⁴² CDs are a buildup of organic molecules and are generally made up of carbon, oxygen, and hydrogen.⁴³ They have defected sp^2 domains which cause the surface states to be flawed, inducing the low energy luminescent emission.⁴⁴ The excitation wavelength can be altered because CDs have excitation-dependent emission properties, which can be used for fluorescence microscopy experiments.⁴⁵ These experiments are often conducted on living cells or even mice for bio-labelling, bio-sensing, and probing of cells.

Bio-labelling is the labelling of a living cell using nanoparticles for organelle and even tumours.^{42, 43} This can help confirm the capability of the cell uptake of CDs.⁴⁸ It can also be used to differentiate between various parts of a cell or organelle. A study conducted by Edward Jan and Nicholas A. Kotov⁴⁹ proved that it is possible to monitor an embryonic

neural stem cell of a mouse as it differentiates into various cells such as neurons, astrocytes, and oligodendrocytes using carbon nanotubes.

Many diseases, including tumours, go undetected because the instruments available are not sensitive enough to detect them at an early stage and subsequently detect the disease once fully developed.⁵⁰ This gives rise to the need for biosensors. Studies have shown that CDs are easily functionalized, which makes it possible to target bio-makers which are present when certain diseases affect the body.^{51, 52} Additionally, CDs have electrical properties, which will make it easier to produce devices that detect bio-makers at high sensitivity.⁴⁷ For instance, CA-125 is a protein in the body encoded by the MUC16 gene, which is routinely used as a biomarker for ovarian cancer because the presence of the protein is increased in the blood when cancer is present.^{47,55} The use of CDs as a biosensor for cancer has shown to have high sensitivity, selectivity, and stability.⁵⁶

The use of CDs in drug delivery for bio-imaging is very popular because the drug can be used on a confocal microscope to monitor the drug released. Doxorubicin is a strong chemotherapeutic drug that diminishes cancer cells or ceases the growth of cancer cells.⁵⁷

CDs have a structure composed of carbon and functional groups found on their surface. CDs' luminous characteristics can be improved via doping. This involves the deliberate insertion of impurities into a substance to change its qualities. Doping CDs can introduce atoms or molecules that act as traps for excited electrons, enhancing the dots' fluorescence quantum yield.

Nitrogen, sulfur, phosphorus, and metal ions such as copper, silver, and gold are common dopants used to improve their luminous properties. The specific dopant utilized will be determined by the desired attributes and the synthesis method used to create the CDs.

2.3.4 N-doped carbon dots decorated with Ge

The properties of CDs can be enhanced by doping with various elements, such as nitrogen. For instance, nitrogen doping of carbon is one of the ways of increasing the luminescent properties of CDs which play an important role in bio-imaging.⁵⁸ CDs have incredible properties, and the use of dopants and co-doping assists in tuning their properties to suit a particular application.⁵⁹ For example, Fatemeh Khodadadei, and co-workers fabricated 10 nm nitrogen-doped graphene quantum dots (N-GQDs) containing ten graphitic layers.⁶⁰

Additionally, the N-GQDs were loaded with the Methotrexate (MTX) cargo by strong π - π stacking interaction. The layered structure of N-GQDs was exploited to render a high specific area to intercalate the MTX between the layers generating a comprehensive anti-cancer drug delivery system.

On the other hand, the properties of CDs have also been modified using additives such as germanium. Germanium is a group 4 metalloid and is prone to have sp^3 like bonding which alters the properties of CDs differently compared to nitrogen.⁶¹ Studies have shown that Ge-CD (carbon dots doped with germanium) are biocompatible with exceptional emission properties and can be used for bio-application.⁶²

Organogermanium compounds have been shown to have anti-cancer properties.⁶³ Spirogermanium compounds, 2-carboxyethyl germanium sesquioxide (Ge-132), amino acid germanium, and lactic acid-Citrate germanium are organogermanium compounds that have been proven to have activity against tumours and have been incorporated in drugs.⁶³ A study conducted by Mark G. Mainwaring and colleagues demonstrated that treatment of Spindle cell carcinoma with orally administered germanium sesquioxide could cause complete remission.⁶⁴ This implies that it can be hypothesized that the N-doped CDs decorated with germanium can have anti-cancer properties.⁶⁵ When germanium is introduced into the N-doped carbon dot, then germanium oxide, amino acid germanium, and other organogermanium compounds will be present in the CDs, which can cause the CDs to have antitumor properties.⁶⁶

2.4 Peptides and their bio-application

Peptides are molecules containing two or more amino acids and are the building blocks of proteins, as demonstrated in **Figure 2.3**.⁶⁷ Attaching a natural peptide ligand or peptidomimetic to an active therapeutic agent can be a direct targeting strategy achieved by binding a receptor that is expressed on the target organ or tumour and undergoes endocytosis.⁶⁸

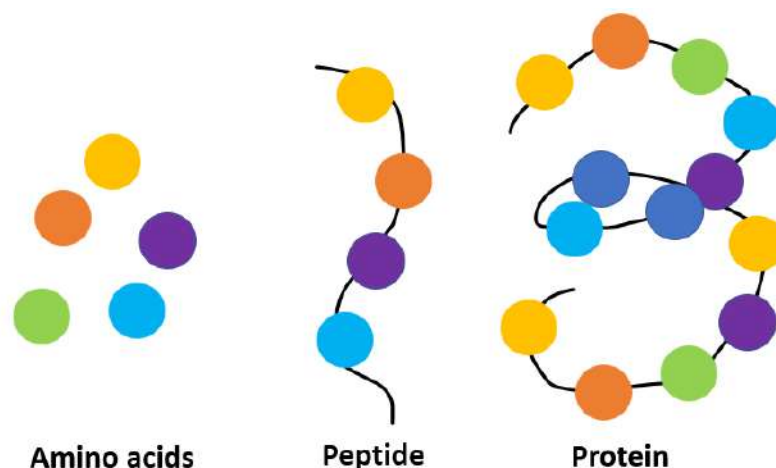


Figure 2.3 Illustration of how amino acids are building blocks of peptides and how proteins are much larger forms of peptides.

On the other hand, stereoisomers can be evaluated as potential ligands for receptors utilizing computation and analysis of peptide libraries. These are employed to elucidate the arrangement of atoms of the peptide in crystalline form, with the prediction of ligand conformation and orientation of the peptides.⁶⁹ This kind of approach bypasses the necessity for the manual synthesis of numerous peptide libraries and screening. However, the disadvantage is that they primarily hinge on data about the receptor targets, and unknown targets that may exist cannot be screened.^{70, 71} Moreover, peptides that demonstrate biological activity *in silico* don't necessarily have activity *in vivo*.⁷² Nonetheless, these strategies can supply an excellent foundation for constructing targeting peptide libraries.⁷³

2.4.1 Cell-penetrating peptides

There are several obstacles that most therapeutic agents need to overcome before reaching the intended site to achieve their physiological function. The ability to permeate membranes is a crucial feature that most therapeutic agents require to reach the targeted site. Therefore, molecules or biomolecules that enhance the cell-penetrating properties must be incorporated into active compounds.

For example, the GKPIILFF fragment as well as the synthetic retro peptide (FFLIPKG) both originate from a cathepsin D enzyme, a lysosomal aspartyl protease and are penetration accelerating sequences (Pas).⁷⁴ Literature has shown that they were able to substantially enhance cellular uptake when bonded to a bioactive agent.⁷⁵

In a study by Woldetsadik and co-workers,⁷⁶ the selectivity of a peptide correlating to the mitochondrial membrane-binding N-terminus sequence of HKII (pHK) was investigated by evaluating the apoptotic selective detachment of HKII from mitochondria in cancerous cells. To further improve the properties of the peptide, the penetration-accelerating GKPILFF peptide was covalently coupled to the pHK peptide resulting in enhanced cellular uptake and cell-death efficacy.

In another study by Kristensen and co-workers,⁷⁷ the peptides NR2B9c and N-dimer were fashioned to hinder binding forces between the N-methylated D-aspartate and the postsynaptic density-95 (PSD-95)/disc large/ZO-1 (PDZ) domains of PSD-95 for relieving neuronal damage. Each of these peptides was covalently incorporated into the cell-penetrating peptide TAT to promote permeation and neuro-cell absorption in the highly selective semipermeable barrier of the brain. The peptide conjugates not only effectively penetrated the vascular system of the mouse brain's central nervous system but also exhibited a similar level of accumulation in brain tissue as other significant polymeric nanomedicines.⁷⁸

2.4.2 Cell homing peptides

Targeted delivery increases the efficacy and decreases the side effects caused by the overall toxicity of the corresponding therapeutic compound, and can function either by direct conjugation or via promoted co-administrative effects.⁷⁹ Most therapeutic agents rely on passive diffusion as transportation across cell membranes; however, one of the main limitations of most pharmaceutical agents, especially highly toxic substances such as chemo drugs, is the inability to target the diseased site without affecting the normal tissue.⁸⁰ This has led to a need for a new class of drugs that incorporate targeting molecules or biomolecules. Peptides that possess cell-targeting properties often bind to receptors and other molecules present in the cell membrane, which makes them attractive candidates for targeted drug delivery systems for bio-imaging.⁸⁰

One of the notable tumour-homing peptides is the Cys-Arg-Glu-Lys-Ala (CREKA) peptide sequence which has been shown to bind to clotted plasmas proteins present in tumour vessels.⁸¹

For instance Zhang, Y. and co-workers designed a nano-vehicle containing the liposome conjugated to the CREKA peptide and loaded with a reversible platelet inhibitor ticagrelor (T). Primarily they showed that platelets could induce the transition of tumour cells, into

invasive phenotypes in vitro and thereafter demonstrated that CREKA-Lipo-T can eliminate this effect. These findings showed that CREKA-Lipo-T could efficiently block tumour cell acquisition of an invasive phenotype.⁸²

Other well-known tumour-homing molecules include folic acid and arginine-glycine-aspartate, which strongly target the folate receptor and integrin $\alpha v \beta 3$, respectively.^{83,84} To harness the properties of both previously mentioned tumour targeting molecules, Yan, H. and co-workers designed the PTX@MSNs-NH₂-FA-RGD conjugate to assess its potential to kill MCF-7 cells, and was found to be 1.6 times effective in contrast to the pristine PTX compound, indicating that PTX@MSNs-NH₂-FA-RGD had enhanced tumour selective cytotoxicity.⁸⁵

2.4.3 Cell homing and penetrating peptides

Cell homing and penetrating peptides are peptides that have a high affinity to certain receptors or sites found in the cell. Additionally, they also can permeate the cell so that they can perform their physiological function.⁸⁶

Cell homing and penetrating peptides can also be designed by combining peptides or molecules that can facilitate each function, respectively. This strategy can be accomplished in several ways by utilizing specific moieties such as carboxylic groups, amines, and thiols to name a few.⁸⁷

Iwasaki, T., and co-workers designed enantiomeric antimicrobial peptide (AMP) analogues derived from beetle defensins and observed their anti-cancer properties in several cancer cell lines. These results demonstrated a negative charge-dependent selectivity in cancer cells attributed to the d-9-mer peptides. Therefore, they have selective cytotoxicity and target anionic phosphatidylserine on the cell surface in the cancer cell membrane. Furthermore, combinations of d-peptide B (RLRLRIGRR-NH₂) and the anti-cancer agent dexamethasone demonstrated synergic growth inhibitory activity against mouse myeloma.⁸⁸

For example, Myrberg, H constructed a cell-homing and penetrating peptide that contained the cyclized peptide sequence, which is a targeting peptide, and the cell-penetrating peptide pVEC. Although the anti-cancer cCPGPEGAGC (PEGA) sequence is unable to penetrate the plasma membrane, it can enter many breast cancer cells in vitro when coupled to the peptide pVEC. Additionally, the conjugate accumulates mostly in the blood veins of tumorous breast

tissue, where it is subsequently taken up, and the PEGA-in pVEC's *in vivo* cell-directing ability is maintained.⁸⁹

2.5 Peptide-Carbon dot conjugates

Nanostructured delivery systems can be used as a delivery system for peptides.⁹⁰ Research from over the years has demonstrated that this strategy is capable of improving the absorption of the peptide and half-life.⁹¹ Different types of nanoparticles (NPs), such as carbon nanoparticles, micelles, and dendrimers, to name a few, can be used for enhanced selective targeted therapy of peptides facilitated by several chemical interactions. Polymetric and mesoporous silica nanoparticles are the most popular class of nanoparticles utilized for active agents and peptide drugs. Moreover, the exterior of nanoparticles can be modified with specific biomolecules, such as carbohydrates, polypeptides, synthetic polymers, enzymes, glycans, antibodies, nucleic acids, and oligonucleotides (ONTs), to name a few, which can feasibly be employed as targeted delivery moieties or cargo.^{92, 93}

While bioconjugation involves the fusion of two molecules, of which one of them is a biomolecule, normal conjugation occurs when two molecules are bonded together. Both strategies can be facilitated by covalent and non-covalent bonds. Fabrication of bioconjugates often involves straightforward, easy, and normally a one-step reaction which is generally confirmed using characterization techniques such as zeta potential, UV-vis, NMR, and FTIR.⁹⁴ This technique is also used for the incorporation of synthetic labels, for instance, isotope labels, fluorescent dyes, affinity tags, and biotin, to biological molecules for monitoring the internalization and bioavailability.^{95, 96}

For instance, Lei Yang and co-workers designed conjugates that comprised CDs that were functionalized with polyethylene glycol (PEG),⁹⁷ which facilitated the covalent conjugation of a nuclear localization signal peptide. Their research showed that the conjugate was biocompatible, and the quantum yield was found to be 75.8%. Furthermore, intracellular localization of the NLS-CDs conjugate in MCF7 and A549 cells was confirmed using confocal laser scanning microscopy images.

In a similar study by Yu Cao and co-workers,⁹⁸ polyethene glycol (PEG) was also used to provide covalent interactions between the graphene quantum dots; however, rather than conjugating a peptide, an aptamer was used to promote internalization and targeted cell-

specific therapeutic effects. The GQD-PEG-P conjugate demonstrated effective discrimination of cancer cells from somatic cells and the capacity to detect cancer-related miRNA.

In recent years, the integration of diagnostic and therapeutic agents that provide multipurpose functions in one individual nanostructure has proven to be critical to developing a new class of theranostic (a term used to describe the combination of diagnostic and therapeutic).⁹⁹ These platforms have received profound attention, especially in the area of personalized nanomedicine and clinical application.

In another study where the cRGD peptide was coupled to nanoparticles, the cRGD@TAT-DINPs showed increased cytotoxicity on both MDA-MB-231 and A549 cells, according to an in vitro investigation. In particular, combination chemo/photothermal/photodynamic therapy significantly increased the therapeutic efficacy of the produced nanoparticles under NIR light.¹⁰⁰

Intricate and versatile platforms can be facilitated by these multifunctional nanostructures, which can open a new gate towards a diagnostic, site-homing, and active treatment using a single multifunctional system. Unfortunately, some of the limitations of such technologies include a limited stability profile, high cost, and short storage life. Additionally, they faced major intrinsic hurdles caused by production batches which led to harsh bench-to-bedside transformation and poor reproducibility. To address these limitations, researchers are finding various ways to improve on these limitations by using cheap resources such as wasted CDs synthesis and facile one-step synthesis to enhance reproducibility.¹⁰¹

At present, another alternative to nanoparticulate delivery systems is bioconjugates. Reports show that medicinal business sector and academic scholars are investing heavily in bioconjugate structures due to the appealing potential features. Bioconjugation is a pervasive technique that finds a multitude of applications in different branches of life sciences, including selective targeted therapy, biosensing biological assays, imaging, and gene delivery applications.

Therefore, the intracellular concentrations for each construct must be determined separately before using cell-permeable peptide/cargo constructs for intracellular investigations of structure-function connections. This will ensure that the improved biological activity solely relies on the conjugation of each component. The conjugation to homing peptides is known to

bind to the specific receptors present in the cells; however, it may not necessarily penetrate the cells, as seen in **Figure 2.4**.¹⁰² To address this potential challenge, conjugation to a cell-penetrating peptide may be necessary. Another method would be to use a peptide that has both the targeting properties and cell-penetrating properties to deliver the drug cargo.

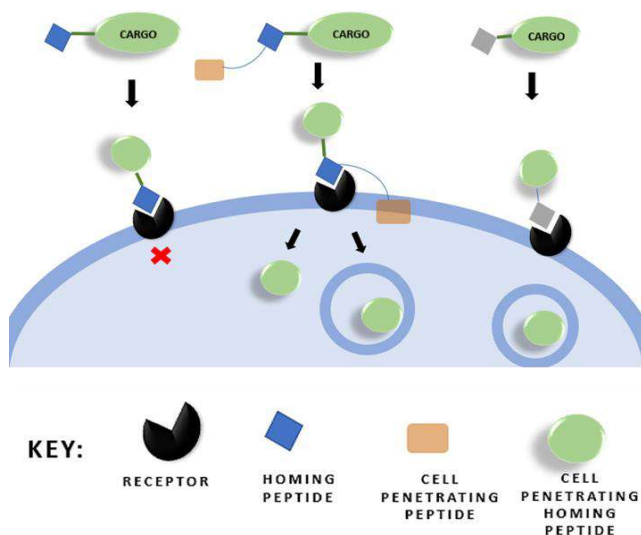


Figure 2.4 Multipurpose functions in one individual nanostructure

2.6 Derivatization of carbon dots and peptide and their effects

2.6.1 Functionalization of carbon dots

To facilitate CD modification, molecules with unique chemical characteristics and reducing groups on the surface of CDs can be employed.¹⁰³ Luminescence properties can be enhanced using synthetic approaches based on known organic chemistry reactions desirable functional groups that can be covalently conjugated to CDs. External functional groups, including carbonyl and carboxyl groups, can facilitate trapping states with different energy levels by treatment of CDs utilizing polymers and organic compounds, producing luminescent CDs that emit light with various energies.¹⁰⁴ Additionally, covalent modifications can accomplish enhanced control of the size, stabilization of surface energy traps, and physical features of CDs.¹⁰⁵ On the other hand, non-covalent modifications rely on interactions between CDs and the substance being added, known as the London force or the -dispersion force. Non-covalent alterations also have a less detrimental impact on the CDs' structure than covalent modifications, making it possible to tailor the photoluminescence and physicochemical characteristics.^{106, 107} Significantly improved biocompatibility and optical properties can be

observed after modification with these molecules, and typically the synthesis is straightforward. For instance, if the surface of the CDs readily contains moieties such as amino groups, then generally non-local π orbital and molecular orbital resonance structures are found, which is associated with the enhanced QY and adjustable photoluminescence.¹⁰⁸ Furthermore, normal CDs, which have been subsequently modified with amino-containing compounds, are known to possess distinctly improved QY.¹⁰⁹ As an alternative, oxygenated moieties on the surface of CDs are affiliated with the photoluminescence properties usually caused by electron-hole recombination and surface energy traps.¹¹⁰ The relationship between the surface functionality of CDs and the different properties produced, oxidation on the surface of CDs and photoluminescence can be customized by fine-tuning the amount of oxygenated and amino groups to achieve specific properties.

Moreover, the main feature of CDs that makes them widely applicable in selectively targeted therapy applications is functionalizing with bioactive molecules.¹¹¹ Pharmaceutical agent and receptor targeting molecules can also be functionalized together to the surface of CDs, resulting in a nanohybrid that can recognize the ailing sites and discharge medicinal agents.

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CDs and similar nanostructures have been utilized as drug delivery systems (DDS) with a focus on promoting properties such as drug solubility, half-life, and accumulation.¹¹³ Targeting and controlling drug release at the tumorous area results in diminishing the drugs' side effects and improving their bioavailability and tolerance.¹¹⁴

2.6.2 Different types of interaction for the functionalization of carbon dots

The covalent and non-covalent interaction methods of functionalizing CDs are both readily used and have different advantages, which are high on the application of the material.¹¹⁵ Both techniques can improve selective targeted therapy and control; however, covalent bonds are known to be very strong, and the integrity of the conjugates may be superior.¹¹⁶ On the other hand, in applications where the attached molecule is required to release and bind to receptors, non-covalent interactions may potentially allow better drug release of the cargo drug attributed to the high affinity to the target site.¹¹⁷

One of the most popular methods of covalently coupling peptides to CDs is EDC/NHS coupling owing to its high efficiency and stability.¹¹⁸ This technique achieves conjugation

through activation of the carboxylic acid groups, which allows it to react with the free amine groups present on the peptide, as seen in **Figure 2.5**.¹¹⁹

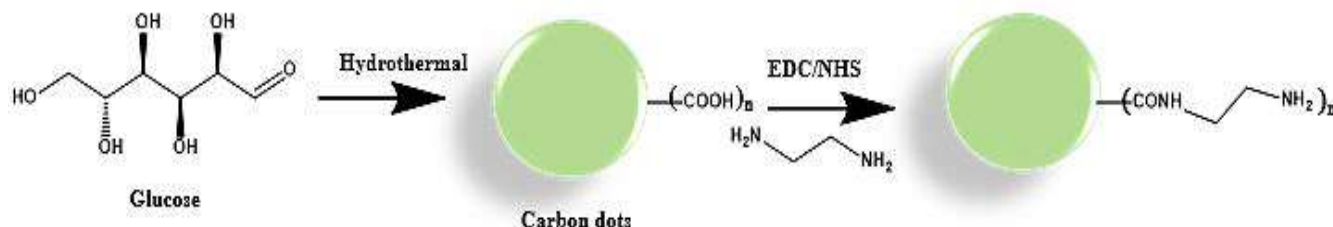


*Figure 2.5 Covalent modification of carbon dots*¹¹⁹

For example, Dada, S. N. and co-workers¹²⁰ fabricated folic acid-modified carbon dots (FA-CDs) conjugated to doxorubicin (Dox) using the EDC/NHS strategy and subsequently compared the covalently and noncovalently bonded nanohybrids as cancer-selective targeted therapy system. For targeting cancer cells, folic acid was integrated into the surface of CDs to attach to the overexpressing folate receptors. The data demonstrated that the non-covalent FA-CD-Dox displayed superior efficiency against MDA-MB-231 in contrast to the CD-Dox and covalent FA-CD-Dox.¹²⁰ Similar work was conducted by Qinghui Zeng and co-workers, and their data demonstrated successful delivery of CD-DOX conjugates to HepG2 cancer cells and HL-7702 normal.¹¹⁴

The various non-covalent modification of CDs includes dipole-dipole interactions, complex formation, or π - π forces between CDs, to name a few.¹⁰⁶ One of the benefits of these non-covalent interactions include maintaining the crucial properties of the CDs while introducing a new feature that enhances or adds new properties.¹⁰³ Furthermore, non-covalent modifications present minimal adverse influence on the pristine framework of CDs and also help provide new moieties, targeting molecules, or metal nanoparticles on the exterior of the CD.¹²¹ Consequently, these new features can establish a supplementary dimension in controlling the interfacial properties and render a powerful link to connect nanoparticles to biological molecules. The integrity of the nanostructure is the supreme virtue of non-covalent interaction, which can be fabricated based on inherent functional groups and dispersion force from van der Waals forces present on the CDs. For instance, in a study conducted by BR. Liu,

successful delivery of CdSe/ZnS quantum dots by cell penetration peptides (PR9) was reported. The intracellular trafficking data and functional assay evidence revealed that the ingestion of the quantum dots was predominantly through endocytosis.¹²² The electrostatic interaction between the peptides and the quantum dots was found to play a key role in the complex formation during conjugation and these interactions were analyzed using Zeta potential.

One of the toughest membranes in the body is the blood-brain barrier (BBB) which is a protective layer separating the brain's blood vessels and the cells and other components that makeup brain tissue.¹²³ This barrier provides a defense mechanism against bacteria, viruses, and toxins that may exist in our blood. The selective permeability of this membrane causes major medical drawbacks for drug-delivery systems that target the brain, which has led to high demand for drug-delivery systems that can penetrate the brain. Shanghai Li, and co-workers observed the penetration of transferrin-conjugated C-Dots in the central nervous system (CNS) by crossing the BBB *in vivo* using a zebrafish model study.¹²⁴ This *in vivo* study revealed that the CDs alone could not pass through the blood vessels that vascularize the central nervous system and that the conjugation to transferrin glycoprotein is crucial for permeability. The diameter of CDs was approximately 5 nm, with plenty of carboxylic groups dispersed on the surface for feasible chemical interaction with the biological molecule.

In another study by Ullah, Z. and co-workers, a comprehensive theoretical study on adsorption performance investigated the Pyrimidin-2-amine drug molecule on graphene quantum dots (GQD).¹²⁵ The computational simulations investigated non-covalent interactions, density functional theory (DFT), and time-dependent density functional theory (TD-DFT), and the data revealed that the enhanced interaction with doping (N, B) quantum dot complexes with a ΔG value of -13.38 kcal/mole, ΔE of -23.49 , and ΔH of 22.60 kcal/mol. This shows that non-covalent conjugation is a good alternative for successfully functionalizing carbon nanodots or other carbon-based nanomaterials with drug molecules, targeting agents, and biomolecules.

2.6.3 Manipulation of the Amino Acid Sequence

The L- configuration of the stereogenic alpha carbon in amino acids is the most predominant one found in nature, which polarizes light anticlockwise (Levorotation), as seen in **Figure**

targeted the C-X-C chemokine receptor type 4 (CXCR-4) and were found to attach to CXCR-4 with IC₅₀ values of 32 and 439 nM, respectively.

2.6.4 C-Terminal Amidation

Peptide degradation or aggregation plays a significant role in reduced activity and solubility.¹³¹ As a result, numerous techniques have been employed to improve the stability and pharmacological activity of peptides. One of the common ways to achieve this is by converting the natural, free carboxyl group (-COOH) C-terminal of the peptide into an amide group (-CONH₂).¹³² Although this technique occasionally causes the peptide to lose activity, several studies have shown that C-terminal amidated peptides have increased biological activity and bioavailability which is why it remains a popular peptide modification.¹³³ Studies have demonstrated that the uncharged C-terminal amide ends approximately imitates the natural protein and consequently amplifies the pharmacological activity of a peptide.¹³⁴ Additionally, the stability and shelf life of the peptide may be further increased. In a recent study conducted by Keon-Hyoung Song and co-workers, C-terminal-amidated, N-terminal-acetylated, and a combination of the two modifications were applied to the FCIGRL peptide modulator. The FCIGRL-NH₂, Ac-FCIGRL, Ac-FCIGRL-NH₂, and FCIGRL-COOH peptides were analyzed. Data revealed that the C-terminal-amidated had superior performance in enhancing nasal drug delivery.¹³⁵ Other peptide modifications on the C-terminal end include the incorporation of benzylamine and memantine functional groups that have been shown to enhance the cell viability and cell internalization of peptides.¹³⁶

2.6.5 N-Terminal modification

Although peptides are naturally occurring biomolecules that are generally bioavailable, natural proteolysis of the peptide through proteases, in addition to fairly inadequate blood-brain-barrier (BBB) permittivity, give rise to difficulties in producing peptides that are “drug-like”.¹³⁷

To broaden the application of peptides for a wide range of biotechnological applications and essential biophysical studies, it is critical to include specific chemical moieties in peptides.¹³⁸ For modifications requiring chemoselectivity, the N-terminus is often easily targeted and can therefore achieve site-specific bioconjugation.¹⁴⁰ The N-terminus position is another attractive target because there is normally only one present in a single-chain peptide, and

examples include attachment of fluorophore, PEGylation, and glycosylation to name a few.⁹⁶ Chemical enhancers are a class of chemical modifiers that improve the permeableness of drug molecules.¹⁴⁰ This includes esters, fatty acids, amides, surfactants, and glycols, to name a few.¹⁴¹ In certain circumstances, modifications of the peptide structure are by covalently bonding to the chosen chemical enhancer, consequently altering its physicochemical features.¹⁴² When it comes to fatty acids, coupling on the N-terminus of a peptide, for example, palmitoylation of a peptide sequence, is a popular molecular modification that can increase the permittivity through membrane layers.¹⁴³ Furthermore, the peptides such as pal-GHK tripeptide and Palmitoyl Dipeptide-6 (Pal-Lys-Val-Dab-OH) are commercially available well-established cosmetic palmitoyl peptides known to have anti-wrinkle and wound-healing properties.¹⁴⁴

Since the central nervous system (CNS) continues to be a hindrance to the delivery of therapeutic peptides and large hydrophilic molecules, it is necessary to explore other compounds that assist in crossing the brain barrier.¹⁴⁵ Sharareh Eskandari and co-workers designed an amphiphilic dalargin derivative peptide containing the palmitic moiety. The Dalargin (Y-DA-G-F-L-R) peptide sequence is an opioid receptor (μ receptor) agonist; however, it has difficulty penetrating the blood-brain-barrier. Their study revealed that the fatty acid modification conquered this major challenge, and additionally, the enhanced stability of the drug in the plasma was observed, promoting endocytosis transportation across the blood-brain-barrier.¹⁴⁶ The integration of fatty acids such as palmitic acid into peptide biomolecules is a powerful technique and has an incredible prospect of increasing the stability and penetrability of peptides.

Another effective technique to enhance pharmaceutical peptide properties is the covalent conjugation of poly(ethylene glycol) which has proven to hinder recognition by inhibitors, proteases, and antibodies.¹⁴⁷ In a study by P Esposito, isomers of mono-poly (ethylene glycol)-modified with a mol. wt. of PEG = 5000 polymer chain was linked to Lys 12 or Lys 21 residues of the synthetic GRF1–29 (Sermorelin).¹⁴⁸ The finding revealed that high biological activity was exhibited *in vitro*, which is similar to that of hGRF1–29. However, it had greater pharmacodynamic activity in comparison to unmodified GRF peptide. Adamantane is yet another moiety that offers appealing opportunities to manufacture diverse adamantane-based compounds for biomedical applications.¹⁴⁹ These tricyclic alkane building block molecules are very bulky and rigid and be exceptionally lipophilic, which can enhance

the BBB penetrability of peptides and prevent proteolysis of peptides.¹⁵⁰ In another study by Li, B. and co-workers, adamantane was integrated into the peptidomimetic compound (Ac-DGGL-NH₂) to investigate its ability to penetrate the highly selective semipermeable brain barrier for Alzheimer's disease treatment. The data revealed that the adamantane-derivatized peptide led to improved hippocampus-dependent learning and memory in normal adult mice. Moreover, an increased generation and maturation of new neurons were observed in the brain's granular layer and the interface region between the hilus and granular layer within the hippocampus of the dentate gyrus.¹⁵¹

2.6.6 Structure-Activity Relationships (SAR)

Biological activity can be predicted from molecular structure thanks to the link between activity and structure. To direct the acquisition or synthesis of desirable novel compounds as well as to further analyze existing molecules, tools like CCD vault and Amber18 are strong technologies utilized in drug development.

It is frequently possible to infer a new chemical compound's biological effects from its molecular structure by applying knowledge of related chemicals. This is due to the possibility that related molecules may share physical and biological characteristics. The idea known as the "Structure-Activity Relationship" states that there is a connection between molecular structures and their biological activity (SAR).¹⁵²

SAR techniques like CDD Vault, which can develop models to assess new chemical compounds and forecast their biological function, can find correlations that can then be used to create and characterize desirable novel molecules. SAR can be used to further assess existing drugs for potential side-effect activities.

Understanding which structural traits are correlated with chemical and biological reactivity is essential for SAR. As a result, to make inferences about an unknown drug, it is necessary to compare it to both database of known molecules and structural properties that can be characterised. When used in conjunction with sound professional judgment, SAR can be an effective tool for comprehending the functional significance of similarities.¹⁵³

For example, in a review study by Tony Velkov,¹⁵⁴ the mechanism of the nonribosomal peptides polymyxin B (PMB) and its analogues were explored, as seen in **Figure 2.7**. The known mechanisms behind the microbial activity and bacterial resistance were correlated to

the structure-activity interactions. According to the research, changes that neutralize the positive charges on the Dab side chains and cyclic ring expansion or decrease frequently result in a complete loss of antibacterial action. The ideal chain length of polymyxins is between seven and nine carbons, and both lipopolysaccharides (LPS) binding and antibacterial action depends on their distinctive three-dimensional design.

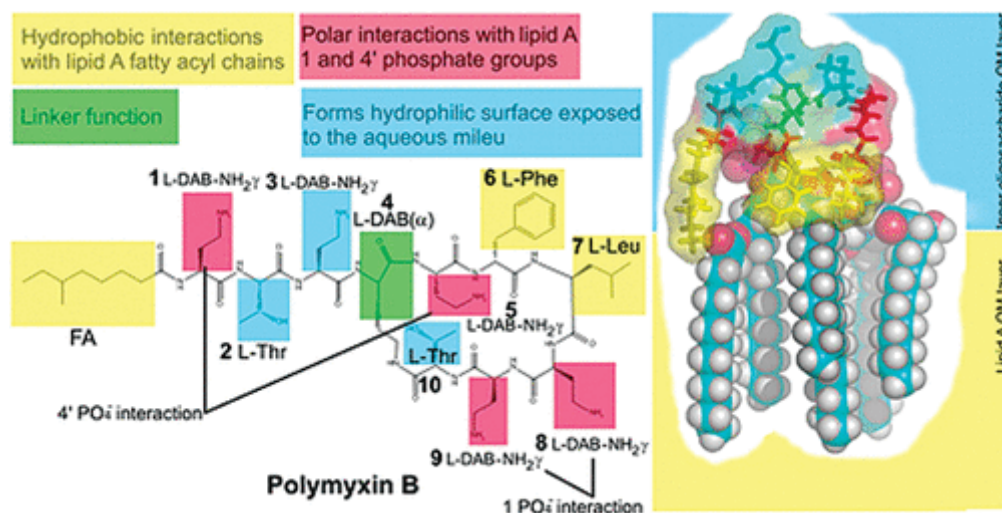


Figure 2.7 Shows a schematic diagram with essential contacts for the development of a complex between PMB and the lipid A component of the LPS colour-coded fatty acyl chain at the end, or FA.¹⁵⁴

In summary, when CDs are coupled with peptides, the resulting nanoparticles can be used for fluorescence imaging, confocal microscopy, and even *in vivo* imaging. CDs emit intense and steady fluorescence signals in fluorescence imaging, which may be identified and observed with specialist equipment. Peptides can be used to target cancer cells selectively and increase the permeability of their cargo. Overall, CDs coupled to peptides are a promising technique for cancer bio-imaging that is now being studied in preclinical investigations. However, more research is required to enhance the design of the conjugates as well as to assess their safety and effectiveness.

2.7 References

1. Cao, W., Chen, H.-D., Yu, Y.-W., Li, N. & Chen, W.-Q. Changing profiles of cancer burden worldwide and in China: a secondary analysis of the global cancer statistics 2020. *Chin. Med. J. (Engl.)* **134**, 783–791 (2021).
2. Irigaray, P., Newby, J. A., Clapp, R., Hardell, L., Howard, V., Montagnier, L., Epstein, S. & Belpomme, D. Lifestyle-related factors and environmental agents causing cancer: An overview. *Biomed. Pharmacother.* **61**, 640–658 (2007).
3. Anand, P., Kunnumakkara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., Sung, B. & Aggarwal, B. B. Cancer is a preventable disease that requires major lifestyle changes. *Pharm. Res.* **25**, 2097–2116 (2008).
4. Cancer – the second leading cause of death worldwide. *Med. Brief* (2016). at <<https://www.medicalbrief.co.za/archives/cancer-second-leading-cause-death-worldwide/>>
5. Reid, A., Merler, E., Peters, S., Jayasinghe, N., Bressan, V., Franklin, P., Brims, F., Klerk, N. H. de & Musk, A. W. Migration and work in postwar Australia: mortality profile comparisons between Australian and Italian workers exposed to blue asbestos at Wittenoom. *Occup. Env. Med.* oemed-2017-104322 (2017). doi:10.1136/oemed-2017-104322
6. Ndlovu, N., Nelson, G., Vorajee, N. & Murray, J. 38 years of autopsy findings in South African mine workers. *Am. J. Ind. Med.* **59**, 307–314 (2016).
7. Young, G. P. & K Le Leu, R. Preventing cancer: dietary lifestyle or clinical intervention? *Asia Pac. J. Clin. Nutr.* **11**, S618–S631 (2002).
8. Cancer on the Global Stage: Incidence and Cancer-Related Mortality in South Africa - The ASCO Post. at <<https://ascopost.com/issues/september-10-2021/cancer-on-the-global-stage-incidence-and-cancer-related-mortality-in-south-africa/>> (Last accessed: 21 Oct. 2023)
9. What Is Cancer? *Natl. Cancer Inst.* at <<https://www.cancer.gov/about-cancer/understanding/what-is-cancer>> (Last accessed: 21 Oct. 2023)
10. Clark, W. H. Tumour progression and the nature of cancer. *Br. J. Cancer* **64**, 631–644 (1991).
11. Marusyk, A. & Polyak, K. Tumor heterogeneity: Causes and consequences. *Biochim. Biophys. Acta BBA - Rev. Cancer* **1805**, 105–117 (2010).

12. Sadoughi, A. & Miller, E. Role of Interventional Pulmonology in Lung Cancer Care. 7
13. Queen, L. Causes, Prevention, and Treatment. *SKIN CANCER* 29
14. Yang, F., Jin, C., Subedi, S., Lee, C. L., Wang, Q., Jiang, Y., Li, J., Di, Y. & Fu, D. Emerging inorganic nanomaterials for pancreatic cancer diagnosis and treatment. *Cancer Treat. Rev.* **38**, 566–579 (2012).
15. Alexis, F., Rhee, J.-W., Richie, J. P., Radovic-Moreno, A. F., Langer, R. & Farokhzad, O. C. New frontiers in nanotechnology for cancer treatment. *Urol. Oncol. Semin. Orig. Investig.* **26**, 74–85 (2008).
16. Li, H., Yan, X., Kong, D., Jin, R., Sun, C., Du, D., Lin, Y. & Lu, G. Recent advances in carbon dots for bioimaging applications. *Nanoscale Horiz.* **5**, 218–234 (2020).
17. Song, Y., Zhu, S. & Yang, B. Bioimaging based on fluorescent carbon dots. *RSC Adv.* **4**, 27184–27200 (2014).
18. De, B. & Karak, N. A green and facile approach for the synthesis of water soluble fluorescent carbon dots from banana juice. *RSC Adv.* **3**, 8286–8290 (2013).
19. Mansuriya, B. D. & Altintas, Z. Carbon Dots: Classification, Properties, Synthesis, Characterization, and Applications in Health Care—An Updated Review (2018–2021). *Nanomaterials* **11**, 2525 (2021).
20. Zhang, M., Bai, L., Shang, W., Xie, W., Ma, H., Fu, Y., Fang, D., Sun, H., Fan, L., Han, M., Liu, C. & Yang, S. Facile synthesis of water-soluble, highly fluorescent graphene quantum dots as a robust biological label for stem cells. *J. Mater. Chem.* **22**, 7461–7467 (2012).
21. Iannazzo, D., Ziccarelli, I. & Pistone, A. Graphene quantum dots: multifunctional nanoplatforms for anticancer therapy. *J. Mater. Chem. B* **5**, 6471–6489 (2017).
22. Kang, Z. & Lee, S.-T. Carbon dots: advances in nanocarbon applications. *Nanoscale* **11**, 19214–19224 (2019).
23. Yang, S.-T., Wang, X., Wang, H., Lu, F., Luo, P. G., Cao, L., Meziani, M. J., Liu, J.-H., Liu, Y., Chen, M., Huang, Y. & Sun, Y.-P. Carbon Dots as Nontoxic and High-Performance Fluorescence Imaging Agents. *J. Phys. Chem. C* **113**, 18110–18114 (2009).
24. Zhao, X., Zhang, J., Shi, L., Xian, M., Dong, C. & Shuang, S. Folic acid-conjugated carbon dots as green fluorescent probes based on cellular targeting imaging for recognizing cancer cells. *RSC Adv.* **7**, 42159–42167 (2017).

25. Hola, K., Zhang, Y., Wang, Y., Giannelis, E. P., Zboril, R. & Rogach, A. L. Carbon dots—Emerging light emitters for bioimaging, cancer therapy and optoelectronics. *Nano Today* **9**, 590–603 (2014).
26. Salata, O. Applications of nanoparticles in biology and medicine. *J. Nanobiotechnology* **2**, 3 (2004).
27. Sun, Y.-P., Zhou, B., Lin, Y., Wang, W., Fernando, K. A. S., Pathak, P., Mezziani, M. J., Harruff, B. A., Wang, X., Wang, H., Luo, P. G., Yang, H., Kose, M. E., Chen, B., Veca, L. M. & Xie, S.-Y. Quantum-Sized Carbon Dots for Bright and Colorful Photoluminescence. *J. Am. Chem. Soc.* **128**, 7756–7757 (2006).
28. Li, Z. F. & Ruckenstein, E. Water-Soluble Poly (acrylic acid) Grafted Luminescent Silicon Nanoparticles and Their Use as Fluorescent Biological Staining Labels. *Nano Lett.* **4**, 1463–1467 (2004).
29. Yang, J., Gao, G., Zhang, X., Ma, Y.-H., Chen, X. & Wu, F.-G. One-step synthesis of carbon dots with bacterial contact-enhanced fluorescence emission: Fast Gram-type identification and selective Gram-positive bacterial inactivation. *Carbon* **146**, 827–839 (2019).
30. Wang, W., Damm, C., Walter, J., J. Nacken, T. & Peukert, W. Photobleaching and stabilization of carbon nanodots produced by solvothermal synthesis. *Phys. Chem. Chem. Phys.* **18**, 466–475 (2016).
31. Yao, J., Yang, M. and Duan, Y., 2014. Chemistry, biology, and medicine of fluorescent nanomaterials and related systems: new insights into biosensing, bioimaging, genomics, diagnostics, and therapy. *Chemical reviews.* **114**, 6130-6178(2014).
32. Chen, B. B., Liu, M. L., Li, C. M. & Huang, C. Z. Fluorescent carbon dots functionalization. *Adv. Colloid Interface Sci.* **270**, 165–190 (2019).
33. Pantarotto, D., Singh, R., McCarthy, D., Erhardt, M., Briand, J.-P., Prato, M., Kostarelos, K. & Bianco, A. Functionalized Carbon Nanotubes for Plasmid DNA Gene Delivery. *Angew. Chem.* **116**, 5354–5358
34. Bianco, A., Kostarelos, K. & Prato, M. Applications of carbon nanotubes in drug delivery. *Curr. Opin. Chem. Biol.* **9**, 674–679 (2005).
35. Bao, H., Pan, Y., Ping, Y., Sahoo, N. G., Wu, T., Li, L., Li, J. & Gan, L. H. Chitosan-Functionalized Graphene Oxide as a Nanocarrier for Drug and Gene Delivery. *Small* **7**, 1569–1578

36. Li, Y., Cu, Y. T. H. & Luo, D. Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. *Nat. Biotechnol.* **23**, 885–889 (2005).
37. Manus, L. M., Mastarone, D. J., Waters, E. A., Zhang, X.-Q., Schultz-Sikma, E. A., MacRenaris, K. W., Ho, D. & Meade, T. J. Gd (III)-Nanodiamond Conjugates for MRI Contrast Enhancement. *Nano Lett.* **10**, 484–489 (2010).
38. Wu, H., Liu, G., Zhuang, Y., Wu, D., Zhang, H., Yang, H., Hu, H. & Yang, S. The behavior after intravenous injection in mice of multiwalled carbon nanotube / Fe₃O₄ hybrid MRI contrast agents. *Biomaterials* **32**, 4867–4876 (2011).
39. McCarthy, J. R. & Weissleder, R. Multifunctional magnetic nanoparticles for targeted imaging and therapy. *Adv. Drug Deliv. Rev.* **60**, 1241–1251 (2008).
40. Johnson, R. R., Johnson, A. T. C. & Klein, M. L. Probing the Structure of DNA–Carbon Nanotube Hybrids with Molecular Dynamics. *Nano Lett.* **8**, 69–75 (2008).
41. Kong, B., Zhu, A., Ding, C., Zhao, X., Li, B. & Tian, Y. Carbon Dot-Based Inorganic–Organic Nanosystem for Two-Photon Imaging and Biosensing of pH Variation in Living Cells and Tissues. *Adv. Mater.* **24**, 5844–5848
42. Du, F., Gong, X., Lu, W., Liu, Y., Gao, Y., Shuang, S., Xian, M. & Dong, C. Bright-green-emissive nitrogen-doped carbon dots as a nanoprobe for bifunctional sensing, its logic gate operation and cellular imaging. *Talanta* **179**, 554–562 (2018).
43. Choi, Y., Choi, Y., Kwon, O.-H. & Kim, B.-S. Carbon Dots: Bottom-Up Syntheses, Properties, and Light-Harvesting Applications. *Chem. – Asian J.* **13**, 586–598
44. Vijesh, K. R., Sony, U., Ramya, M., Mathew, S., Nampoori, V. P. N. & Thomas, S. Concentration dependent variation of thermal diffusivity in highly fluorescent carbon dots using dual beam thermal lens technique. *Int. J. Therm. Sci.* **126**, 137–142 (2018).
45. Ali, H., Ghosh, S. & Jana, N. R. Biomolecule-derived Fluorescent Carbon Nanoparticle as Bioimaging Probe. *MRS Adv.* **3**, 779–788 (2018).
46. Kim, T., Momin, E., Choi, J., Yuan, K., Zaidi, H., Kim, J., Park, M., Lee, N., McMahon, M. T., Quinones-Hinojosa, A., Bulte, J. W. M., Hyeon, T. & Gilad, A. A. Mesoporous Silica-Coated Hollow Manganese Oxide Nanoparticles as Positive T_1 Contrast Agents for Labeling and MRI Tracking of Adipose-Derived Mesenchymal Stem Cells. *J. Am. Chem. Soc.* **133**, 2955–2961 (2011).
47. Hamd-Ghadareh, S., Salimi, A., Fathi, F. & Bahrami, S. An amplified comparative fluorescence resonance energy transfer immunosensing of CA125 tumor marker and

- ovarian cancer cells using green and economic carbon dots for bio-applications in labeling, imaging and sensing. *Biosens. Bioelectron.* **96**, 308–316 (2017).
48. Enhanced photoluminescence properties of a carbon dot system through surface interaction with polymeric nanoparticles - ScienceDirect. at <https://www.sciencedirect.com/science/article/pii/S0021979718301206> (Last accessed: 21 Oct. 2023)
 49. Jan, E. & Kotov, N. A. Successful Differentiation of Mouse Neural Stem Cells on Layer-by-Layer Assembled Single-Walled Carbon Nanotube Composite. *Nano Lett.* **7**, 1123–1128 (2007).
 50. Issaq, H. J., Waybright, T. J. & Veenstra, T. D. Cancer biomarker discovery: Opportunities and pitfalls in analytical methods. *Electrophoresis* **32**, 967–975 (2011).
 51. Sharma, B. & Kanwar, S. S. Phosphatidylserine: A cancer cell targeting biomarker. *Semin. Cancer Biol.* **52**, 17–25 (2018).
 52. Bhattacharyya, D., Sarswat, P. K. & Free, M. L. Quantum dots and carbon dots based fluorescent sensors for TB biomarkers detection. *Vacuum* **146**, 606–613 (2017).
 53. Huang, Q., Hu, S., Zhang, H., Chen, J., He, Y., Li, F., Weng, W., Ni, J., Bao, X. & Lin, Y. Carbon dots and chitosan composite film-based biosensor for the sensitive and selective determination of dopamine. *Analyst* **138**, 5417–5423 (2013).
 54. Cui, X., Zhu, L., Wu, J., Hou, Y., Wang, P., Wang, Z. & Yang, M. A fluorescent biosensor based on carbon dots-labeled oligodeoxyribonucleotide and graphene oxide for mercury (II) detection. *Biosens. Bioelectron.* **63**, 506–512 (2015).
 55. Yin, B. W. T. & Lloyd, K. O. Molecular Cloning of the CA125 Ovarian Cancer Antigen identification as a new mucin, muC16. *J. Biol. Chem.* **276**, 27371–27375 (2001).
 56. Mansouri Majd, S. & Salimi, A. Ultrasensitive flexible FET-type aptasensor for CA 125 cancer marker detection based on carboxylated multiwalled carbon nanotubes immobilized onto reduced graphene oxide film. *Anal. Chim. Acta* **1000**, 273–282 (2018).
 57. Doxorubicin (Adriamycin) | Cancer drugs | Cancer Research UK. at <http://www.cancerresearchuk.org/about-cancer/cancer-in-general/treatment/cancer-drugs/drugs/doxorubicin> (Last accessed: 21 Oct. 2023)
 58. Xu, Q., Zhao, J., Liu, Y., Pu, P., Wang, X., Chen, Y., Gao, C., Chen, J. & Zhou, H. Enhancing the luminescence of carbon dots by doping nitrogen element and its application in the detection of Fe(III). *J. Mater. Sci.* **50**, 2571–2576 (2015).

59. Zhang, Q., Xu, W., Han, C., Wang, X., Wang, Y., Li, Z., Wu, W. & Wu, M. Graphene structure boosts electron transfer of dual-metal doped carbon dots in photooxidation. *Carbon* **126**, 128–134 (2018).
60. Khodadadei, F., Safarian, S. and Ghanbari, N. Methotrexate-loaded nitrogen-doped graphene quantum dots nanocarriers as an efficient anticancer drug delivery system. *Materials Science and Engineering: C*. **79**, 280-285(2017).
61. Wang, S. A comparative first-principles study of orbital hybridization in two-dimensional C, Si, and Ge. *Phys. Chem. Chem. Phys. PCCP* **13**, 11929–11938 (2011).
62. Huan Yuan, Y., Sheng Li, R., Wang, Q., Lian Wu, Z., Wang, J., Liu, H. & Zhi Huang, C. Germanium-doped carbon dots as a new type of fluorescent probe for visualizing the dynamic invasions of mercury (ii) ions into cancer cells. *Nanoscale* **7**, 16841–16847 (2015).
63. Li, L., Ruan, T., Lyu, Y. & Wu, B. Advances in Effect of Germanium or Germanium Compounds on Animals—A Review. *J. Biosci. Med.* **05**, 56 (2017).
64. Mainwaring, M. G., Poor, C., Zander, D. S. & Harman, E. Complete remission of pulmonary spindle cell carcinoma after treatment with oral germanium sesquioxide. *Chest* **117**, 591–593 (2000).
65. Emanuele, A., Cailotto, S., Campalani, C., Branzi, L., Raviola, C., Ravelli, D., Cattaruzza, E., Trave, E., Benedetti, A., Selva, M. & Perosa, A. Precursor-Dependent Photocatalytic Activity of Carbon Dots. *Molecules* **25**, 101 (2020).
66. Goodman, S. Therapeutic effects of organic Germanium. *Med. Hypotheses* **26**, 207–215 (1988).
67. Neri, D. & Lerner, R. A. DNA-Encoded Chemical Libraries: A Selection System Based On Endowing Organic Compounds with Amplifiable Information. *Annu. Rev. Biochem.* **87**, null (2018).
68. Aina, O. H., Sroka, T. C., Chen, M.-L. & Lam, K. S. Therapeutic cancer targeting peptides. *Pept. Sci.* **66**, 184–199 (2002).
69. Claros, M. G. & Vincens, P. Computational Method to Predict Mitochondrially Imported Proteins and their Targeting Sequences. *Eur. J. Biochem.* **241**, 779–786 (1996).
70. Mansbach, R. A., Travers, T., McMahon, B. H., Fair, J. M. & Gnanakaran, S. Snails In Silico: A Review of Computational Studies on the Conopeptides. *Mar. Drugs* **17**, 145 (2019).

71. Schaduangrat, N., Nantasenamat, C., Prachayasittikul, V. & Shoombuatong, W. ACPred: A Computational Tool for the Prediction and Analysis of Anticancer Peptides. *Molecules* **24**, 1973 (2019).
72. Mulligan, V. K. The emerging role of computational design in peptide macrocycle drug discovery. *Expert Opin. Drug Discov.* **15**, 833–852 (2020).
73. Svensen, N., Walton, J. G. A. & Bradley, M. Peptides for cell-selective drug delivery. *Trends Pharmacol. Sci.* **33**, 186–192 (2012).
74. Liu, B. R., Lo, S.-Y., Liu, C.-C., Chyan, C.-L., Huang, Y.-W., Aronstam, R. S. & Lee, H.-J. Endocytic Trafficking of Nanoparticles Delivered by Cell-penetrating Peptides Comprised of Nona-arginine and a Penetration Accelerating Sequence. *PLOS ONE* **8**, e67100 (2013).
75. Takayama, K., Nakase, I., Michiue, H., Takeuchi, T., Tomizawa, K., Matsui, H. & Futaki, S. Enhanced intracellular delivery using arginine-rich peptides by the addition of penetration accelerating sequences (Pas). *J. Controlled Release* **138**, 128–133 (2009).
76. Woldetsadik, A. D., Vogel, M. C., Rabeh, W. M. & Magzoub, M. Hexokinase II–derived cell-penetrating peptide targets mitochondria and triggers apoptosis in cancer cells. *FASEB J.* **31**, 2168–2184 (2017).
77. Kristensen, M., Kucharz, K., Felipe Alves Fernandes, E., Strømgaard, K., Schallburg Nielsen, M., Cederberg Helms, H. C., Bach, A., Ulrikkaholm Tofte-Hansen, M., Irene Aldana Garcia, B., Lauritzen, M. & Brodin, B. Conjugation of Therapeutic PSD-95 Inhibitors to the Cell-Penetrating Peptide Tat Affects Blood–Brain Barrier Adherence, Uptake, and Permeation. *Pharmaceutics* **12**, 661 (2020).
78. Fahmy, T. M., Fong, P. M., Goyal, A. & Saltzman, W. M. Targeted for drug delivery. *Mater. Today* **8**, 18–26 (2005).
79. Browning, R. J., Reardon, P. J. T., Parhizkar, M., Pedley, R. B., Edirisinghe, M., Knowles, J. C. & Stride, E. Drug Delivery Strategies for Platinum-Based Chemotherapy. *ACS Nano* **11**, 8560–8578 (2017).
80. Ganipineni, L. P., Danhier, F. & Préat, V. Drug delivery challenges and future of chemotherapeutic nanomedicine for glioblastoma treatment. *J. Controlled Release* **281**, 42–57 (2018).
81. Zanuy, D., Flores-Ortega, A., Casanovas, J., Curco, D., Nussinov, R. & Aleman, C. The energy landscape of a selective tumor-homing pentapeptide. *J. Phys. Chem. B* **112**, 8692–8700 (2008).

82. Zhang, Y., Liu, G., Wei, J. & Nie, G. Platelet membrane-based and tumor-associated platelet-targeted drug delivery systems for cancer therapy. *Front. Med.* **12**, 667–677 (2018).
83. Battistini, L., Bugatti, K., Sartori, A., Curti, C. & Zanardi, F. RGD Peptide-Drug Conjugates as Effective Dual Targeting Platforms: Recent Advances. *Eur. J. Org. Chem.* **2021**, 2506–2528 (2021).
84. Low, P. S., Henne, W. A. & Doorneweerd, D. D. Discovery and Development of Folic-Acid-Based Receptor Targeting for Imaging and Therapy of Cancer and Inflammatory Diseases. *Acc. Chem. Res.* **41**, 120–129 (2008).
85. Yan, H., You, Y., Li, X., Liu, L., Guo, F., Zhang, Q., Liu, D., Tong, Y., Ding, S. & Wang, J. Preparation of RGD Peptide/Folate Acid Double-Targeted Mesoporous Silica Nanoparticles and Its Application in Human Breast Cancer MCF-7 Cells. *Front. Pharmacol.* **11**, (2020).
86. Kondo, E., Saito, K., Tashiro, Y., Kamide, K., Uno, S., Furuya, T., Mashita, M., Nakajima, K., Tsumuraya, T., Kobayashi, N., Nishibori, M., Tanimoto, M. & Matsushita, M. Tumour lineage-homing cell-penetrating peptides as anticancer molecular delivery systems. *Nat. Commun.* **3**, 951 (2012).
87. Alsina, J. & Albericio, F. Solid-phase synthesis of C-terminal modified peptides. *Pept. Sci.* **71**, 454–477 (2003).
88. Iwasaki, T., Ishibashi, J., Tanaka, H., Sato, M., Asaoka, A., Taylor, D. & Yamakawa, M. Selective cancer cell cytotoxicity of enantiomeric 9-mer peptides derived from beetle defensins depends on negatively charged phosphatidylserine on the cell surface. *Peptides* **30**, 660–668 (2009).
89. Myrberg, H., Zhang, L., Mäe, M. & Langel, Ü. Design of a Tumor-Homing Cell-Penetrating Peptide. *Bioconjug. Chem.* **19**, 70–75 (2008).
90. Tesaro, D., Accardo, A., Diaferia, C., Milano, V., Guillon, J., Ronga, L. & Rossi, F. Peptide-Based Drug-Delivery Systems in Biotechnological Applications: Recent Advances and Perspectives. *Molecules* **24**, 351 (2019).
91. Chakraborty, K., Biswas, A., Mishra, S., Mallick, A. M., Tripathi, A., Jan, S. & Sinha Roy, R. Harnessing Peptide-Functionalized Multivalent Gold Nanorods for Promoting Enhanced Gene Silencing and Managing Breast Cancer Metastasis. *ACS Appl. Bio Mater.* **6**, 458–472 (2023).

92. Pirsaeheb, M., Mohammadi, S., Salimi, A. & Payandeh, M. Functionalized fluorescent carbon nanostructures for targeted imaging of cancer cells: a review. *Microchim. Acta* **186**, 231 (2019).
93. Svensen, N., Walton, J. G. A. & Bradley, M. Peptides for cell-selective drug delivery. *Trends Pharmacol. Sci.* **33**, 186–192 (2012).
94. Sarkar, P., Ghosh, S. & Sarkar, K. Folic acid based carbon dot functionalized stearic acid-g-polyethyleneimine amphiphilic nanomicelle: Targeted drug delivery and imaging for triple negative breast cancer. *Colloids Surf. B Biointerfaces* **197**, 111382 (2021).
95. Wang, L., Li, C., Luo, Y. & Jiang, Z. Silver nanosol SERS quantitative analysis of ultratrace biotin coupled N-doped carbon dots catalytic amplification with affinity reaction. *Food Chem.* **317**, 126433 (2020).
96. Ning, X., Liu, D., Liu, S., Zhang, M. & Shen, H. Modification of Oligopeptides on Aspartic Acid or Lysine Residues by Solid-Phase Synthesis through On-Resin Side-Chain Conjugation. *Synlett* **29**, 2588–2594 (2018).
97. Yang, L., Jiang, W., Qiu, L., Jiang, X., Zuo, D., Wang, D. & Yang, L. One pot synthesis of highly luminescent polyethylene glycol anchored carbon dots functionalized with a nuclear localization signal peptide for cell nucleus imaging. *Nanoscale* **7**, 6104–6113 (2015).
98. Cao, Y., Dong, H., Yang, Z., Zhong, X., Chen, Y., Dai, W. & Zhang, X. Aptamer-Conjugated Graphene Quantum Dots/Porphyrin Derivative Theranostic Agent for Intracellular Cancer-Related MicroRNA Detection and Fluorescence-Guided Photothermal/Photodynamic Synergetic Therapy. *ACS Appl. Mater. Interfaces* **9**, 159–166 (2017).
99. Schroeder, K. L., Goreham, R. V. & Nann, T. Graphene Quantum Dots for Theranostics and Bioimaging. *Pharm. Res.* **33**, 2337–2357 (2016).
100. Wu, H., You, C., Chen, F., Jiao, J., Gao, Z., An, P., Sun, B. & Chen, R. Enhanced cellular uptake of near-infrared triggered targeted nanoparticles by cell-penetrating peptide TAT for combined chemo/photothermal/photodynamic therapy. *Mater. Sci. Eng. C* **103**, 109738 (2019).
101. Ming, F., Hou, J., Hou, C., Yang, M., Wang, X., Li, J., Huo, D. & He, Q. One-step synthesized fluorescent nitrogen doped carbon dots from thymidine for Cr (VI) detection in water. *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.* **222**, 117165 (2019).

102. Ruoslahti, E. Peptides as targeting elements and tissue penetration devices for nanoparticles. *Adv. Mater. Deerfield Beach Fla* **24**, 3747–3756 (2012).
103. Chen, B. B., Liu, M. L., Li, C. M. & Huang, C. Z. Fluorescent carbon dots functionalization. *Adv. Colloid Interface Sci.* **270**, 165–190 (2019).
104. Guo, X., Wang, C.-F., Yu, Z.-Y., Chen, L. & Chen, S. Facile access to versatile fluorescent carbon dots toward light-emitting diodes. *Chem. Commun.* **48**, 2692 (2012).
105. Tetsuka, H., Asahi, R., Nagoya, A., Okamoto, K., Tajima, I., Ohta, R. & Okamoto, A. Optically Tunable Amino-Functionalized Graphene Quantum Dots. *Adv. Mater.* **24**, 5333–5338 (2012).
106. Georgakilas, V., Tiwari, J. N., Kemp, K. C., Perman, J. A., Bourlinos, A. B., Kim, K. S. & Zboril, R. Noncovalent Functionalization of Graphene and Graphene Oxide for Energy Materials, Biosensing, Catalytic, and Biomedical Applications. *Chem. Rev.* **116**, 5464–5519 (2016).
107. Chen, R. J., Zhang, Y., Wang, D. & Dai, H. Noncovalent Sidewall Functionalization of Single-Walled Carbon Nanotubes for Protein Immobilization. *J. Am. Chem. Soc.* **123**, 3838–3839 (2001).
108. Tachi, S., Morita, H., Takahashi, M., Okabayashi, Y., Hosokai, T., Sugai, T. & Kuwahara, S. Quantum Yield Enhancement in Graphene Quantum Dots via Esterification with Benzyl Alcohol. *Sci. Rep.* **9**, 14115 (2019).
109. Zhang, Y., Liu, X., Fan, Y., Guo, X., Zhou, L., Lv, Y. & Lin, J. One-step microwave synthesis of N-doped hydroxyl-functionalized carbon dots with ultra-high fluorescence quantum yields. *Nanoscale* **8**, 15281–15287 (2016).
110. Mishra, L., Behera, R. K., Mondal, S., Panigrahi, A. & Sarangi, M. K. Surface Charge Alteration in Carbon Dots Governs the Interfacial Electron Transfer and Transport. *J. Phys. Chem. C* **125**, 23398–23408 (2021).
111. Guo, J., Gao, X., Su, L., Xia, H., Gu, G., Pang, Z., Jiang, X., Yao, L., Chen, J. & Chen, H. Aptamer-functionalized PEG–PLGA nanoparticles for enhanced anti-glioma drug delivery. *Biomaterials* **32**, 8010–8020 (2011).
112. Wu, Y.-F., Wu, H.-C., Kuan, C.-H., Lin, C.-J., Wang, L.-W., Chang, C.-W. & Wang, T.-W. Multi-functionalized carbon dots as theranostic nanoagent for gene delivery in lung cancer therapy. *Sci. Rep.* **6**, 21170 (2016).
113. Kong, T., Hao, L., Wei, Y., Cai, X. & Zhu, B. Doxorubicin conjugated carbon dots as a drug delivery system for human breast cancer therapy. *Cell Prolif.* **51**, e12488 (2018).

114. Zeng, Q., Shao, D., He, X., Ren, Z., Ji, W., Shan, C., Qu, S., Li, J., Chen, L. & Li, Q. Carbon dots as a trackable drug delivery carrier for localized cancer therapy *in vivo*. *J. Mater. Chem. B* **4**, 5119–5126 (2016).
115. Ding, C. & Li, Z. A review of drug release mechanisms from nanocarrier systems. *Mater. Sci. Eng. C* **76**, 1440–1453 (2017).
116. Scicluna, M. C. & Vella-Zarb, L. Evolution of Nanocarrier Drug-Delivery Systems and Recent Advancements in Covalent Organic Framework–Drug Systems. *ACS Appl. Nano Mater.* (2020). doi:10.1021/acsanm.9b02603
117. Kaminskas, L. M., McLeod, V. M., Porter, C. J. H. & Boyd, B. J. Association of Chemotherapeutic Drugs with Dendrimer Nanocarriers: An Assessment of the Merits of Covalent Conjugation Compared to Noncovalent Encapsulation. *Mol. Pharm.* **9**, 355–373 (2012).
118. Wang, C., Yan, Q., Liu, H.-B., Zhou, X.-H. & Xiao, S.-J. Different EDC/NHS Activation Mechanisms between PAA and PMAA Brushes and the Following Amidation Reactions. *Langmuir* **27**, 12058–12068 (2011).
119. Keleştemur, S., Altunbek, M. & Culha, M. Influence of EDC/NHS coupling chemistry on stability and cytotoxicity of ZnO nanoparticles modified with proteins. *Appl. Surf. Sci.* **403**, 455–463 (2017).
120. Dada, S. N., Babanyinah, G. K., Tetteh, M. T., Palau, V. E., Walls, Z. F., Krishnan, K., Croft, Z., Khan, A. U., Liu, G., Wiese, T. E., Glotser, E. & Mei, H. Covalent and Noncovalent Loading of Doxorubicin by Folic Acid-Carbon Dot Nanoparticles for Cancer Theranostics. *ACS Omega* **7**, 23322–23331 (2022).
121. Yan, F., Jiang, Y., Sun, X., Bai, Z., Zhang, Y. & Zhou, X. Surface modification and chemical functionalization of carbon dots: a review. *Microchim. Acta* **185**, 424 (2018).
122. Liu, B. R., Winiarz, J. G., Moon, J.-S., Lo, S.-Y., Huang, Y.-W., Aronstam, R. S. & Lee, H.-J. Synthesis, characterization and applications of carboxylated and polyethylene-glycolated bifunctionalized InP/ZnS quantum dots in cellular internalization mediated by cell-penetrating peptides. *Colloids Surf. B Biointerfaces* **111**, 162–170 (2013).
123. Su, Y. & Sinko, P. J. Drug delivery across the blood–brain barrier: why is it difficult? how to measure and improve it? *Expert Opin. Drug Deliv.* **3**, 419–435 (2006).
124. Li, S., Peng, Z., Dallman, J., Baker, J., Othman, A. M., Blackwelder, P. L. & Leblanc, R. M. Crossing the blood–brain–barrier with transferrin conjugated carbon dots: A zebrafish model study. *Colloids Surf. B Biointerfaces* **145**, 251–256 (2016).

125. Ullah, Z., Kim, H. J., Mary, Y. S., Zhan, X. & Kwon, H. W. Adsorption of Pyrimidin-2-amine (PA) on Graphene Quantum Dots (GQDs): Non-covalent Interaction Study. *J. Mol. Liq.* **368**, 120654 (2022).
126. Luo, Y.-C., Zhang, H.-H., Wang, Y. & Xu, P.-F. Synthesis of α -Amino Acids Based on Chiral Tricycloiminolactone Derived from Natural (+)-Camphor. *Acc. Chem. Res.* **43**, 1317–1330 (2010).
127. Tverdislov, V. A. & Malyshko, E. V. On regularities in the spontaneous formation of structural hierarchies in chiral systems of nonliving and living matter. *Phys.-Uspekhi* **62**, 354 (2019).
128. Carmona, G., Rodriguez, A., Juarez, D., Corzo, G. & Villegas, E. Improved Protease Stability of the Antimicrobial Peptide Pin2 Substituted with d-Amino Acids. *Protein J.* **32**, 456–466 (2013).
129. Li, C. M., Haratipour, P., Lingeman, R. G., Perry, J. J. P., Gu, L., Hickey, R. J. & Malkas, L. H. Novel Peptide Therapeutic Approaches for Cancer Treatment. *Cells* **10**, 2908 (2021).
130. Aliashkevich, A., Alvarez, L. & Cava, F. New Insights Into the Mechanisms and Biological Roles of D-Amino Acids in Complex Eco-Systems. *Front. Microbiol.* **9**, (2018).
131. Dennison, S. R. & Phoenix, D. A. Influence of C-Terminal Amidation on the Efficacy of Modelin-5. *Biochemistry* **50**, 1514–1523 (2011).
132. Fairman, R., Shoemaker, K. R., York, E. J., Stewart, J. M. & Baldwin, R. L. Further studies of the helix dipole model: Effects of a free α -NH₃⁺ or α -COO[−] group on helix stability. *Proteins Struct. Funct. Bioinforma.* **5**, 1–7 (1989).
133. Nguyen, L. T., Chau, J. K., Perry, N. A., Boer, L. de, Zaat, S. A. J. & Vogel, H. J. Serum Stabilities of Short Tryptophan- and Arginine-Rich Antimicrobial Peptide Analogs. *PLOS ONE* **5**, e12684 (2010).
134. Arispe, N., Diaz, J. C. & Flora, M. Efficiency of Histidine-Associating Compounds for Blocking the Alzheimer's A β Channel Activity and Cytotoxicity. *Biophys. J.* **95**, 4879–4889 (2008).
135. Song, K.-H., Kim, S.-B., Shim, C.-K., Chung, S.-J., Kim, D.-D., Rhee, S.-K., Choi, G. J., Kim, C.-H. & Kim, K. Paracellular permeation-enhancing effect of AT1002 C-terminal amidation in nasal delivery. *Drug Des. Devel. Ther.* **9**, 1815–1823 (2015).

136. Soleymani-Goloujeh, M., Nokhodchi, A., Niazi, M., Najafi-Hajivar, S., Shahbazi-Mojarrad, J., Zarghami, N., Zakeri-Milani, P., Mohammadi, A., Karimi, M. & Valizadeh, H. Effects of N-terminal and C-terminal modification on cytotoxicity and cellular uptake of amphiphilic cell penetrating peptides. *Artif. Cells Nanomedicine Biotechnol.* **46**, 91–103 (2018).
137. Chopineau, J., Robert, S., Fénart, L., Cecchelli, R., Lagoutte, B., Paitier, S., Dehouck, M.-P. & Domurado, D. Monoacylation of ribonuclease A enables its transport across an in vitro model of the blood–brain barrier. *J. Controlled Release* **56**, 231–237 (1998).
138. León-Buitimea, A., Garza-Cárdenas, C. R., Garza-Cervantes, J. A., Lerma-Escalera, J. A. & Morones-Ramírez, J. R. The Demand for New Antibiotics: Antimicrobial Peptides, Nanoparticles, and Combinatorial Therapies as Future Strategies in Antibacterial Agent Design. *Front. Microbiol.* **11**, (2020).
139. Jiang, H., Chen, W., Wang, J. & Zhang, R. Selective N-terminal modification of peptides and proteins: Recent progresses and applications. *Chin. Chem. Lett.* **33**, 80–88 (2022).
140. Pathan, I. B. & Setty, C. M. Chemical Penetration Enhancers for Transdermal Drug Delivery Systems. *Trop. J. Pharm. Res.* **8**, (2009).
141. Lane, M. E. Skin penetration enhancers. *Int. J. Pharm.* **447**, 12–21 (2013).
142. Kováčik, A., Kopečná, M. & Vávrová, K. Permeation enhancers in transdermal drug delivery: benefits and limitations. *Expert Opin. Drug Deliv.* **17**, 145–155 (2020).
143. Chen, J., Bian, J., Hantash, B. M., Albakr, L., Hibbs, D. E., Xiang, X., Xie, P., Wu, C. & Kang, L. Enhanced skin retention and permeation of a novel peptide via structural modification, chemical enhancement, and microneedles. *Int. J. Pharm.* **606**, 120868 (2021).
144. Jariwala, N., Ozols, M., Bell, M., Bradley, E., Gilmore, A., DeBelle, L. & Sherratt, M. J. Matrikines as mediators of tissue remodelling. *Adv. Drug Deliv. Rev.* **185**, 114240 (2022).
145. Begley, D. J. Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. *Pharmacol. Ther.* **104**, 29–45 (2004).
146. Eskandari, S., Guerin, T., Toth, I. & Stephenson, R. J. Recent advances in self-assembled peptides: Implications for targeted drug delivery and vaccine engineering. *Adv. Drug Deliv. Rev.* **110–111**, 169–187 (2017).

147. Pai, S. S., Hammouda, B., Hong, K., Pozzo, D. C., Przybycien, T. M. & Tilton, R. D. The Conformation of the Poly(ethylene glycol) Chain in Mono-PEGylated Lysozyme and Mono-PEGylated Human Growth Hormone. *Bioconjug. Chem.* **22**, 2317–2323 (2011).
148. Esposito, P., Barbero, L., Caccia, P., Caliceti, P., D'Antonio, M., Piquet, G. & Veronese, F. M. PEGylation of growth hormone-releasing hormone (GRF) analogues. *Adv. Drug Deliv. Rev.* **55**, 1279–1291 (2003).
149. Lamanna, G., Grillaud, M., Macri, C., Chaloin, O., Muller, S. & Bianco, A. Adamantane-based dendrons for trimerization of the therapeutic P140 peptide. *Biomaterials* **35**, 7553–7561 (2014).
150. Grillaud, M. & Bianco, A. Multifunctional adamantane derivatives as new scaffolds for the multipresentation of bioactive peptides. *J. Pept. Sci.* **21**, 330–345 (2015).
151. Li, B., Wanka, L., Blanchard, J., Liu, F., Chohan, M. O., Iqbal, K. & Grundke-Iqbal, I. Neurotrophic peptides incorporating adamantane improve learning and memory, promote neurogenesis and synaptic plasticity in mice. *FEBS Lett.* **584**, 3359–3365 (2010).
152. McKinney, J. D., Richard, A., Waller, C., Newman, M. C. & Gerberick, F. The Practice of Structure Activity Relationships (SAR) in Toxicology. *Toxicol. Sci.* **56**, 8–17 (2000).
153. Bemis, G. W. & Murcko, M. A. The Properties of Known Drugs. 1. Molecular Frameworks. *J. Med. Chem.* **39**, 2887–2893 (1996).
154. Velkov, T., Thompson, P. E., Nation, R. L. & Li, J. Structure–Activity Relationships of Polymyxin Antibiotics. *J. Med. Chem.* **53**, 1898–1916 (2010).

3. Physicochemical properties of in situ microwave-assisted germanium oxide modified nitrogen-doped carbon dots

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3.1 Abstract

Preparation of carbon dots (CDs) and nitrogen-doped carbon dots (NCDs) with germanium oxide (GeO_2/GeO) using a facile one-step microwave-assisted synthesis procedure is presented. Iso-ascorbic acid, urea and Ge-132 were used as carbon/nitrogen and germanium sources, respectively. Studies have shown that the CDs, NCDs and GeO_2/GeO nanoparticles individually have excellent qualities for bio-imaging applications including photoluminescence, biocompatibility, good stability and low toxicity. While CDs have been thoroughly explored in literature, CDs with germanium oxide on their surface are an avenue that has scarce information. Hence the synthesis and physicochemical characterizations of CDs- /NCDs- GeO_2/GeO composites were studied using different microscopic and spectroscopic techniques to explore their compatibility with bio-imaging applications. In the presence of Ge, the approximate particle sizes of both the CDs and NCDs changed from 2.5 nm to 5.8 nm and the spherical or quasi-spherical shape was maintained. The X-ray photoelectron spectroscopy survey spectrum showed that the surface of *Iso-N-Ge-CDs* contained nitrogen and germanium, indicating the successful synthesis of nitrogen-doped carbon dots (NCDs) with germanium oxide (GeO_2/GeO). Photoluminescence emissions of longer wavelengths suitable for cell imaging were observed for *Iso-N-Ge-CDs* (350 nm) and *Iso-Ge-CDs* (430 nm) and the *Iso-N-Ge-CDs* are able to fluoresce under the red and blue wavelengths. The *Ge-NCDs* demonstrated excitation-dependent emission wavelength behaviour, pH sensitivity and Fe^{3+} sensitivity which may be used as a probe for Fe^{3+} metal ions and intracellular pH measurements.

Keywords: nitrogen-doped carbon dots; germanium; photoluminescence; wavelength excitation-dependence; bio-imaging.

3.2 Introduction

Traditionally, carbon dots (CDs) are classified as nanoparticles that are generally spherical or quasi-spherical possessing a diameter that is 10 nm or smaller.¹⁻³ There are four classes of CDs known thus far, these are carbonized polymer dots (CPDs), graphene quantum dots (GQDs), and carbon quantum dots (CQDs).^{4,5} CDs are considered the most promising allotrope of carbon for drug delivery, bioimaging, biosensors, tissue engineering and cancer therapy. This is due to their versatile physicochemical properties including good water solubility, superior quantum yields, low cytotoxicity, favorable biocompatibility, good photostability, high sensitivity, great cell membrane permeability, tunable fluorescence emission and excitation, and selectivity for target analytes.^{6, 7} These qualities make them far better candidates for aforementioned biomedical applications compared to other carbon allotropes and metal-based quantum dots. For example, to activate the fluorescent qualities of carbon nanotubes (CNTs) and graphene, further modification of their structures and surface chemistry is required, whereas the as-prepared CDs already possess these fluorescent qualities without any modification. On the other hand, metal-based quantum dots are traditionally synthesized from heavy metals that may be toxic, however, CDs have shown to overcome these limitations.⁸ Additionally, the synthesis of CDs is facile and can be fabricated using low energy, and a broad variety of carbon sources can be exploited including waste such as banana waste, peanut shell and rose petal, carbohydrates, polymers and biomass.⁸ As such, there has been an exponential growth in the number of CD publications in biomedical applications over the past few years.⁹

Another important quality of the CDs that makes them attractive is the fact that they allow structure and interface modification which enhance their features for a specific application. This is done by doping the carbon lattice with foreign atoms such as nitrogen. This has shown significant improvement in the electrical dispersion and appropriate electronic energy levels of CDs and thereby improving their fluorescent and other features.¹⁰ For example, Wang *et al.* synthesized CDs plus nitrogen-doped CDs (or NCDs) with blue fluorescence, and utilized them to enhance the light-harvesting qualities during the plant's photosynthesis process.¹¹ The authors reported that the NCDs had better light conversion and electron donor features which

contributed to the promotion of the photoelectron transfer rate. Liao *et al.* synthesized NCDs with a high fluorescence and higher quantum yield (28.46 %) compared to undoped CDs (quantum yield = 5.3 %).¹² Additionally, the NCDs showed quick and extreme sensitivity while also having a selective fluorescence quenching effect toward Au³⁺ ions.

Composites of CDs with metal atoms also form nanomaterials with attractive features for various applications.¹³ For example, Lou *et al.*, were able to produce a highly efficient photocatalyst for hydrogen generation using Pt-NCDs.¹⁴ The catalyst synthesis strategies also showed great potential for electrocatalysis, heterogeneous catalysis and drug delivery applications. In another study, Safardoust-Hojaghan and Salavati-Niasari prepared composites of NCDs with TiO₂ and showed that by introducing the NCDs nanoparticles, photodegradation efficacy of TiO₂ to methylene blue under UV light (400 W UV tube) is increased from 40% to 85%.¹⁵ Li *et al.*, showed that Mg- and N-codoped CDs have enhanced photoluminescence traits compared to CDs alone.¹⁶ Furthermore, biocompatibility, superior photostability and reduced cytotoxicity are some of the properties of the Mg- and N-codoped CDs which showed great potential for bioimaging applications. Indeed, studies show that there has been a growing interest in these metal-CDs nanocomposites, especially in clean energy research.¹⁷

Other interesting composites of CDs and metal to study for biomedical applications are the CDs and germanium (Ge)/germanium oxide (GeO₂/GeO) composites. This is because both CDs and Ge have excellent features for biomedical applications. Like the CDs, Ge nanoparticles also possess quantum confinement, a broader optical range and powerful visible photoluminescence.¹⁸ Germanium is a member of group 4 elements. It is a non-metal similar to C and Si, and it is believed that it may feasibly have sp³-like bonding which may also impact the electronic and structural features of carbon-based material but in a unique way compared with other dopants such as N or S.¹⁹ Germanium is a semi-metal that is mostly used as a semiconductor. Elemental germanium is similar in appearance to pure silicon, and it readily forms complexes when reacted with oxygen. A trace amount of germanium can be found naturally in food such as broccoli, celery, garlic and ginseng but it is not an essential mineral.^{20–23} Germanium oxide nanoparticle is also reported to have both photoluminescent as well as antimicrobial traits.^{24,25} Anticancer activity of synthetic organo-germanium compounds such as bis-carboxyethyl germanium sesquioxide or Ge132, has several biological applications and is reported in numerous cancer cell models and clinical trials due

to their antioxidant features.²⁶ Other widely exploited metal-containing anticancer pharmaceuticals including cisplatin may be circumvented by organogermanium compounds such as Ge-132 that enhance pro-inflammatory responses by boosting interferon- γ (IFN- γ), natural killer cell and T-cell activity, and are substantially less harmful. Therefore, great synergy and enhanced photoluminescence, higher biostability, higher activity and selectivity can be expected. This could potentially broaden the application of both CDs and Ge.

Only a few journal articles have reported on the synthesis of CDs-Ge/GeO₂ composites. However according to literature, they are biocompatible and have low toxicity. Yuan *et al.* reported that the Ge-CDs composite for the detection of mercury ions in cancer cells which demonstrated that bio-application is feasible for these compounds.²⁷ The composite exhibited a blue-green luminescence, excitation-dependent photoluminescence traits, as well as a high sensitivity towards mercury ions. Another study by Yu-Cing Juho *et al.* evaluated the improvement of blood circulation and sexual satisfaction using a unique FIR fiber called Ge-Ti-textile made of germanium, titanium, and various polymers.²⁸ After three months of therapy, the Ge-Ti-underpants treatment considerably raised the average penile blood flow velocity and enhanced sexual function, according to the findings. On the other hand, in a different study, the activity of composites of CDs-Ge-GeO₂ was investigated for the photocatalytic degradation of pendimethalin and dinotefuran pesticides.²⁹ It was found that the CDs-Ge-GeO₂ can degrade ~100% of both pendimethalin and dinotefuran pesticides under visible light illumination (>440 nm) in less than 30 min. This high photocatalytic degradation was ascribed to the synergy between the composite of the components. To enlarge the scope of these CDs-Ge/GeO₂ composites in practical applications, this study reports on a simple microwave reaction for the synthesis of germanium oxide-modified NCDs. Ge-NCDs produced were fluorescent, water-soluble, and emitted different colours due to the different sizes. To the best of our knowledge, the effect of both germanium and nitrogen on the photoluminescent traits of CDs has not been reported. We found that the CDs modified with germanium oxide have photoluminescent traits which are crucial for bio-imaging and are sensitive towards Fe³⁺.

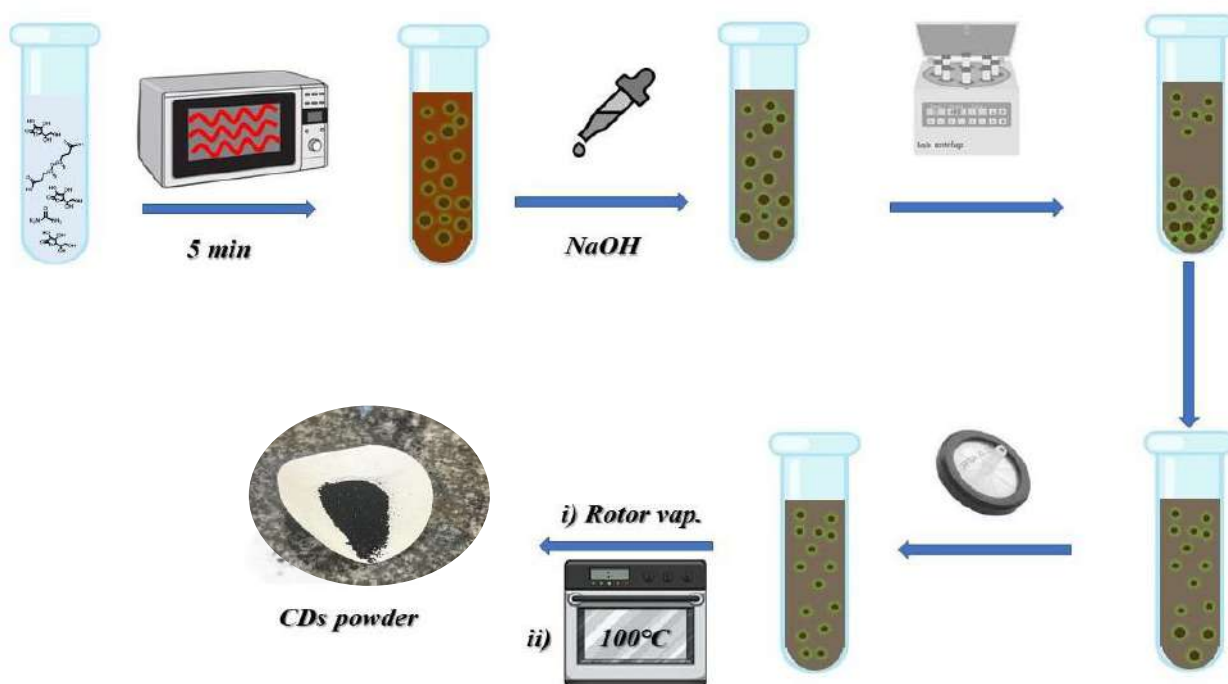
3.3 Experimental

3.3.1 Materials

Iso-ascorbic acid ($C_6H_8O_6$), urea (CH_4N_2O) and bis (2-carboxyethyl germanium (IV) sesquioxide) (Ge-132) were all purchased from Sigma Aldrich, South Africa, and used as received. Distilled water and hydrochloric acid (HCl 32 %, Sigma Aldrich, South Africa) were utilized as solvents in the experiments.

3.3.2 Synthesis of various carbon dots

Carbon dots (CDs) and carbon dots composites were synthesized using previously reported iso-ascorbic acid and urea-derived CDs technique but with modifications^{30, 31}. The schematic representation of the CDs preparation is summarized in **Scheme 3.1** below. The experiments were carried out in a CEM Discovery SP microwave operating under closed vessel conditions at a maximum power and pressure output of 150 W and 250 psi, respectively. Before the reaction, the precursors were dispersed in 8.0 mL distilled water and 2 mL 0.1 M HCl until a homogenous mixture was formed. Thereafter, the mixture was transferred into a 30 mL microwave reaction vessel, placed in the microwave oven, and irradiated at 150 W for 5 min until an infrared temperature of 125 °C was reached. After the reaction, it was observed that the reaction mixture changed colour from clear to black-brown, which symbolized carbonization.³⁰ The reaction vessel was allowed to cool down to room temperature before the acid was neutralized by adding 0.1 M NaOH. The resulting solution was purified by centrifugation at 14000 rpm for 20 min to separate the supernatant. The supernatant was then filtered using a hydrophilic 0.22 μ m nylon syringe filter membrane. Finally, a rotary evaporator was then employed to evaporate the water from the CDs solution. The obtained product was allowed to dry in an oven at 100 °C overnight. The quantity of precursors used in each reaction as well as the product yields are summarized in **Table 3.1** below. The products were named *Iso*-CDs, *Iso-Ge*-CDs, *Iso-N*-CDs and *Iso-N-Ge*-CDs depending on the precursors utilized in each experiment, as explained in **Table 3.1**.



Scheme 3.1 A simple illustration of the fabrication process for the various CDs and Ge-CDs composites presented in this work.

Table 3.1 CDs microwave-assisted synthesis conditions

Product name	Precursor amount (g)			Final product	
	Iso-ascorbic acid	Ge132	Urea	Mass (g)	% Yield
Iso-CDs	2.00	-	-	0.19	9.5
Iso-Ge- CDs	2.00	0.50	-	1.16	47
Iso-N- CDs	2.00	-	0.50	1.2	48
Iso-N-Ge-CDs	2.00	0.50	0.50	0.95	38

“-”= this precursor was not used for that particular experiment.

3.4 Characterization techniques

The morphology of the synthesized CDs and modified CDs was probed using transmission electron microscopy (TEM) which uses a FEI Technai G2 Spirit electron microscope operated at 120 kV. The particle size distribution was analysed using the ImageJ software.³² A Bruker D2 phaser with Cu-K α radiation ($\lambda=1.5405$ Å) was employed to determine the powder X-ray diffraction (PXRD) patterns of the samples. The data was collected from 10° to 70° 2 θ . The Agilent Technologies Cary Series UV-Vis spectrophotometer was used, acquiring data using an absorption spectrum with a range from 200 to 800 nm and varying at an excitation wavelength of 245 nm to 288 nm. The Photoluminescence (PL) spectroscopy data were obtained from Varian Cary Eclipse EL04103870 fluorescence spectrophotometer at a range between 320 and 380 nm, by exciting the sample solutions at 300 nm to 400 nm wavelength. The analysis of the functional groups on the CDs was explored using a Bruker Tensor 27 FTIR analyser. The X-ray photoelectron spectroscopy (XPS) data was collected using a quantum 2000 scanning ESCA lab 250Xi microprobe system operated at 300 W with monochromatic Al K α (1486.7 eV). The images were captured using the Olympus BX63 Fluorescence Microscope (OFM). The processing of the captured images was conducted on Fiji ImageJ software.

3.5 Results and discussion

3.5.1 Morphological characterization

Carbon dots (CDs) were synthesized using a microwave-assisted technique with commonly used precursors for CDs synthesis such as iso-ascorbic acid as a carbon source and urea for nitrogen doping.³¹ However, for the reactions reported here, HCl was utilized as one of the solvents to help accelerate the formation of the CDs during the reaction.³³ Typically, microwave-assisted irradiation of ascorbic acid produces CDs with approximate average particle sizes of 2 to 3 nm when iso-ascorbic acid is irradiated at microwave powers between 600 and 900W for ≥ 5 min.^{30, 31} Herein, it is shown that CDs from iso-ascorbic acid, with average sizes of 3.2 ± 1.2 nm and an average size ranging between 2.0 and 8.0 nm (**Figure 3.1a**) and the corresponding Ge-modified (**Figure 3.1b**), can be produced under reduced temperature, power and reaction time by simply adding HCl to the reaction mixture. Likewise, nitrogen-doped CDs synthesized from equal amounts of iso ascorbic acid and urea,

when irradiated at 600W for 6 min were produced with particle sizes of ~ 4 nm.³⁴ The synthesis of CDs is traditionally in harsh conditions however under these milder reaction conditions mentioned in this work, the average particle sizes were 2.2 ± 1.88 nm, with an approximate size range between 1.0 nm and 5.5 nm as seen in **Figure 3.1c**. Other studies have shown that machine-learning-based techniques can be used to further fine-tune the microwave-assisted reaction for optimum CDs productions (i.e., production of CDs with high % yield, quantum yield and other properties).³⁵

The incorporation of germanium oxide (GeO_2/GeO) led to a slight enlargement in the CDs particle sizes as shown by the size distribution graphs in **Figure 3.1b**) and **3.d**). For example, the *Iso-Ge-N-CD*'s approximate average particle sizes were 5.8 ± 0.78 nm **Figure 3.1d**), which is ~ 3 nm larger than the *Iso-N-CDs* (i.e., without GeO_2/GeO modification). The enlargement in the CDs particle sizes may be ascribed to the large sizes of Ge atomic radius (211 pm) on the carbon lattices or surface functionalization, which will be further discussed in the XPS analysis. The quasi-spherical morphology of the CDs did not change when germanium was incorporated in both *Iso-Ge-CDs* and *Iso-Ge-N-CDs*. High-resolution TEM images were taken to determine the lattice fringes of the prepared samples (if any), and the results obtained from each sample are inserted on images in **Figure 3.1**. The lattice d -spacing of *Iso-CDs* was found to be 0.18 nm corresponding to the (004) plane of a graphitic structure (insert **Figure 3.1a** and supplementary information **Figure S3.1**).³⁶ Surprisingly, the *Iso-Ge-CDs* sample did not show any lattices even at higher resolution (insert **Figure 3.1b** and **S3.2**), which implies that these nanoparticles are highly amorphous compared to the other samples. *Iso-N-CDs* and *Iso-N-Ge-CDs* had lattice d -spacing of 0.21 nm, corresponding to the (100) plane lattice of graphite as seen in insert **Figure 3.1c, 3.1d** and **Figures S3.3 and S3.4**.³⁷ The d -spacing of the CDs were consistent with the literature,³⁸ the slight changes in size may be attributed to doping with nitrogen and germanium. Moreover, in order to comprehend structural morphology, quantify qualities, measure particle size distributions, validate findings, and conduct comparative analysis³⁹, a number of TEM images were obtained for the CDs and are presented in **Figures S3.5 – S3.8** for *Iso-CDs*, *Iso-Ge-CDs*, *Iso-N-CDs* and *Iso-N-Ge-CDs* respectively.

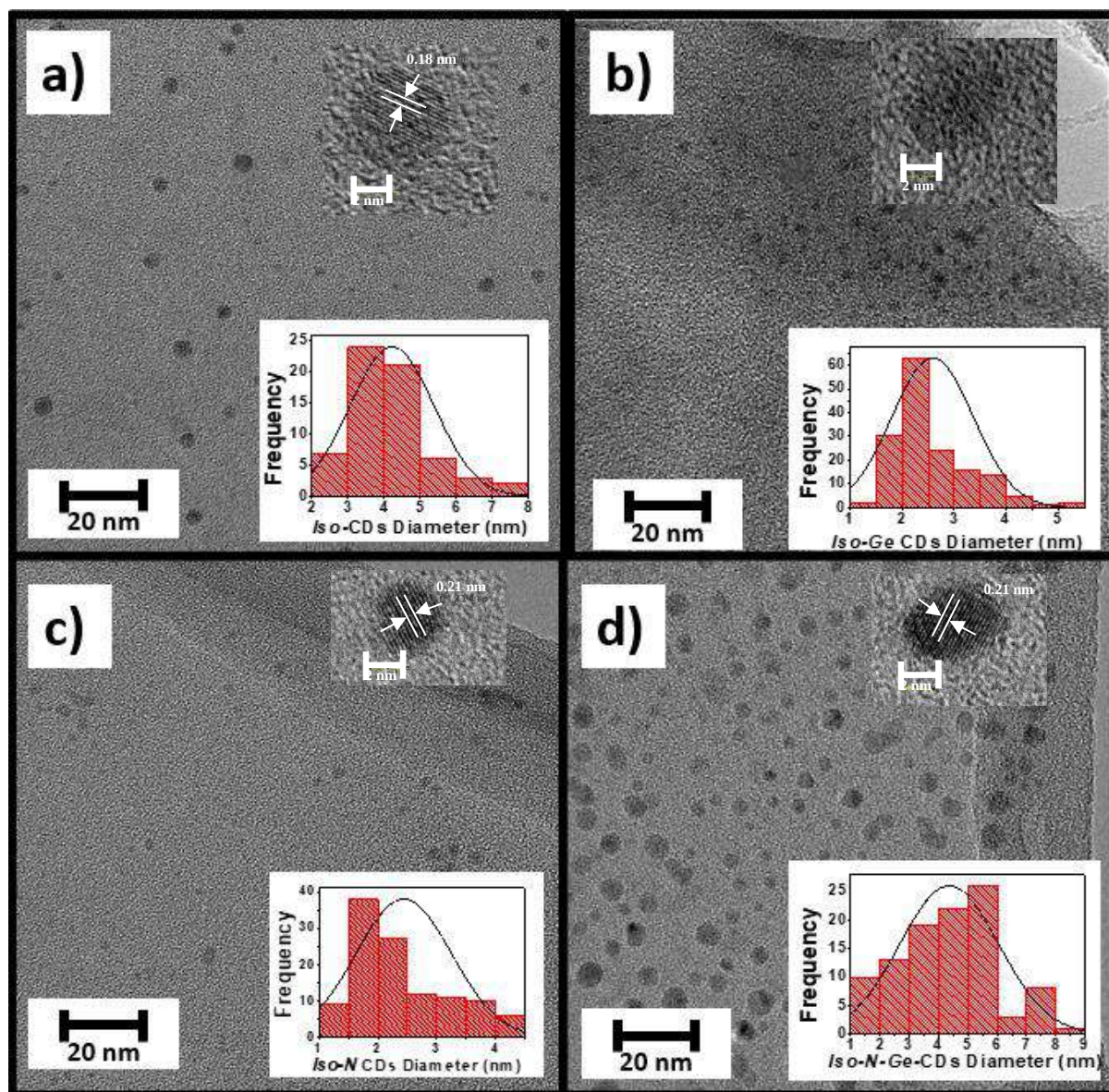


Figure 3.1 TEM micrographs and (inserted) size distribution curves of (a) Iso-CDs, (b) Iso-Ge-CDs, (c) Iso-N-CDs and (d) Iso-N-Ge-CDs.

3.5.2 Chemical properties

Figure 3.2 below displays the FT-IR spectra of Iso-CDs, Iso-Ge-CDs, Iso-N-CDs and Iso-N-Ge-CDs. The absorption peaks of all the samples showed a broad intense absorption band at approximately 3247 cm^{-1} indicating the existence of -O-H or O-H/N-H for N-doped CDs. The peak at approximately 2939 cm^{-1} was due to the C-H stretching vibrations and the peak at approximately 1747 cm^{-1} and 1606 cm^{-1} can be ascribed to C=O stretching vibrations and C=C stretching vibrations, respectively. The characteristic absorption band of C-N stretching at approximately 1392 cm^{-1} and the absorption band at approximately 1053 cm^{-1} is ascribed

to C–O–C stretching vibrations. Interestingly, the Ge-containing samples showed a significant reduction in the C=C bands, which could imply that the incorporation of Ge somehow reduces the graphitic nature of the CDs.

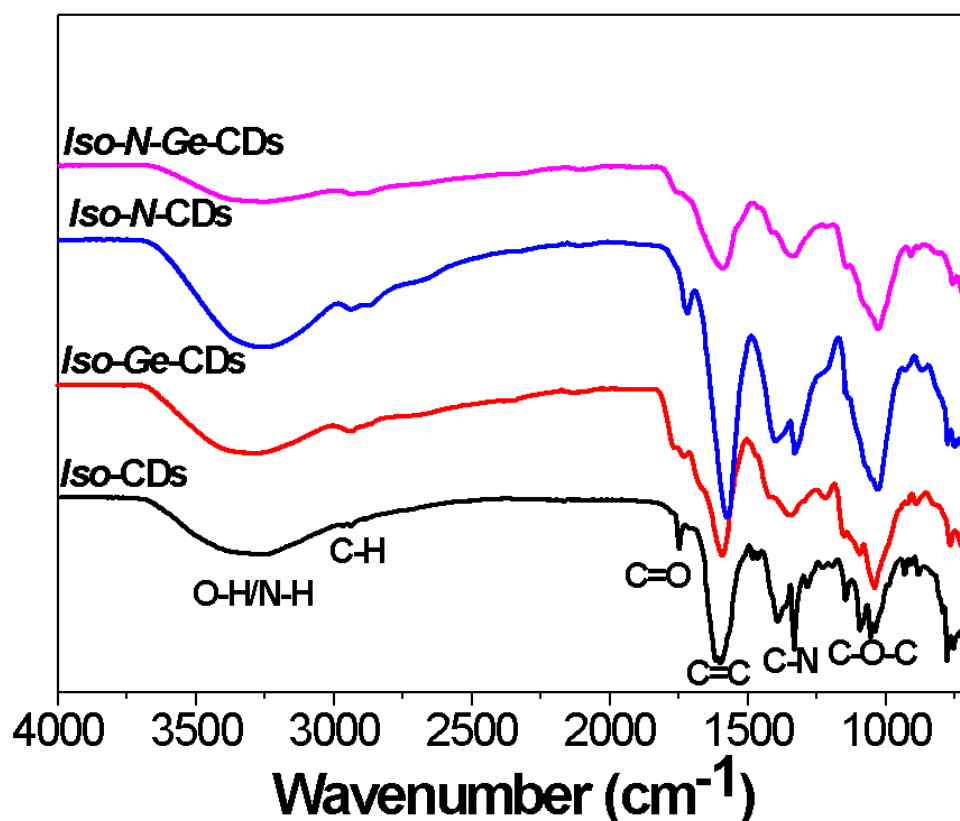


Figure 3.2 FT-IR spectra of (a) Iso-CDs, (b) Iso-N-CDs, (c) Iso-N-Ge-CDs and (d) Iso-N-Ge-CDs.

XPS analysis was further conducted to study the surface elemental analysis of the doped and Ge modified CDs. The XPS survey spectrum comparison of Iso-CDs and Iso-N-Ge-CDs shown in **Figure 3.3a**) clearly shows the carbon (C1s) and oxygen (O1s) surface functionalities of Iso-CDs (in black), versus the Iso-N-Ge-CDs (in blue) which further contains nitrogen (N1s) and germanium (Ge2p3). The relative abundance of C1s, O1s, N1s and Ge2p3 for Iso-N-Ge-CDs were estimated to be 55.4, 25.9, 15.9 and 2.8 w%, respectively, as summarized in **Table S3.1**.⁴⁰ The Iso-N-Ge-CDs expanded XPS spectra of the C1s (**Figure 3.3b**) showed peaks at 284.7, 286.0, 287.9 and 288.7 eV, matching the sp^2 C-C and/or N-C, sp^3 C-C/N-C, C=O, and O-C=O and/or N-C=O bonds, respectively.³⁴ The O1s peak

deconvolution (**Figure S3.9**) revealed the presence of O-C as well as the O-C=O and/or N-C=O bonds at binding energies of 531.5 and 532.7 eV, respectively. N1s spectrum (**Figure 3.3c**) revealed the presence of graphitic-N (399.6 eV) and NO_x (401.3 eV).³⁴ Finally, the Ge2p3 spectrum (**Figure 3.3d**) showed a peak at 1219.6 eV, which is evident in the presence of Ge=O and Ge-O.⁴¹ This data suggest that nitrogen was successfully incorporated into the carbon lattice as proven by the presence of graphitic-N, however, the absence of a Ge-C bond suggests that there were no bonds formed between the core of CDs and germanium. The XPS data of *Iso*-CDs (**Figure S3.10**), *Iso-Ge*-CDs (**Figure S3.11**) and *Iso-Ge*-CDs (**Figure S3.12**) showed the expanded survey scan consistent with literature.^{27, 41}

The atomic abundance of carbon in *Iso*-CDs, *Iso-Ge*-CDs, *Iso-N*-CDs and *Iso-N-Ge*-CDs, was found to be 64.6%, 63.4%, 58.9% and 55.4% respectively (**Table S3.1, S3.3, S3.5, and S3.7**). This data illustrated that when nitrogen or germanium is incorporated in the sample the abundance of carbon is reduced. As a result, *Iso-N-Ge*-CDs which contain both nitrogen and germanium has the lowest carbon content. Additionally, the incorporation of nitrogen affected the atomic abundance of carbon more significantly. This may be attributed to the fact that nitrogen replaces some of the graphitic carbons in addition to the surface functional groups as seen in the breakdown of C, N, O and Ge species found in *Iso-N-Ge*-CDs in **Table S2** and species breakdown of C, N, and O *Iso-N*-CDs **Table S3.8** found in the supplementary information. The comparison of the atomic species between *Iso*-CDs and *Iso-Ge*-CDs seen in the supplementary information **Table S3.4** and **S3.6** respectively demonstrated that germanium only affected the surface functional groups since no Ge-C bond were detected.

Interestingly, the CDs containing nitrogen (*Iso-N*-CDs and *Iso-N-Ge*-CDs) had a higher C-O content compared to the CDs which did not contain nitrogen (*Iso*-CDs and *Iso-Ge*-CDs) and a higher content of C=O was observed (supplementary information). This may be due to the fact that urea (the nitrogen source) is also known to be a reducing agent.⁴² Carboxyl groups (O=C-OH) can undergo reduction reactions to form various reduced products, depending on the reaction conditions and the nature of the reducing agent used. This suggests that carboxylic groups were further reduced by urea to form functional groups such as alcohols, esters, and aldehydes, which resulted in an increase of the C-O content in the samples.

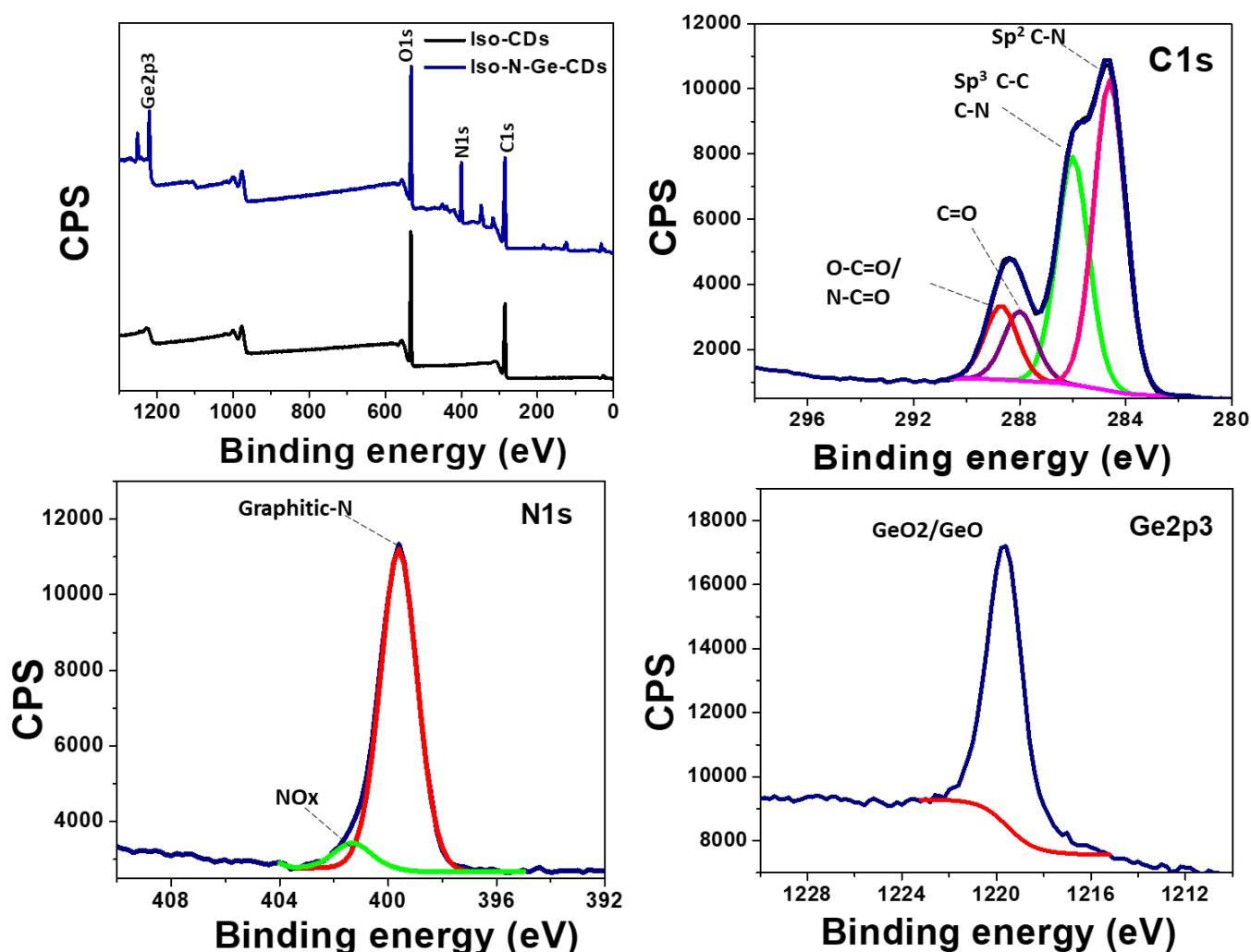


Figure 3.3 (a) Survey scans of Iso-CDs and Iso-N-Ge-CDs showing four major peaks of carbon, oxygen, nitrogen and germanium, and the expanded survey scan of (b) C1s (c) N1s and (d) Ge2p3.

3.5.3 XRD analysis

The PXRD pattern (**Figure 3.4**) of the synthesized CDs showed one broad peak centred at 2θ of 17° , 20° , 23° and 22° for Iso-CDs, Iso-Ge-CDs, Iso-N-CDs and Iso-N-Ge-CDs, respectively. The peaks that appear at these 2θ ranges are typically observed for amorphous carbon.^{43,44} The incorporation of nitrogen and (especially) germanium resulted in a significant broadening of the peak for Iso-N-CDs, Iso-Ge-CDs and Iso-N-Ge-CDs, which implied that the incorporation of heteroatoms in the CDs structure makes them more amorphous. Interestingly, Iso-Ge-CDs appear to produce the broadest peak, indicating that it

is more amorphous compared to the other CDs.⁴⁵ Moreover, this highly amorphous nature correlates to the lack of lattice fringes previously observed in the high-resolution TEM.

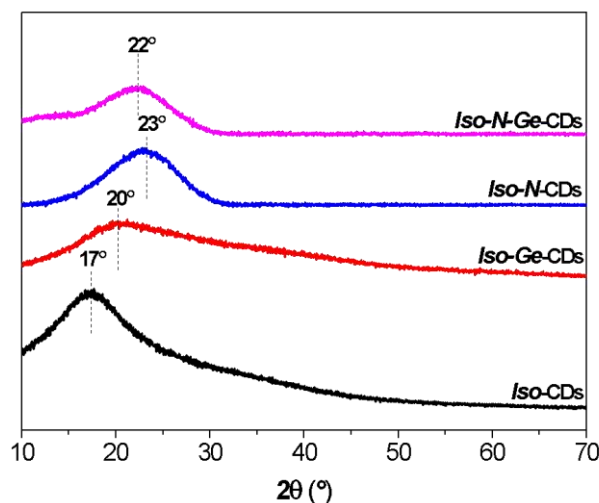


Figure 3.4 XRD spectra of the various CDs.

3.5.4 Photophysical properties

CDs have distinct optical characteristics that make them ideal for photoluminescence (PL) research. Additional investigations, such as PL excitation mapping, PL decay dynamics, or PL imaging, might be investigated depending on the unique research objectives and applications. For example, a study by Pyng Yu and co-workers investigated the temperature-dependent fluorescence measurements on highly fluorescent CDs between 77 and 300 K.⁴⁶ Their data indicated that CDs have metallic-nanocluster-like temperature behavior. Herein, the excitation dependence, pH sensitivity and quenching capacity were investigated using photoluminescence spectroscopy.

The CDs showed very intriguing luminescent qualities. **Figure 3.5** demonstrates the PL spectra of the newly prepared 5 $\mu\text{g/ml}$ CDs aqueous solutions. Each PL spectrum revealed a single band, indicating the origin of luminescence should be the same. A redshift was observed in the spectrum for all the CDs while increasing the excitation wavelength.⁴⁷ The maximum emission (and full width at half maximum) was observed for **(a)** Iso-CDs **(b)** Iso-Ge-CDs **(c)** Iso-N-CDs **(d)** Iso-N-Ge-CDs an excitation of 330 nm (95 nm), 340 nm (110 nm), 330 nm (120 nm) and 330 nm (90 nm), respectively, as seen in **Table 3.2**. Moreover, the defects that are present in the material and the inhomogeneity of the material are known to

affect the full width at half maximum (FWHM) which may give more insight regarding the fabricated samples.⁴⁸ Literature has shown that when the material is doped and the maximum emission as well as the FWHM in PL is affected³⁸, this can be ascribed to the changes in the width of the absorption line due to the lattice vibrations and lattice imperfections.⁴⁹ The intensity of the peaks appear to be low compared to literature, however, their ability to emit photons after the excitation and recombination processes demonstrated that they do possess photoluminescent properties.⁵⁰ The photoluminescence and quantum yield are closely related as they are both linked to the efficiency of the light emission process.⁵¹

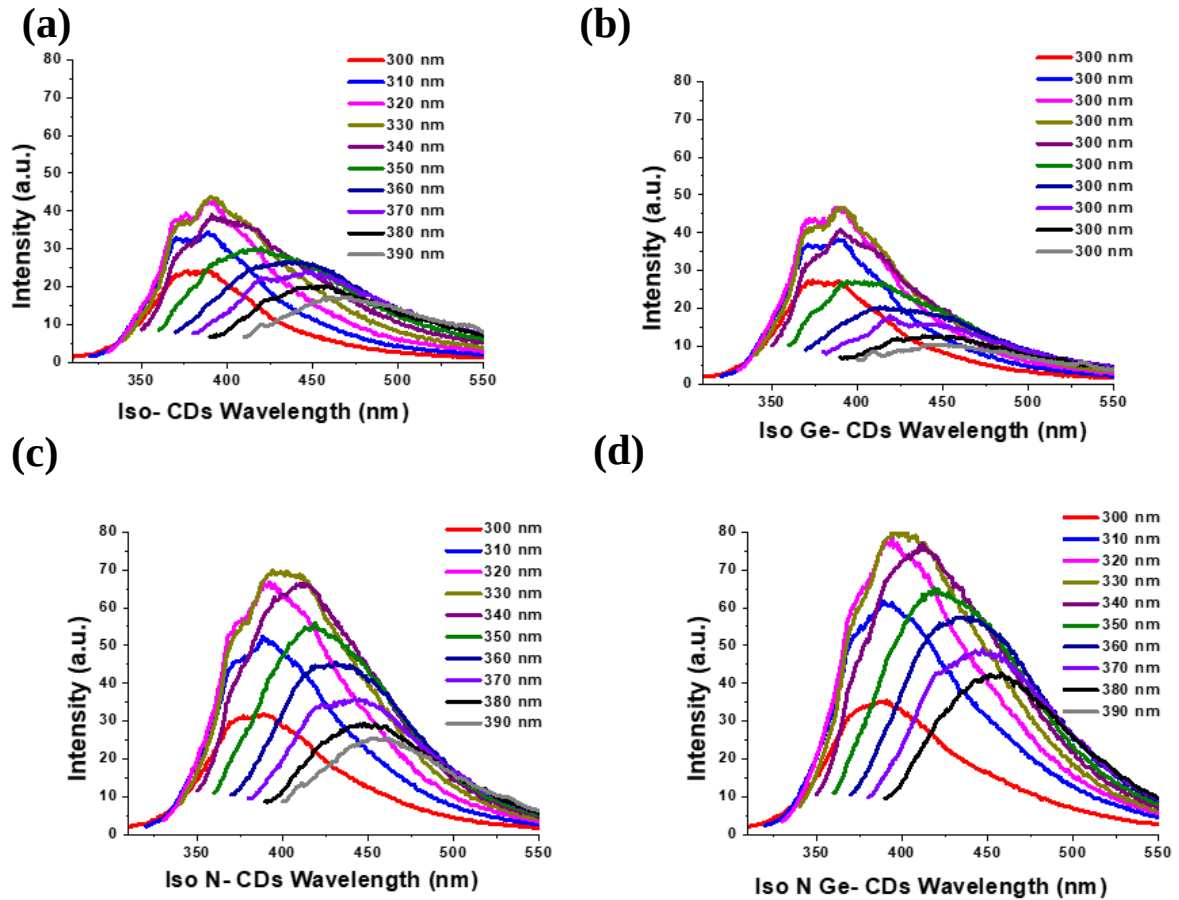


Figure 3.5 Photoluminescence (PL) of (a) Iso-CDs (b) Iso-Ge-CDs (c) Iso-N-CDs d) Iso-N-Ge-CDs

Table 3.2 Data derived from the Photoluminescence data.

Name	PL data		
	Maximum excitation (nm)	Emission (nm)	FWHM (nm)
<i>Iso</i> - CDs	380	457	95
<i>Iso-Ge</i> - CDs	320	370	120
<i>Iso-N</i> - CDs	380	417	110
<i>Iso-N-Ge</i> -CDs	320	383	90

Interestingly, PL emission with a longer wavelength is more favourable because the cell nuclei generally emit blue light. Hence, the CDs presented in this work are more suitable for cell imaging applications.⁵²

CDs PL characteristics can be greatly impacted by N-doping. CDs can have their electrical and surface properties changed via N-doping, which entails adding nitrogen atoms to the carbon lattice. CDs photoluminescence efficiency can be increased by N-doping, which can also cause their emission spectra to shift toward the red. Although the exact mechanism underlying the PL enhancement in N-doped carbon dots is unknown, it is thought to be connected to the entry of new energy levels into the bandgap of the CDs as illustrated in **Figure S3.13**. When nitrogen atoms are added, N-doped graphitic domains can emerge, which can produce new energy levels inside the CD bandgap. This could explain why the intensity of **Figures 3.5 (a) and (b)** appears to be lower than the nitrogen-doped CDs in **Figures 3.5 (c) and (d)**.

Since the CD concentrations were kept constant, the intensities were compared, and the *Iso-N-Ge*-CDs exhibited the best photoluminescent properties. This sample was then selected for the pH and quenching study. The surface charge, size, and chemical makeup of CDs can all be influenced by the pH, which can have an impact on how they behave.⁵³ The protonation or deprotonation of surface functional groups, changes in the electronic structure of CDs, and changes in the local microenvironment of the CD are just a few examples of the many

variables that can affect the PL properties of carbon dots.⁵⁴ Emission wavelength, intensity, and quantum yield, change as a function of pH CDs may be used in bioimaging, sensing, and medication delivery, all of which depend on pH for optimal functioning.⁵⁵

Several studies have shown that C-dots with pH-sensitive PL show tremendous potential for monitoring pH changes in living cells, tissues, and animals, demonstrating that they can be used as a useful research tool in biomedical studies. For instance, CDs can be utilized as pH sensors in bioimaging because of their pH-dependent photoluminescence characteristic. Liu's group created two fluorescent CDs with pH-sensitive red fluorescence in the pH range of 1.0 to 9.0 and a linear range of 3.5 to 6.5. This was shown to be useful for measuring the pH value in living cells MCF-7 cells and were used to test the ability of CDs to monitor intracellular pH fluctuations.⁵⁶

Figure 3.6 shows the pH-sensitive properties of *Iso-N-Ge-CDs*. The maximum excitation is observed at a pH of 3, and the minimum is pH 11. Interestingly, there appears to be a linear increase from pH 4 to 6. Since tumour tissues have an acidic extracellular pH of 6.2–6.8, and *Iso-N-Ge-CDs* seem to have stronger photoluminescent properties at low pH, then this suggests that the *Iso-N-Ge-CDs* possess good prospects for monitoring pH changes in living cells or releasing pH sensitive cargo.⁵⁷

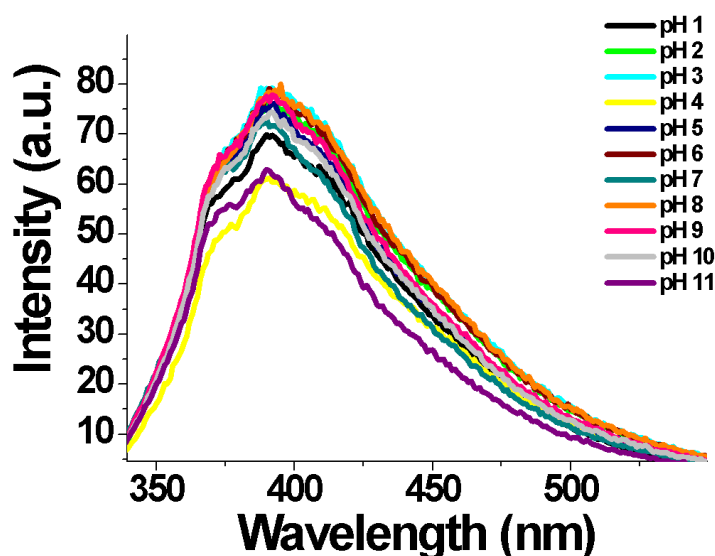


Figure 3.6 Photoluminescence spectra of the *Iso-N-Ge-CDs* at pH ranging from 1 to 11.

Carbon dots can interact with ion metals due to their great surface area and variety of surface functional groups. Ion metals and carbon dots may interact, which may change some characteristics, including photoluminescence, stability, and toxicity which can be used for sensing.³⁵ Comprehending the mechanism of interaction between carbon dots and ion metals can be aided by ion metal investigations. For instance, ion metals can snuff out CDs photoluminescence, which can be exploited for sensing purposes. The photoluminescence of carbon dots, on the other hand, can be enhanced by ion metals and employed for bioimaging and sensing applications. For example, the human body cannot usually metabolize Fe^{3+} ions and too much of them can lead to cancerous cell growth.⁵⁸ As a result, it is crucial to find Fe^{3+} ions. With an excitation wavelength of 340 nm, the quenching capabilities of 12 different metal ions (0.01 mM) were tested to clarify the fluorescence sensing of 1mg/ml CDs for Fe^{3+} ions. This study was evaluated by adding 0.5 ml of the CD solution and 0.5 ml of the metal ions at room temperature. The emission peak intensity of *Iso-N-Ge*-CDs with different metal ions, such as Cu^{2+} , Fe^{3+} , Co^{3+} , Zn^{3+} , Ni^{2+} , Cd^{2+} , Ca^{2+} , Na^+ , Al^{3+} , K^+ , As^{3+} and As^{2+} ions, was depicted in **Figure 3.7 (a-b)**. According to the findings, when compared to other metal ions, Fe^{3+} ions had the highest level of quenching for *Iso-N-Ge*-CDs.

In **Figure 3.7 (c)**, the samples containing the metal ions were placed under a UV lamp, and the data reveal that the photoluminescence spectroscopy matched the fluorescent intensity seen and UV light. The concentration of the Fe^{3+} was then varied from 0.01 mM to 0.09 mM, and 5 $\mu\text{g/ml}$ was used for the study in **Figure 3.7 (d)**. A consistent decrease was detected as the concentration of Fe^{3+} was increased. The quenching effect of Fe^{3+} can be reversible, and in a similar study by Yan Liang *et al.*, an “on-off-on” photoluminescent properties using glutathione (GSH) of Fe^{3+} was demonstrated.⁵⁶

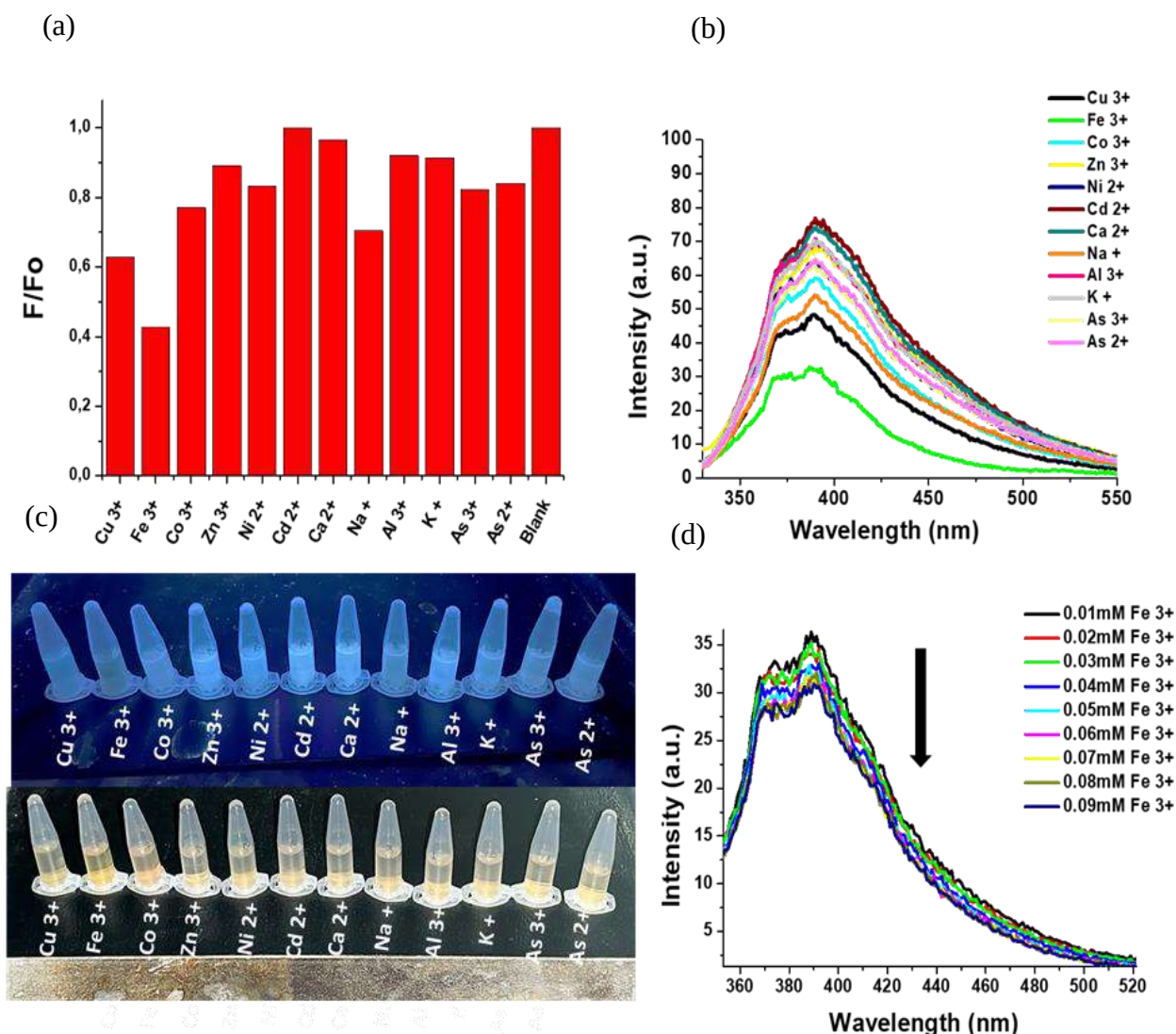


Figure 3.7 (a) The emission peak intensity ratio of Iso-N-Ge-CDs with Cu^{2+} , Fe^{3+} , Co^{3+} , Zn^{3+} , Ni^{2+} , Cd^{2+} , Ca^{2+} , Na^{+} , Al^{3+} , K^{+} , As^{3+} and As^{2+} ions, (b) Photoluminescence (PL) of carbon dots of Iso-N-Ge-CDs with different metal ions, (c) fluorescent properties of t

According to prior studies, the intramolecular photoinduced electron transfer process from excited CDs to Fe^{3+} ions is a plausible mechanism for the fluorescence quenching of CDs.⁵⁷ The d-orbital of Fe^{3+} splits when electron-deficient Fe^{3+} complexes containing functional groups on the surface of the N-CDs (such as carboxyl groups and amino groups) are added as shown in **Figure S3.14**. As a result, a portion of the electrons in the excited state of the CDs were transported to the d-orbital of the Fe^{3+} . Fluorescence quenching resulted from the radiation forms restrained electron transition (FL emission).

The absorption spectra of Iso-CDs, Iso-Ge-CDs, Iso-N-CDs, and Iso-N-Ge-CDs are shown in Figure 3.8 below. Iso-CDs showed absorption peaks at ~ 204 and 291 nm, which correlated to

the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transition of C=C and C=O bonds, respectively. The absorption peaks for Iso-Ge-CDs were observed at ~ 204 and 267 nm, correlating to the $\pi \rightarrow \pi^*$, characteristic of the aromatic sp^2 domains. Iso-N-CDs showed peaks at ~ 214 and 243 nm, correlating to the $\pi \rightarrow \pi^*$ transition of the C=C bond. Finally, similar to the Iso-Ge-CDs, Iso-N-Ge-CDs absorbed UV-Vis radiation at ~ 204 and 264 nm, correlating to the $\pi \rightarrow \pi^*$, characteristic of the aromatic sp^2 domains. The absorption edges of all four samples extend into the visible band. The products were dispersed in water and were exposed to UV light and each exhibited greenish luminescence (**Figure S3.15**).

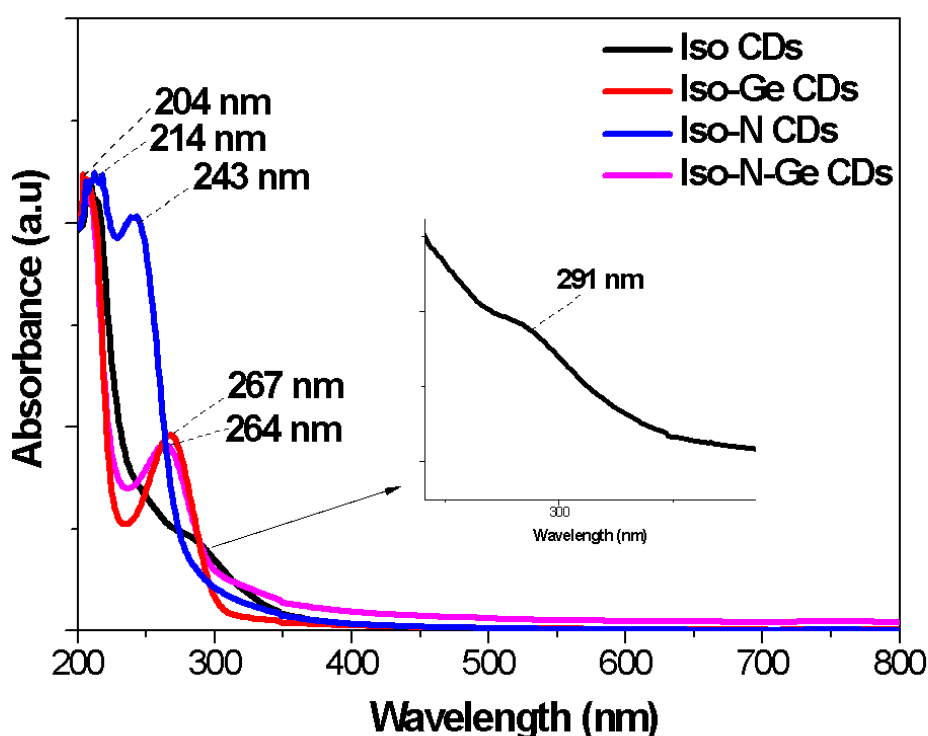


Figure 3.8 UV-Vis absorption spectra of Iso-CDs, Iso-Ge-CDs, Iso-N-CDs and Iso-N-Ge-CDs

The BX63 Upright Microscope was then utilized to analyse the fluorescent qualities of the Iso-N-Ge-CDs to determine their capacity for bio-imaging applications. A single grain of the Iso-N-Ge-CD powder was placed on a glass slide and analysed under the microscope, and this revealed that the Iso-N-Ge-CD can fluoresce under the red and blue wavelengths, **Figure 3.9**. Most CDs fluoresce in the blue only however the Iso-N-Ge-CD fluoresces in longer

wavelength regions as well. CDs which are red display high resistance to photobleaching, intense absorption in the red/NIR region, and biodegradability.⁵⁸

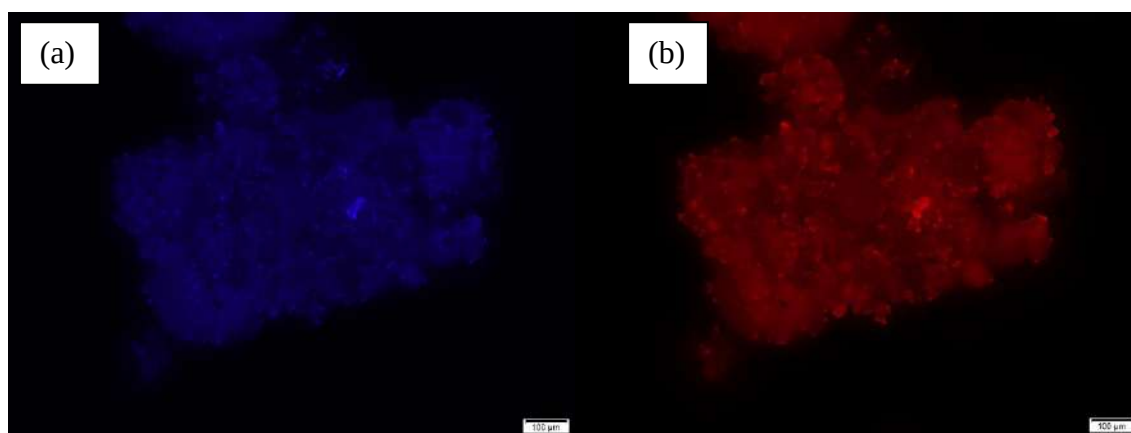


Figure 3.9 Fluorescent Microscopy of Iso-N-Ge-CDs under blue (a) and red (b) wavelength

3.6 Conclusion

A successful synthesis of CDs (diameter ≤ 10 nm) using a simple microwave assisted method was confirmed using TEM. The FTIR spectra showed that the CDs were successfully doped with nitrogen for the *Iso-N-Ge-CDs*, as shown by the characteristic band at approximately 3247 cm^{-1} (N-H) and 1392 cm^{-1} (C-N) and further confirmed by the XPS data with the relative abundance of N1s estimated to be 15.9 w %. The presence of GeO_2/GeO on the carbon dots was also confirmed by the XPS data however, the lack of C-Ge interaction suggests that the incorporation of germanium only affected the surface functionalities. The effects were more notable in the size increase of the CDs shown in TEM as well as the significant shift and broadening of the peaks on the XRD. The photoluminescent qualities of the CDs show that the CDs are fluorescent, and when exposed to UV light, the CDs exhibited a wide range of colors. PL data also showed that the CDs have photoluminescent qualities. Data from the BX63 Upright Microscope showed that the *Iso-N-Ge-CDs* exhibit fluorescence, which can be applied for bio-imaging applications. The *Iso-N-Ge-CDs* demonstrated the best photoluminescent capabilities, although for future work, further studies may be conducted to improve these properties. The linear rise in pH from 4 to 6 in the pH-sensitivity study may be exploited because tumor tissues have an acidic extracellular pH of 6.2-6.8. This implies that *Iso-N-Ge-CDs* have promising applications for monitoring pH

changes. Moreover, the quenching study of the different metal ions revealed that Fe^{3+} ions showed the highest quenching effect for *Iso-N-Ge-CDs*.

3.7 References

1. Yao, B., Huang, H., Liu, Y. & Kang, Z. Carbon Dots: A Small Conundrum. *Trends Chem.* **1**, 235–246 (2019).
2. Kang, Z. & Lee, S.-T. Carbon dots: advances in nanocarbon applications. *Nanoscale* **11**, 19214–19224 (2019).
3. Mkhari, O., Ntuli, T. D., Coville, N. J., Nxumalo, E. N. & Maubane-Nkadimeng, M. S. A comparison of fluorescent N-doped carbon dots supported on the surface of hollow and solid carbon spheres, and solid silica spheres. *Diam. Relat. Mater.* **118**, 108500 (2021).
4. Xia, C., Zhu, S., Feng, T., Yang, M. & Yang, B. Evolution and Synthesis of Carbon Dots: From Carbon Dots to Carbonized Polymer Dots. *Adv. Sci.* **6**, 1901316 (2019).
5. Sarkar, S., Banerjee, D., Ghorai, U. K., Das, N. S. & Chattopadhyay, K. K. Size dependent photoluminescence property of hydrothermally synthesized crystalline carbon quantum dots. *J. Lumin.* **178**, 314–323 (2016).
6. Hu, L. *et al.* Multifunctional carbon dots with high quantum yield for imaging and gene delivery. *Carbon* **67**, 508–513 (2014).
7. Su, W. *et al.* Carbon dots: A booming material for biomedical applications. *Mater. Chem. Front.* **4**, 821–836 (2020).
8. El-Shafey, A. M. Carbon dots: Discovery, structure, fluorescent properties, and applications. *Green Process. Synth.* **10**, 134–156 (2021).
9. Wang, J., Choi, H. S. & Wáng, Y.-X. J. Exponential growth of publications on carbon nanodots by Chinese authors. *J. Thorac. Dis.* **7**, E201-205 (2015).
10. Li, F., Yang, D. & Xu, H. Non-Metal-Heteroatom-Doped Carbon Dots: Synthesis and Properties. *Chem. – Eur. J.* **25**, 1165–1176 (2019).
11. Wang, C. *et al.* Nitrogen-Doped Carbon Dots Increased Light Conversion and Electron Supply to Improve the Corn Photosystem and Yield. *Environ. Sci. Technol.* **55**, 12317–12325 (2021).

12. Liao, J., Cheng, Z. & Zhou, L. Nitrogen-Doping Enhanced Fluorescent Carbon Dots: Green Synthesis and Their Applications for Bioimaging and Label-Free Detection of Au³⁺ Ions. *ACS Sustain. Chem. Eng.* **4**, 3053–3061 (2016).
13. Lin, L., Luo, Y., Tsai, P., Wang, J. & Chen, X. Metal ions doped carbon quantum dots: Synthesis, physicochemical properties, and their applications. *TrAC Trends Anal. Chem.* **103**, 87–101 (2018).
14. Luo, H. *et al.* Pt single-atoms supported on nitrogen-doped carbon dots for highly efficient photocatalytic hydrogen generation. *J. Mater. Chem. A* **8**, 14690–14696 (2020).
15. Safardoust-Hojaghan, H. & Salavati-Niasari, M. Degradation of methylene blue as a pollutant with N-doped graphene quantum dot/titanium dioxide nanocomposite. *J. Clean. Prod.* **148**, 31–36 (2017).
16. Li, F. *et al.* Mg/N double doping strategy to fabricate extremely high luminescent carbon dots for bioimaging. *RSC Adv.* **4**, 3201–3205 (2014).
17. Xu, Q. *et al.* Recent progress of quantum dots for energy storage applications. *Carbon Neutrality* **1**, 13 (2022).
18. Xu, Y. *et al.* Group IV nanodots: synthesis, surface engineering and application in bioimaging and biotherapy. *J. Mater. Chem. B* **8**, 10290–10308 (2020).
19. Chroneos, A., Uberuaga, B. P. & Grimes, R. W. Carbon, dopant, and vacancy interactions in germanium. *J. Appl. Phys.* **102**, 083707 (2007).
20. Goodman, S. Therapeutic effects of organic Germanium. *Med. Hypotheses* **26**, 207–215 (1988).
21. Jiang, S. l., Shi, C. h. & Wu, J. g. Determination of trace amount for germanium (ge) by atomic fluorescence spectrometry in rice (ORYZA SATIVA L.). *J. Food Qual.* **30**, 481–495 (2007).
22. Stanisz, E. & Krawczyk-Coda, M. ZnO nanoparticles as an adsorbent in ultrasound-assisted dispersive micro solid-phase extraction combined with high-resolution continuum source electrothermal atomic absorption spectrometry for determination of trace germanium in food samples. *Microchem. J.* **132**, 136–142 (2017).

23. Zaijun, L., Jian, T., Huizhen, L., Xia, Z. & Rui, Y. Determination of trace amounts of germanium in food and fruit by spectrophotometry with p-methybenzeneazosalicylflurone. *J. Food Compos. Anal.* **20**, 1–6 (2007).
24. Hu, J. *et al.* Synthesis of Fluorescent and Water-Dispersed Germanium Nanoparticles and Their Cellular Imaging Applications. *Langmuir* **34**, 8932–8938 (2018).
25. Zaki, M. F. & Tawfik, S. M. Synthesis, Surface Properties and Antimicrobial Activity of Some Germanium Nonionic Surfactants. *J. Oleo Sci.* **advpub**, (2014).
26. Ikemoto, K. *et al.* 2-Carboxyethylgermanium sesquioxide, a synthetic organogermanium compound, as an inducer of contrasuppressor T cells. *Experientia* **52**, 159–166 (1996).
27. Yuan, Y. H. *et al.* Germanium-doped carbon dots as a new type of fluorescent probe for visualizing the dynamic invasions of mercury(II) ions into cancer cells. *Nanoscale* **7**, 16841–16847 (2015).
28. Juho, Y.-C. *et al.* Germanium-Titanium- π Polymer Composites as Functional Textiles for Clinical Strategy to Evaluate Blood Circulation Improvement and Sexual Satisfaction. *Polymers* **13**, 4154 (2021).
29. Dhenadhayalan, N., Chauhan, A., Lin, K.-C. & AlFantazi, A. Architecting 3D prism shaped carbon dots/germanium/germanium oxide nanohybrid for photocatalytic degradation of pendimethalin and dinotefuran pesticides. *Mater. Today Chem.* **24**, 100913 (2022).
30. Mokoloko, L. L. *et al.* Evolution of large-area reduced graphene oxide nanosheets from carbon dots via thermal treatment. *Carbon Trends* **4**, 100074 (2021).
31. Gong, J., Lu, X. & An, X. Carbon dots as fluorescent off–on nanosensors for ascorbic acid detection. *RSC Adv.* **5**, 8533–8536 (2015).
32. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
33. Martínez-Rodríguez, R. A., Vidal-Iglesias, F. J., Solla-Gullón, J., Cabrera, C. R. & Feliu, J. M. Synthesis of Pt Nanoparticles in Water-in-Oil Microemulsion: Effect of HCl on Their Surface Structure. *J. Am. Chem. Soc.* **136**, 1280–1283 (2014).

34. L. Mokoloko, L. *et al.* From 0D to 2D: N-doped carbon nanosheets for detection of alcohol-based chemical vapours. *RSC Adv.* **12**, 21440–21451 (2022).
35. Xu, Q. *et al.* Machine learning guided microwave-assisted quantum dot synthesis and an indication of residual H₂O₂ in human teeth. *Nanoscale* **14**, 13771–13778 (2022).
36. Perikala, M. & Bhardwaj, A. Excellent color rendering index single system white light emitting carbon dots for next generation lighting devices. *Sci. Rep.* **11**, 11594 (2021).
37. Bajpai, S. K., D’Souza, A. & Suhail, B. Blue light-emitting carbon dots (CDs) from a milk protein and their interaction with *Spinacia oleracea* leaf cells. *Int. Nano Lett.* **9**, 203–212 (2019).
38. Atchudan, R., Jebakumar Immanuel Edison, T. N., Perumal, S., Vinodh, R. & Lee, Y. R. Multicolor-emitting carbon dots from *Malus floribunda* and their interaction with *Caenorhabditis elegans*. *Mater. Lett.* **261**, 127153 (2020).
39. Malatesta, M. Transmission Electron Microscopy as a Powerful Tool to Investigate the Interaction of Nanoparticles with Subcellular Structures. *Int. J. Mol. Sci.* **22**, 12789 (2021).
40. Wei, X., Si, X., Han, M. & Bai, C. One-Step Preparation of S, N Co-Doped Carbon Quantum Dots for the Highly Sensitive and Simple Detection of Methotrexate. *Molecules* **27**, 2118 (2022).
41. Kraft, N. *et al.* Phase and grain size engineering in Ge-Sb-Te-O by alloying with La-Sr-Mn-O towards improved material properties. *Mater. Des.* **199**, 109392 (2021).
42. Lei, Z., Lu, L. & S. Zhao, X. The electrocapacitive properties of graphene oxide reduced by urea. *Energy Environ. Sci.* **5**, 6391–6399 (2012).
43. Siddique, A. B., Pramanick, A. K., Chatterjee, S. & Ray, M. Amorphous Carbon Dots and their Remarkable Ability to Detect 2,4,6-Trinitrophenol. *Sci. Rep.* **8**, 9770 (2018).
44. Dlamini, M. W. *et al.* Carbon Spheres Prepared by Hydrothermal Synthesis—A Support for Bimetallic Iron Cobalt Fischer–Tropsch Catalysts. *ChemCatChem* **7**, 3000–3011 (2015).
45. Jiang, L. *et al.* Photoactivated Fluorescence Enhancement in F,N-Doped Carbon Dots with Piezochromic Behavior. *Angew. Chem.* **132**, 10072–10077 (2020).

46. Yu, P., Wen, X., Toh, Y.-R. & Tang, J. Temperature-Dependent Fluorescence in Carbon Dots. *J. Phys. Chem. C* **116**, 25552–25557 (2012).
47. Holá, K. *et al.* Graphitic Nitrogen Triggers Red Fluorescence in Carbon Dots. *ACS Nano* **11**, 12402–12410 (2017).
48. Toyozawa, Y. Further Contribution to the Theory of the Line-Shape of the Exciton Absorption Band. *Prog. Theor. Phys.* **27**, 89–104 (1962).
49. Toyozawa, Y. Theory of Line-Shapes of the Exciton Absorption Bands. *Prog. Theor. Phys.* **20**, 53–81 (1958).
50. Zhu, S. *et al.* The photoluminescence mechanism in carbon dots (graphene quantum dots, carbon nanodots, and polymer dots): current state and future perspective. *Nano Res.* **8**, 355–381 (2015).
51. Fries, F. & Reineke, S. Statistical treatment of Photoluminescence Quantum Yield Measurements. *Sci. Rep.* **9**, 15638 (2019).
52. Ding, H., Cheng, L.-W., Ma, Y.-Y., Kong, J.-L. & Xiong, H.-M. Luminescent carbon quantum dots and their application in cell imaging. *New J. Chem.* **37**, 2515–2520 (2013).
53. Jiang, J., Oberdörster, G. & Biswas, P. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *J. Nanoparticle Res.* **11**, 77–89 (2009).
54. Yu, J. *et al.* Luminescence Mechanism of Carbon Dots by Tailoring Functional Groups for Sensing Fe³⁺ Ions. *Nanomaterials* **8**, 233 (2018).
55. Shen, C., Sun, Y., Wang, J. & Lu, Y. Facile route to highly photoluminescent carbon nanodots for ion detection, pH sensors and bioimaging. *Nanoscale* **6**, 9139–9147 (2014).
56. Liu, C., Zhang, F., Hu, J., Gao, W. & Zhang, M. A Mini Review on pH-Sensitive Photoluminescence in Carbon Nanodots. *Front. Chem.* **8**, 605028 (2021).
57. Das, P. *et al.* Carbon Dots for Heavy-Metal Sensing, pH-Sensitive Cargo Delivery, and Antibacterial Applications. *ACS Appl. Nano Mater.* **3**, 11777–11790 (2020).

58. Liu, T. *et al.* Ferrous-Supply-Regeneration Nanoengineering for Cancer-Cell-Specific Ferroptosis in Combination with Imaging-Guided Photodynamic Therapy. *ACS Nano* **12**, 12181–12192 (2018).

4. Synthesis and characterization of enhanced cell-penetrating peptides derivatives

Machumele, K., Makatini, M. and Maubane-Nkadimeng, M.S., Title: Synthesis and characterization of cell-penetrating peptides derivatives.

4.1 Abstract

Scientists are persistently striving to improve the design of cell-penetrating peptides (CPPs) and grasp their cellular uptake mechanisms, aiming to surmount challenges and fully exploit their potential in diverse biomedical and biotechnological uses. In this section, adamantane, palmitic acid and D-amino acids were used to improve the properties of the GKPILFF and RLRLRIGRR peptide sequences which have cell-penetrating properties. The novel peptides derived from these sequences were synthesized using an optimized procedure, reducing coupling time and increasing purity. The addition of adamantane and palmitic acid increased retention time due to hydrophobic moieties and potential secondary structure changes. The RGDGKP peptide had a lower retention time of 8.75 minutes compared to the GKP peptide. The syntheses and purification of these peptides were successful with yields vary from 20% to 62%.

Keywords: peptide synthesis; adamantane; palmitic acid; peptide permeability; penetration-accelerating segment; enantiomeric 9-mer peptide

4.2 Introduction

The CDs presented in Chapter 3 have demonstrated potential for bioimaging applications due to their unique optical properties, such as strong fluorescence. However, their application on their own in bioimaging is often limited due to low permeability.

To address this limitation, carbon dot-peptide conjugates, where the peptide used is derived from a penetration-enhancing sequence were developed. Modifications were conducted to further increase permeation, and the short length of the sequence is beneficial in terms of cost, synthesis, and purification.

Remarkable attributes of synthetic peptides, such as facile and cost-effective synthesis, higher mechanical stability, good tissue penetration, and less immunogenicity, are ascribed to ultra-short peptides, which have gained popularity over the years. Moreover, better control of the micromolecule and characterization is much more straightforward. Additionally, very short peptides have better tunability, biodegradability, biocompatibility, non-cytotoxicity, and non-immunogenicity in contrast to their longer counterparts.¹

The penetration-accelerating segment (GKPILFF) which is briefly discussed in **section 2.3.1** of Chapter 2, is a peptide that has been used as a cell-penetrating tool. This peptide can be added to other peptides or bioactive molecules for enhancement of the permeability of the pharmaceutical agent.² This short cell-penetrating peptide (CPP) is described as a hydrophobic peptide previously shown to enhance endosomal escape as a membrane transport mechanism.³ Endosomal escape begins with an initial destabilization of the membrane, which subsequently leads to the formation of pores on the membrane surface.⁴ Once an endosome has formed, it either results in endosomal rupture or membrane fusion.⁵ Endosomal escape is the desired effect that provides optimum membrane penetration, which is encouraged by several features, such as the pH-buffering effect of portable groups, which may result in enhanced permeability.⁶

Researchers have found innovative ways to enhance the pharmacokinetic properties of peptides by making slight modifications that affect how the peptide is absorbed and distributed in the body.⁷ Successful peptide-based pharmaceutical drugs have modifications such as N-terminal modifications, replacement of L-amino acids with D-amino acids, N-methylated amino acids, peptoid moieties, etc.^{8, 9} These impact the bioavailability, potency, stability, and activity of the peptide.¹⁰

In this study, we investigated the influence of D-amino acids, lipophilic molecules as well as the incorporation of the tumour-homing RGD moiety on the overall structure of the peptide derivatives using the LCMS and NMR. Structural analysis conducted using the NMR technique is outlined in Chapter 5.

A study by Dorit Avrahami *et al.* described the synthesis of a new class of lipopeptides with strong antifungal and antibacterial properties.¹¹ These lipopeptides were created by palmitoylating (PA), the N terminus of positively charged peptides that include both D- and L-amino acids. The peptides which were investigated had the sequence K₄X₇W, where X stands for Gly, Ala, Val, or Leu (**Table 4.1**). The results showed that KGGGKWGGKGGK-

NH₂ (PA-d-G) and KAAAKWAAKAAK-NH₂ (PA-d-A) (**Table 4.1**) exhibited strong antibacterial and antifungal activity even though both parental peptides had no effect on model phospholipid membranes or microorganisms. In contrast, KLLLKWLLKLLK-NH₂ (PA-d-L) obtained and kept some antifungal activity but lost the ancestral peptide's powerful antibacterial activity.¹¹ This demonstrates the significance of conjugating palmitic acid to peptides and the effect of D-amino acids. It also shows that modifications often enhance certain properties while negatively affecting others. In order to understand the effects of each modification, it is very crucial to know the structural properties of each derivative to provide foresight in designing new peptides.

Table 4.1 K4G7W peptide analogues investigated by Dorit Avrahami et al. conjugated to Palmitic Acid and D-amino acids

PEPTIDE DESIGNATION	PEPTIDE FORMULA	SEQUENCE	MOLECULAR WEIGHT (G/MOL)
PA-d-G	PA-D-K ^{1,5,9,12} - K ₄ G ₇ <u>W</u>	(CH ₃ (CH ₂) ₁₄ CO)- KGGGK <u>W</u> GGKGGK -NH ₂	1353
PA-d-A	PA-D-A ^{3,4,8,10} - K ₄ A ₇ <u>W</u>	(CH ₃ (CH ₂) ₁₄ CO)- KAAAK <u>W</u> AAKAAK- NH ₂	1451
PA-d-V	PA-D-V ^{3,4,8,10} - K ₄ V ₇ <u>W</u>	(CH ₃ (CH ₂) ₁₄ CO)- KVVVK <u>W</u> VVKVVK- NH ₂	1647
PA-d-L	PA-D-L ^{3,4,8,10} - K ₄ L ₇ <u>W</u>	(CH ₃ (CH ₂) ₁₄ CO)- KLLLK <u>W</u> LLKLLK- NH ₂	1746

Each diastereomer contained four D-amino acids at positions 3, 4, 8,10, when X Ala, Val, or Leu, or alternatively at positions 1, 5, 9, 12, when X Gly.¹¹

The original peptide sequence RLRLRIGRR and its derivatives was also investigated in this study. RLRLRIGRR is a synthetic antimicrobial peptide with anticancer properties that consists of 9 amino acids and has a net charge of +5 due to the presence of 5 positively charged arginine residues.¹² The amino acid sequence of RLRLRIGRR contains a high proportion of basic amino acids, that can interact with negatively charged molecules such as nucleic acids or cell membranes.

The synthetic enantiomeric 9-mer peptide named D-peptide B was based on the amino acid sequence of the active site of the 43-mer insect defensins **Figure 4.1**.¹² The D-9-mer peptides

maintained bacterial membrane disruptive activity similar to the original peptides and also showed various extents of growth inhibitory activity against different cancer cell lines.¹³

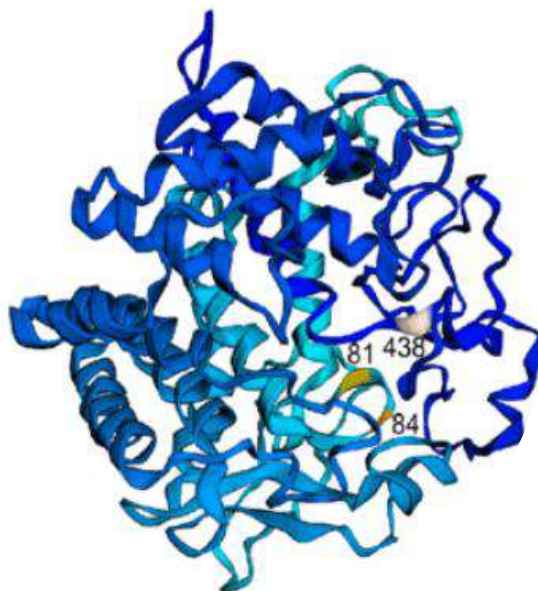


Figure 4.1 Using an information-theoretic technique based on Jensen-Shannon divergence, active sites were predicted at D amino acid 81 (D, Aspartic Acid), 84aa (D, Aspartic Acid), and 438 (E, Glutamic Acid).¹²

In a study by Takashi Iwasaki and coworkers., the selectivity of the RLRLRIGRR peptide towards cancer cells was shown to be due to its high affinity for negatively charged phospholipids found in the cancer cells.¹⁴ Additionally, the peptide has been proven to have selective cancer cell cytotoxicity against the mouse myeloma cell line P3-X63-Ag8.653. Moreover, in various cancer cell line studies, a strong correlation between the negatively charged phosphatidylserine (PS) density in cancer cell membrane surface and sensitivity to D-9-mer peptides was observed. These results suggest that D-9-mer peptides have negative charge-dependent selective cancer cell cytotoxicity and they target the PS in the cell membrane. Hence, they may enhance the cancer-cell selectivity of bio-imaging carbon dots.

In summary, peptides derived from the GKPIILFF and RLRLRIGRR sequences were synthesized and conjugated with CD dots to improve the selectivity and permeability of the CD dots. The anticancer properties of RLRLRIGRR may have a synergistic effect since the imaged sites are tumours or cancer growths.

4.3 Design of the short peptide derivatives

4.3.1 L- and D-amino acids

The foundation of peptide research is predominantly based on L--amino acid peptides. On the other hand, peptides containing D-amino acids are less commonly found in nature. The difference in stereochemistry demonstrated in **Figure 4.2** leads to altered secondary and tertiary structures of peptides with identical sequences.

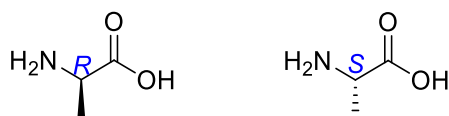


Figure 4.2 D-amino acid ((R)-alanine) and L- amino acid ((S)-alanine)

The synthesized D-peptide have been reported to be resistant to proteolytic degradation, which represents a breakthrough in utilizing D-amino acids to develop biostable inhibitors as potential therapeutics.¹⁵

D-amino acids rarely act as the substrates of endogenous proteases, contrary to L-amino acids hence, the D-peptides end up being resistant to proteolysis. Exploiting this stereochemical advantage is a common strategy to improve *in vivo* proteolytic stability of bioactive molecules.¹⁶ In this chapter, we investigated L-amino acid peptide analogues and D-amino acid peptide analogues of the GKP peptide, as illustrated in **Figure 4.3**, to study the effect it may have on the secondary structure of the peptides. This data may provide some insight into how to achieve optimum activity and information on the features needed to prolong the integrity of the peptide in the body.

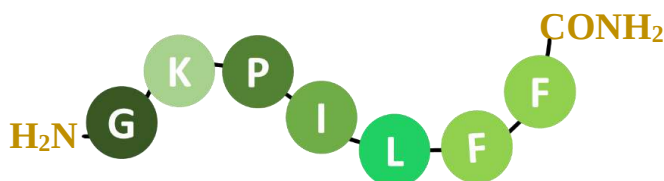


Figure 4.3 Peptide sequence of GKP peptide.

4.3.2 Adamantane and Palmitic acid

The remarkable structural and chemical properties of adamantane afford attractive opportunities to design various adamantane-based scaffolds for biomedical applications. A wide variety of mono-functionalized adamantane compounds have previously been investigated and reviewed, mostly as anti-viral agents such as amantadine and rimantadine, which treats a wide array of influenza strains owing to their ability to increase permeability as discussed in **section 2.5.5**. Moreover, other FDA-approved pharmaceutical agents containing this moiety include vildagliptin, tromantadine, carmantadine, bromatane, adapromine, saxagliptin, and memantine.¹⁷ The four bridgehead positions of the adamantane supply many possibilities to design poly-functional derivatives, and the recent conception of adamantane building blocks with multiple substituents has shown promising applications in several domains.¹⁸ Therefore, the adamantane moiety was added to the peptide to increase permeability, as demonstrated in **Figure 4.4**.

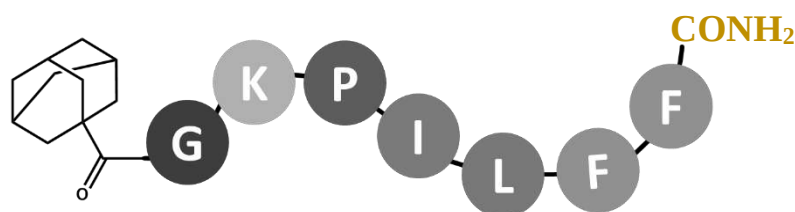


Figure 4.4 Peptide sequence of the L and D- GKP peptides with the adamantane moiety on the N terminus

The most common saturated fatty acid found in the human body is palmitic acid, and it can be supplied through diet or produced through endogenic processes from other fatty acids, carbohydrates, and amino acids.¹⁹ In the membrane, 20–30% of total fatty acids (FA) are in the form of palmitic acids, which is present in phospholipids (PL) and adipose triacylglycerols.²⁰

The treatment and prevention of external skin ageing is the predominant focus for drugs containing palmitic acid.²¹ Skin health has been shown to improve due to synthetic peptides containing the palmitoyl moiety.²² Fatty acid-conjugated peptide drugs, including the long-acting insulin drugs such as Levemir and Tresiba, as well as the anti-diabetic medication liraglutide, have been FDA-approved and are commercially available for clinical use. Additionally, the binding of fatty acids to albumin has been shown to achieve long-lasting

impact by extending the circulation of insulin in the blood.²³ Therefore, the palmitic acid moiety was added to the peptides to investigate the enhancement of the penetration into the cell membrane, as shown in **Figure 4.5**.

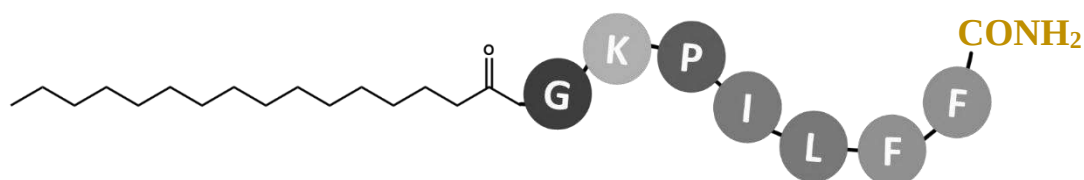


Figure 4.5 Peptide sequence of the GKP peptide with the palmitic acid moiety on the N terminus

4.3.3 RGD homing moiety

The tripeptide arginine-glycine-aspartic acid (RGD) is the first and the most widely used endothelial binding peptide that has high affinity towards integrins $\alpha_v\beta_1$, integrins $\alpha_v\beta_3$, and $\alpha_v\beta_5$, which are almost exclusively overexpressed during angiogenesis and nearly absent in the normal tissue.²⁴

Chemical reactions have been used to couple RGD-peptides to other compounds. Moreover, some of these products have shown impressive results in preclinical animal models.²⁵ For example, an RGD-targeted radiotracer has already successfully been tested in humans for the visualization of $\alpha_v\beta_3$ -integrin, which demonstrates the feasibility of this approach.²⁶ Furthermore, an FDA-approved drug known as Eptifibatide is an RGD-based drug that is a glycoprotein IIb/IIIa class platelet inhibitor drug that successfully minimizes ischemic cardiac events in certain patients that have particular criteria.²⁷ Therefore, the integration of the RGD moiety into the peptide may assist in enhancing the selectivity of the peptide, as shown in **Figure 4.6**.

This design combines the known homing properties of the RGD moiety and penetration-enhancing properties of the GKPILFF sequence to fabricate a selective bio-imaging carbon dot-peptide conjugate with superior permeability properties.

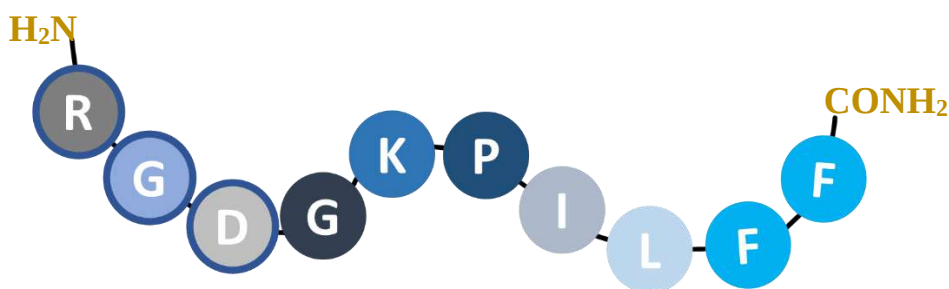


Figure 4.6 Peptide sequence of the GKP peptide with the RGD moiety on the N terminus

Synthesizing peptide analogues with the previously mentioned moieties allowed for the creation of novel molecules with therapeutic potential. These modifications can lead to entirely new functions or improved activities against specific targets. The D-amino acid peptide analogues listed in **table 4.2**, were designed to improve the stability and bioavailability of naturally occurring peptides. Modified peptides containing palmitic acid and adamantane, can enhance their pharmacokinetic properties, such as absorption, distribution, metabolism, and excretion (ADME), as discussed in the section above. As a way to minimize harm to healthy cells, the RGD peptide analogue was synthesized to facilitate targeting of cancer cells or tissues. Listed below are the peptide analogues which were synthesized with the explanation of each modification.

Table 4.2 Peptide analogues and the modification

PEPTIDE ID.	PEPTIDE SEQUENCE	MOLECULAR MASS	MODIFICATIONS
GKP	CONH₂ -GKPILFF- NH₂	819.50	The free carboxylic end is changed to an amide
GKP-ada	ADA -GKPILFF- NH₂	981.61	The N terminus is coupled to adamantane
GKP-pal	PAL -GKPILFF- NH₂	1057.73	The N terminus is coupled to palmitic acid
D-GKP	D-amino acid CONH₂ -GKPILFF- NH₂	819.50	The free carboxylic end is changed to an amide, L-amino acids are replaced with D-amino acids
D-GKP-ada	D-amino acid ADA -GKPILFF- NH₂	981.61	The N terminus is coupled to adamantane, and L-amino acids are replaced with D-amino acids
D-GKP-pal	D-amino acid PAL -GKPILFF- NH₂	1057.73	The N terminus has been modified with palmitic acid, and D-amino acids replace L-amino acids
RGDGKP	CONH₂ -RGDGKPILFF- NH₂	1147.6292	The free carboxylic end is changed to an amide
BP	CONH₂ -RLRLRIGRR- NH₂	1193.81	The free carboxylic end is changed to an amide
BP -ada	ADA -RLRLRIGRR- NH₂	1355.91	The N terminus has been modified with adamantane
BP -pal	PAL -RLRLRIGRR- NH₂	1432.04	The N terminus has been modified with palmitic acid
D- BP - GKP	D-amino acid CONH₂ -RLRLRIGRR - NH₂	1193.81	The free carboxylic end is changed to an amide, and D-amino acids replace L-amino acids
D- BP - ada	D-amino acid ADA -RLRLRIGRR- NH₂	1355.91	The N terminus has been modified with adamantane and D-amino acids to replace L-amino acids
D- BP -pal	D-amino acid PAL -RLRLRIGRR- NH₂	1432.04	The N terminus has been modified with palmitic acid and D-amino acids to

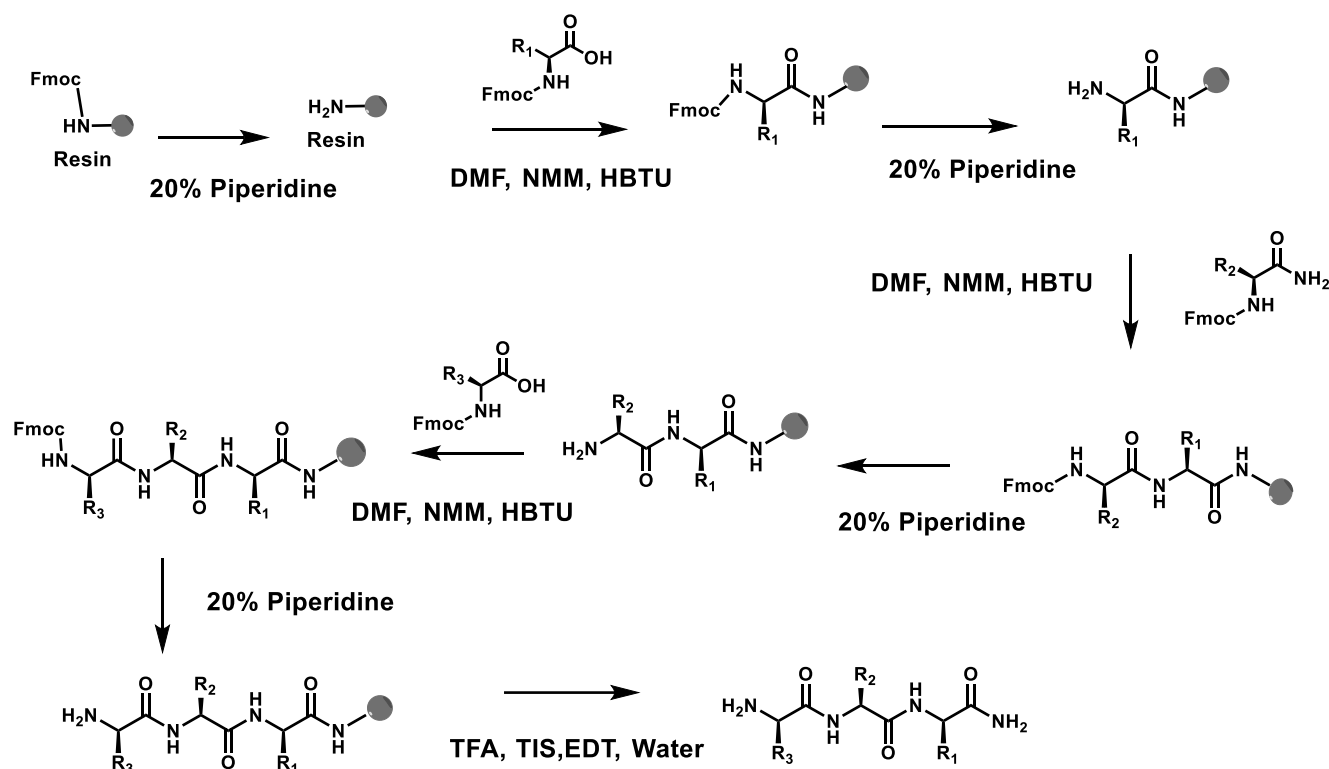
			replace L-amino acids
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4.4 Methods and Procedures for Peptide Synthesis

The landmark studies by an American biochemist Bruce Merrifield introduced solid-phase peptide synthesis (SPPS), which played a monumental role in immensely simplifying the synthesis and the elimination of entities like toxic reagents and solvents in peptide synthesis. This Nobel prize-winning approach opened the doors for automation as subsequent couplings could be iteratively performed by a defined set of unit operations. As a result, many challenging peptides have been successfully synthesized using SPPS. Originally, the N- α -Boc-protected amino acid building blocks were used for peptide synthesis. Subsequently, the 9-Fluorenylmethoxycarbonyl/tert-butyl (Fmoc/tBu) strategy achieved great prominence mainly owing to its avoided use of dangerous liquids such as anhydrous hydrogen fluoride for cleavage from the solid support and final deprotection.²⁸

4.4.1 General peptides synthesis

The Fmoc-based solid phase peptide synthesis process was used to synthesize the L-amino acid and D-amino acid GKPILFF peptides. The rink amide resin (300 mg) was swelled in dimethylformamide (DMF, 10 ml) and placed in a 15 ml centrifuge tube. The resin was then transferred into a fritted reaction vessel, and a suction vacuum was used to remove the solvent. To deprotect the resin, 15 ml of 20% piperidine in DMF was added twice for $\pm$ 5 minutes each time. The reaction was filtered, and the resin was washed with DMF (3 X 10 ml) and dichloromethane (DCM, 3 X 10 ml), as shown in **Scheme 4.1**.



Scheme 4.1 General synthetic scheme for peptides

Subsequently, a solution of N-methylmorpholine (NMM, 1.0 M), O-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium hexafluorophosphate (HBTU, 1.9 M), and the first amino acid phenylalanine (2.0 M) were added to the reaction vessel, and the reaction was mixed by bubbling a gentle stream of nitrogen gas for 15 minutes. Upon filtration, the resin was washed with DMF (3 x 15 ml) and DCM (3 x 15 ml). A 20% piperidine solution (15 ml) was added to the resin to remove the protecting group for $\pm$ 5 minutes twice. The resin was then washed with DMF (3x15 ml) followed by DCM (3x15 ml). This method of deprotection and coupling was repeated for each amino acid added to the growing peptide chain on the resin. Once the full peptide was synthesized, it was then washed with (3 x 15 ml) DMF, and then (3 x 15 ml) DCM dried under suction. A cleavage mixture was prepared by mixing 9.6:2.5:1.5 % (v/v) trifluoroacetic acid (TFA): H₂O: triisopropylsilane (TIPS) in a 15 ml centrifuge tube. The dry resin was then transferred into the 50 ml centrifuge tube containing 20 ml of the cleavage mixture and placed in a shaker for 3 hours. The resin was filtered and washed with the cleavage mixture, and 50 ml of cold diethyl ether was added to the filtrate, which caused the peptide to precipitate. The samples were centrifuged at 5000 rpm for 10 minutes. The

supernatant was discarded, and the precipitate was washed with cold diethyl ether three times.

This method was mostly used in the beginning stages. However, we anticipated that several changes had to be employed to improve the efficacy of the method.

It is also worth noting that the fluorenylmethyloxycarbonyl (Fmoc) amino acids in the sequence GKPIILFF do not contain any side chain protecting groups, except lysine, which has the Boc protecting group.

Protecting groups commonly used to protect specific functional groups on the side chains of amino acids during peptide synthesis play a crucial role in directing the reaction site.²⁹ Protecting groups include:

- Boc (tert-butyloxycarbonyl) - This group is commonly used to protect the amine groups on the side chain of lysine and arginine.
- tBu (tert-butyl) - This group is commonly used to protect the hydroxyl group on the side chain of serine, threonine, and tyrosine.
- Trt (trityl) - This group is commonly used to protect the thiol group on the side chain of cysteine.

These groups block the reactive functional group on the side chain and prevent it from participating in unwanted reactions during peptide synthesis. However, the amino acids with no protecting groups on the side chain, such as Gly, Pro, Ile, Leu, and Phe, may contribute to lower steric hindrance during the reaction and higher efficiency. These protecting groups are acid labile and can be removed using TFA, revealing the original reactive functional group on the side chain.

4.4.2 Peptide purification

An Agilent 1260 Infinity preparative equipment HPLC with a UV/VIS detector (215 nm and 254 nm) and an automated fraction collector were used to purify the peptides on a Phenomenex Kinetex XB-C18 column (5 μ m 100 Å 250 mm x 21.2 mm) column at a flow-rate of 15 mL/min. Formic acid served as the ion-pairing agent in a two-buffer system. Buffers A and B contained 0.1% formic acid/H₂O (v/v) and 0.1% formic acid/acetonitrile (v/v), respectively. The peptide was eluted using a gradient method of 5 - 95% Buffer B for 15 minutes.

The peptide NH₂-GKPILFF-CONH₂ has seven amino acids and a molecular weight of 819,5007 g/mol. The net charge of the peptide is 1+ and is very water immiscible with an average hydrophilicity of -0.08 due to the non-polar groups such as glycine, proline, phenylalanine, leucine, and isoleucine.³⁰ The peptide only has one polar group, which is lysine, which may facilitate some hydrogen bonding which affected the solubility of the peptide and led to use of 1:1 DMSO: ACN to dissolve the peptide. After synthesis and cleavage of the short peptide, the sample was analyzed on the UHPLC-MS, and the mass found was [M+H]⁺ 820,5053 m/z, as shown in **Figure 4.7**. The peptide was eluted with a gradient method from 5 – 95 % ACN in 10 minutes with a 0.3 ml/minute flow rate. A two-buffer system was employed, Buffer A consisted of 0.1 % formic acid/H₂O (v/v), and Buffer B consisted of 0.1 % formic acid/acetonitrile (v/v). A Phenomenex Kinetex XB-C18 column (5 μ m, 100 Å, 250 mm $\times$ 21.2 mm) was used to separate the compounds.

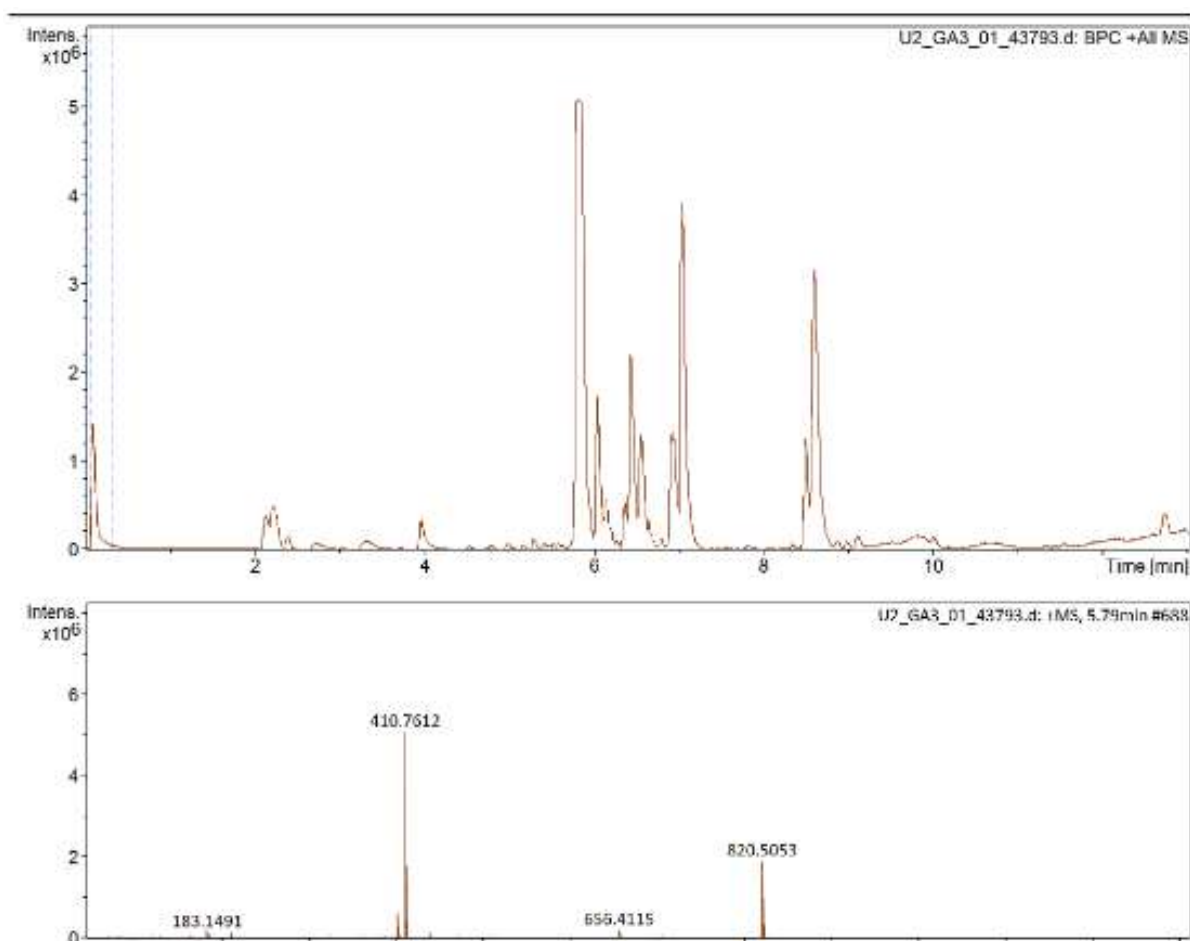


Figure 4.7 UHPLC-MS spectrum of crude GKPILFF peptide on the first attempt

4.4.3 Modifications for optimization

Upon studying the data from UHPLC-MS it was noted that the crude sample contained numerous impurities due to side reactions. However, for a short seven amino acid-containing peptide, this level of contamination was not expected.

Impurities in biological compounds can significantly impact experimental results and therapeutic applications. They can lead to misinterpretation of data, introduce variability and inconsistency in experiments, raise toxicity and safety concerns, cause undesired biological effects, complicate clinical trials, hinder quality control and analysis, result in legal and regulatory issues, and decrease the compound's shelf life and stability. To address these issues, rigorous optimization, thorough purification, and comprehensive characterization are crucial to ensure high purity and safety of biological compounds.

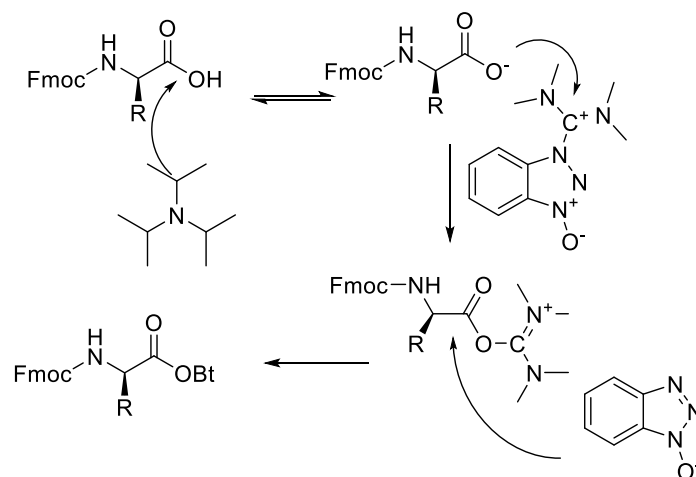
Short peptides such as the present peptide should be at less risk of side-chain reactions. Nonetheless, the mass peak of $[M+H]^+$ 820,5033 m/z detected on the instrument indicated that the peptide was successfully synthesized. The area under each peak in the chromatogram, which is detected using UV absorbance, can be used to determine the purity % of the sample. The crude sample was found to be approximately 25% of the desired peptide, and the rest of the sample contained side products that formed during synthesis. Although the quantity of the desired peptide was low, it remained the major product formed during synthesis, and several changes were employed to optimize the synthesis.

Several aspects can contribute to the formation of side products. The most common side products include:

1. Incompletely deprotected sequences
2. Sequences modified during cleavage (reconnection of protecting groups at other locations on the peptide)
3. Truncation sequences
4. Deletion sequences
5. Other side-reaction products that are formed during synthesis include oxidation reactions and the formation of aspartimide

The coupling reagents can also affect the efficiency of the peptide synthesis. HBTU is a popular coupling reagent used in solid-phase peptide synthesis. It activates carboxylic acids found on the amino acid of the peptide after deprotonation of the carboxylic acid group by diisopropylethylamine, as shown in **Scheme 4.2**. The HBTU produces a HOBT (Hydroxybenzotriazole), which further attacks the carbonyl group on the amino acid, creating a HOBT ester. This ester is more sensitive, which easily allows the incoming amino acid to form a bond.

The use of HATU instead of HBTU increased the efficiency of the peptide synthesis. This coupling reagent is known to be more reactive than HBTU, which also contributed to the lower reaction time; however, caution needs to be taken because the high reactivity can lead to more side reactions if the reaction time is too long or left unattended.



Scheme 4.2 Mechanism of deprotonation with DIPEA and activation with HBTU

Both HBTU and HATU are very effective coupling reagents and have very striking similarities; however, HATU contains a triazolopyridine ring structure rather than a benzotriazole ring structure seen in HBTU as illustrated in **Figure 4.8**, resulting in a much quicker reaction time with lower side reaction production during activation.

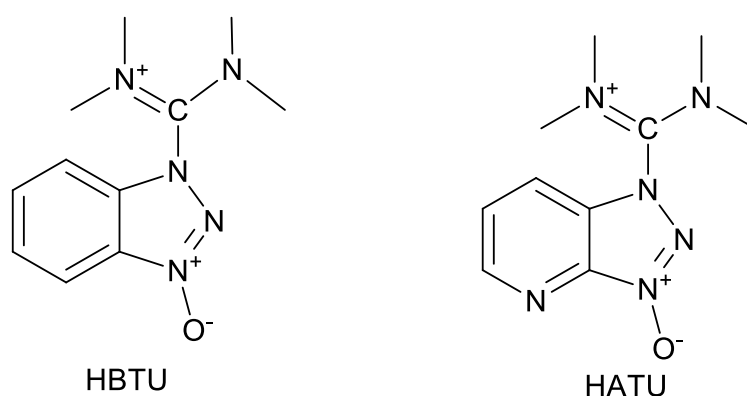
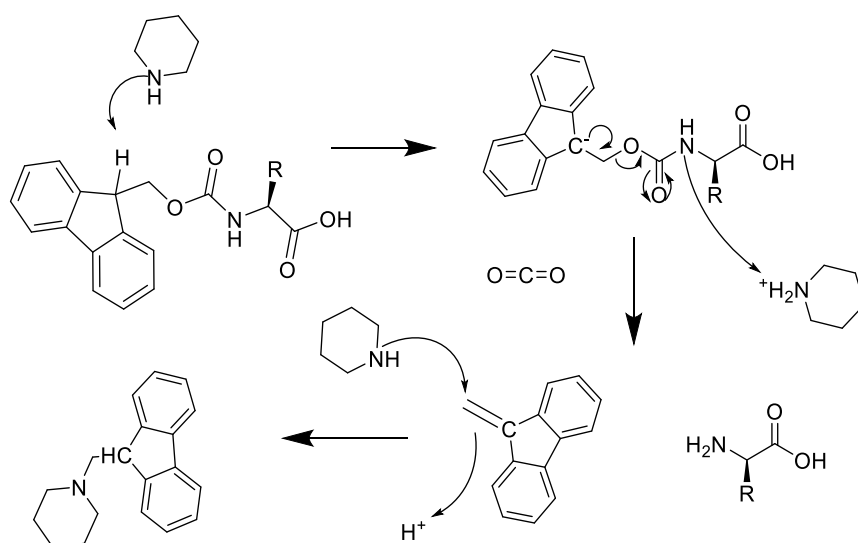


Figure 4.8 Side-by-side comparison of HBTU and HATU

The incorporation of DBU into the deprotection reaction with piperidine was also investigated, which significantly enhanced the efficacy since it is a much stronger base. Generally, a small amount of DBU (<5 %) is added to a 20% piperidine solution because it is highly reactive and may cause unwanted side products. When 1% or less is added, the reaction is improved, and impurities are minimized.

Therefore, the Fmoc (fluorenylmethyloxycarbonyl) removal step mediated by a secondary amine base, which conventionally employs piperidine/DMF (dimethylformamide), was one of the varied steps, and the alternative Fmoc-removal solution 1% DBU (1,8-diazabicyclo[5.4.0]undec-7-ene)/15% piperidine/DMF led to a drastic reduction of side products when compared to 20% piperidine/DMF.³¹ Piperidine is a heterocyclic amine organic reagent with a six-membered ring containing five methylene bridges and an amine bridge. This bulky base extract a proton from the Fmoc, which sets off a chain of reactions that result in the deprotection of the amino acid, as seen in **Scheme 4.3**.



Scheme 4.3 Mechanism of piperidine

Initially, diisopropylethylamine (DIPEA) was used in the preliminary stages of peptide synthesis. At this point, several method variations were being investigated. Diisopropylethylamine is a very bulky base and is relatively hindered. The basicity depends on the nature of the substituents on the nitrogen atom, generally increasing as the number of electron-donating methyl and methylene groups increases. This tertiary amine base is bulkier than N-methylmorpholine (NMM), which is another base we investigated. N-methylmorpholine (**Figure 4.9**) is a cyclic tertiary amine which is also rather bulky, and it was also used to synthesize the peptide.

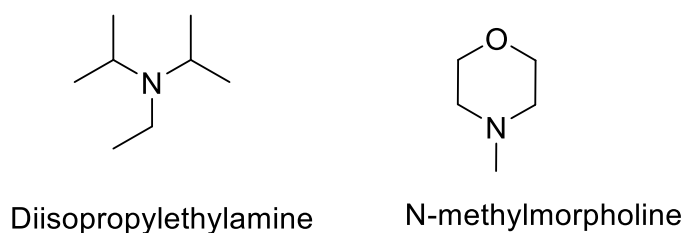


Figure 4.9 Side-by-side comparison of DIPEA and NMM

The intention was to compare the two bases and continue with the best reagent. However, the resulting peptides were very similar, and there was no significant effect when synthesizing these peptides. N-methylmorpholine was utilized moving forward even though, theoretically, it's not as strong a base as diisopropylethylamine. Additionally, diisopropylethylamine was not as readily available commercially, and it had little to no effect on peptide synthesis. In **Table 4.3** below, the variations in the peptide synthesis are summarized, and all the peptide synthesis were further made with NMM, HATU, and 15% piperidine with 1% DBU.

Table 4.3 Modification of the synthesis procedure of GKP peptide for optimization

ATTEMPT NO.	DEPROTECTING REAGENTS	TOTAL DEPROTECTING TIME (MIN)	COUPLING REAGENTS	TOTAL COUPLING TIME (MIN)
1	20% -piperidine	5x2	HBTU	35
2	20% piperidine	5x2	HATU	20
3	15% piperidine+ 1% DBU	5x2	HBTU	20
4	15% piperidine+ 1% DBU	5x2	HATU	15

After the procedure was optimized, the chromatogram of the crude sample was significantly improved compared to the first crude sample. The crude peptide samples were then dissolved in an acetonitrile/DMSO mixture or 100% DMSO. The UHPLC-MS spectrum (**Figure 4.10**) revealed that only two other components were present in the crude sample, which demonstrated that the changes that were introduced to the synthesis drastically decreased the formation of side products and impurities. Not only was the purification of the peptides straightforward, but the time required to synthesize the peptide was also reduced. It is also worth noting that the desired peak appears to be tailing, which implies that the DMSO in the sample needed to be reduced because it was interfering with the stationary phase.

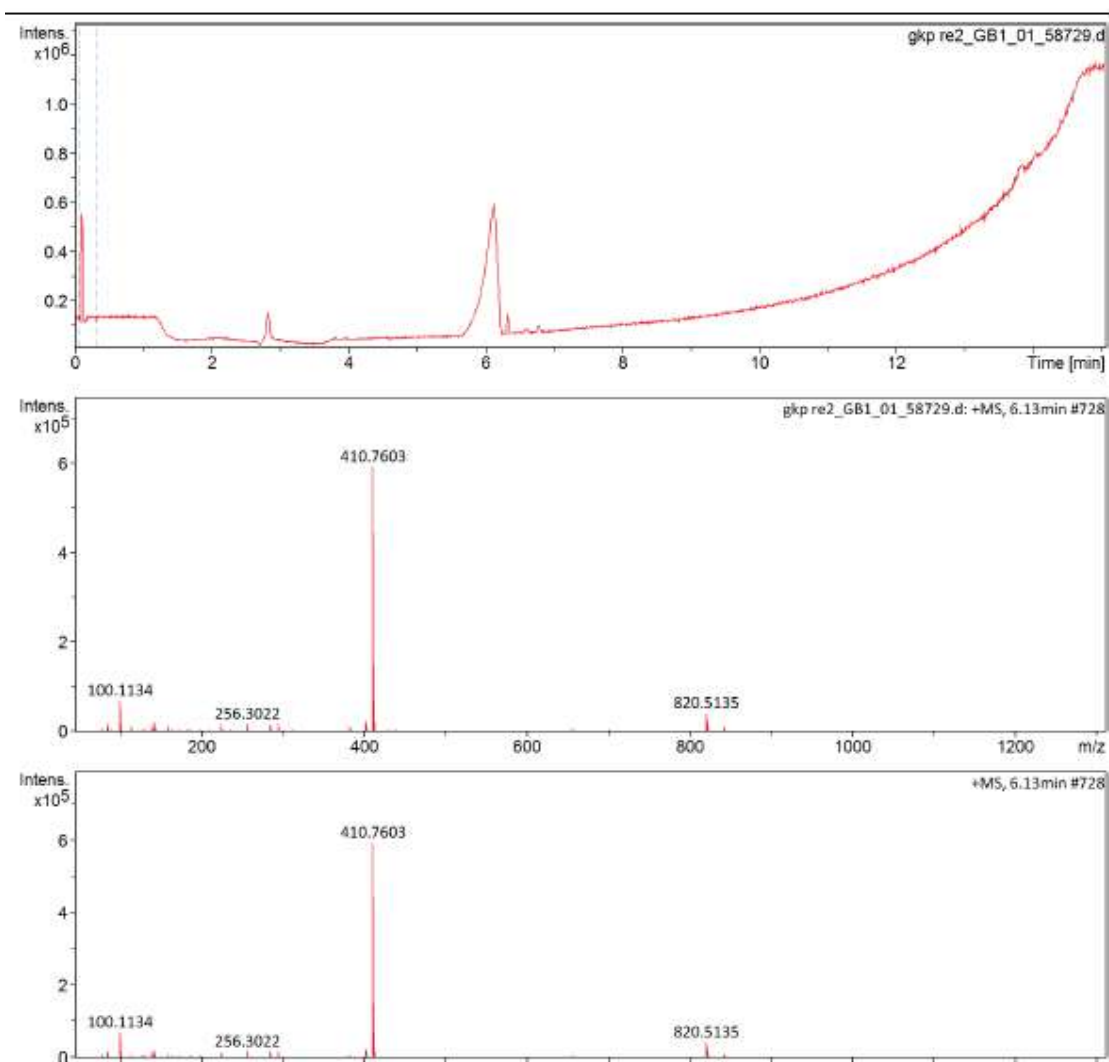
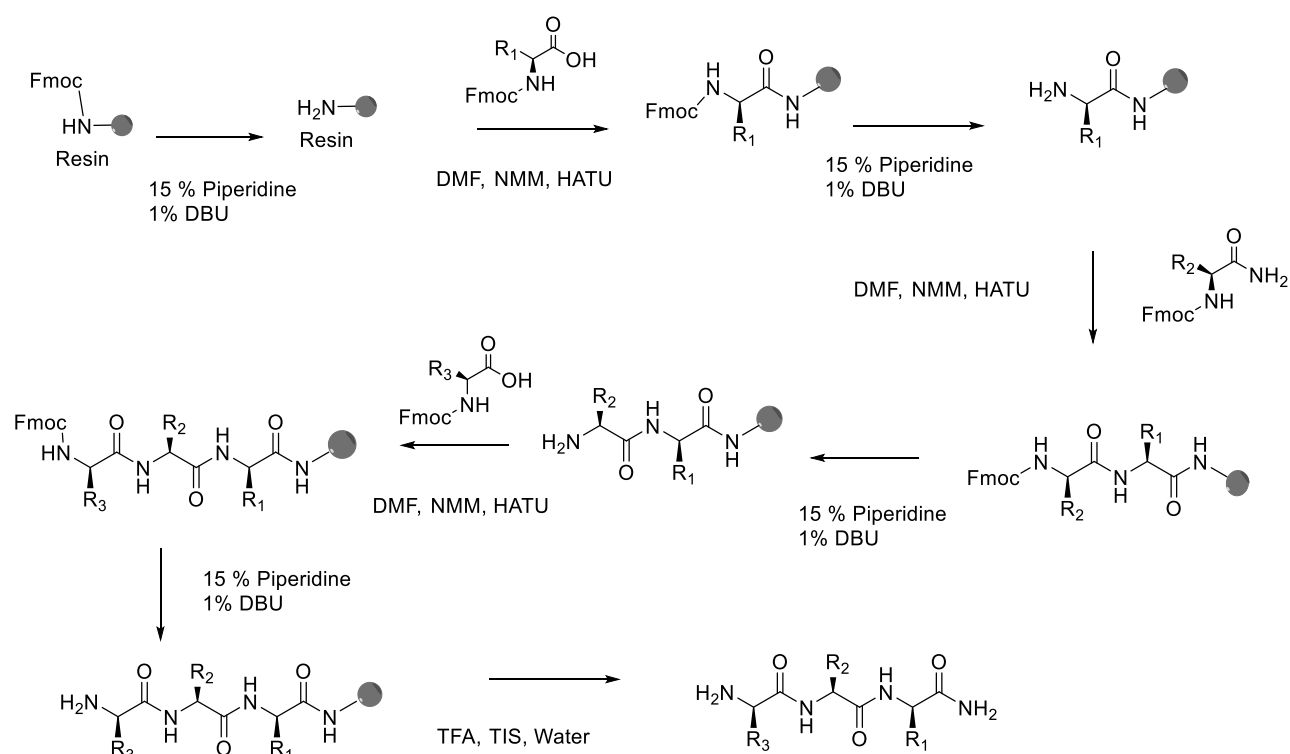


Figure 4.10 LCMS spectrum of the crude sample after optimization of the synthesis

4.5 Synthesis of the GKP peptide derivatives

All the peptides were then synthesized using NMM, HATU, and 15% piperidine with 1% DBU (optimized method mentioned in **section 4.4.3**). The UHPLC-MS data of the second and third trials of the GKP peptide synthesis is presented in the appendix, and the peptide synthesis procedure with the optimized modifications in the synthesis can be seen in **Scheme 4**. This peptide was then scaled up and divided into three parts for the derivatives containing adamantane and palmitic acid.



Scheme 4.4 Optimized procedure for the synthesis of peptides

4.5.1.1 Synthesis of the GKP peptide

The peptide was cleaved off the resin with a TFA solution following the procedure described in **section 4.4.1**. The purity was evaluated using the UHPLC-MS, which was found to be 97%. Once purification was complete, the peptide was dried using the rotary evaporator resulting in a mass of 7.0 mg and a yield of 35%. Thereafter, the synthesis was repeated and scaled up, which resulted in 80 mg of the GKPIILFF sequence peptide and a yield of 62% using the optimized procedure mentioned in **section 4.4.3**. The sample was dissolved in 100% DMSO, and the theoretical yield was calculated using the equations described in **Section 8.2.6**. From the UHPLC-MS spectrum (**Figure 4.11**) of the scaled-up GKP peptide, the desired mass of $[M+H]^+$ 820,5069 m/z was observed with a retention time of 8.94 minutes. This matched the molecular mass calculated (not shown) using ChemDraw which was 819.5007 g/mol and the chemical formula C₄₃H₆₅N₉O₇.

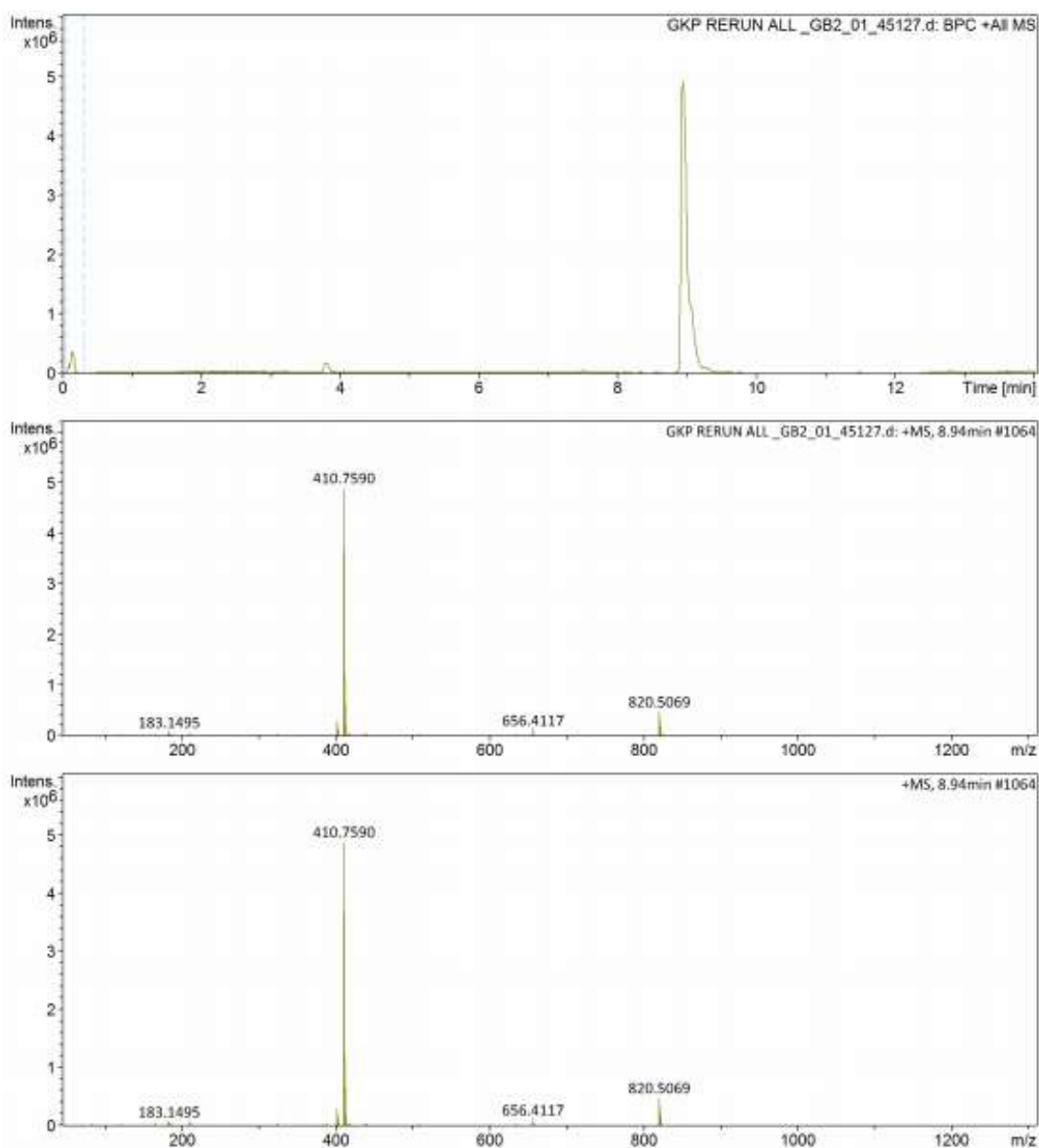


Figure 4.11 LCMS of purified GKPILFF

4.5.1.2 Synthesis of the GKP-ada peptide

The N-terminal adamantane analogues were synthesized by mixing the resin-bound peptide with 1-Adamantanecarboxylic acid (0.2 M, 0.432 g), NMM (1.0 M, 12.0 ml), and HBTU (0.863 g 0.19 M) in DMF. After 30 minutes of reaction time and gentle mixing, the solvent was removed using vacuum suction, and the resin was washed with DMF (3 x 15.0 ml) followed by DCM (3 x 15.0) and then dried under vacuum. The peptide was then cleaved off

the resin with a TFA solution following the procedure described in **section 4.4.1**. A 15 mg (yield of 38 %) of the GKP-ada peptide was obtained using the optimized procedure mentioned in **section 4.4.3**. The sample was dissolved in 100% DMSO. The HPLC-MS retention time was found to be 8.10 minutes as seen in **Figure 4.12**, and the mass found was $[M+H]^+$ 982.6120 m/z. The molar mass calculated using ChemDraw was 981.6052 g/mol and the chemical formula $C_{54}H_{79}N_9O_8$. The derivatives containing palmitic acid were also successfully synthesized and the final chromatograms are presented in the appendices.

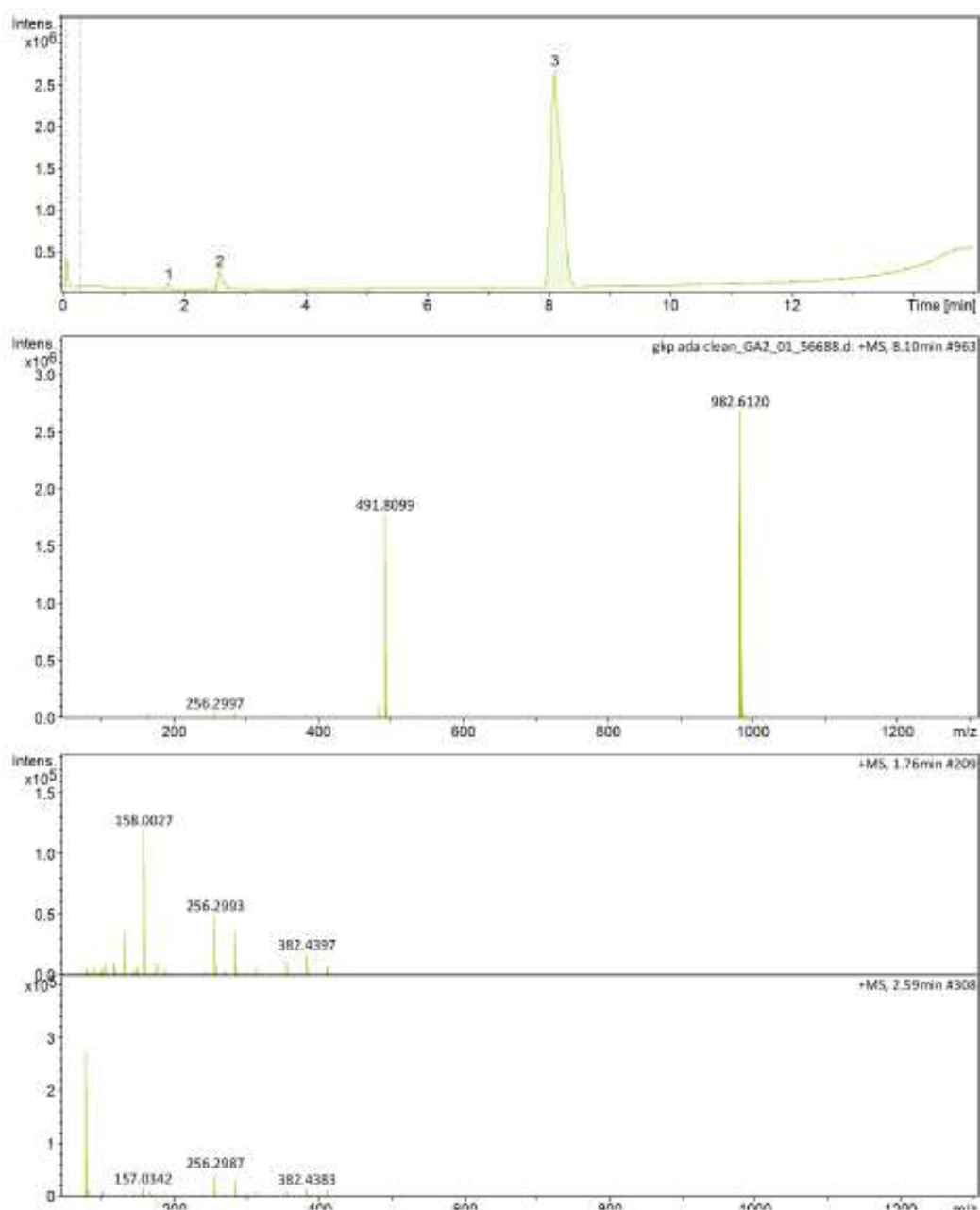


Figure 4.12 LCMS of crude GKPILFF-ada peptide

4.5.1.3 *Synthesis of the RGD-GKP peptide*

For the synthesis of the RGD-GKP peptide, the same optimized synthesis process outlined in **section 4.4.3** was utilized. After purification on prep-HPLC, a yield of 43% (21mg) was obtained. Peaks 2 and 3 were both the desired peptide, and the mass found was $[M+H]^+$ 1148.6233 m/z. The calculated mass determined using ChemDraw provided a value of 1147.6292 g/mol and the chemical formula $C_{55}H_{85}N_{15}O_{12}$. This was consistent with the expected mass. The different retention times may suggest the presence of conformational isomers, and the peptide eluted at 8.75- and 9.00-minutes retention time, as seen in **Figure 4.13**. Peak 1 was an impurity which eluted very close to the desired peptide and cause challenge during purification. Several strategies and conditions were attempted such as increasing the duration of prep_HPLC method and varying the ratio between acetonitrile and water. Moreover, smaller amounts of sample were loaded to avoid overloading and the fraction collecting for the peak of interest was reduced. Other prep-HPLC strategies such as varied colounm temperature and pH adjustment could be tried in future.

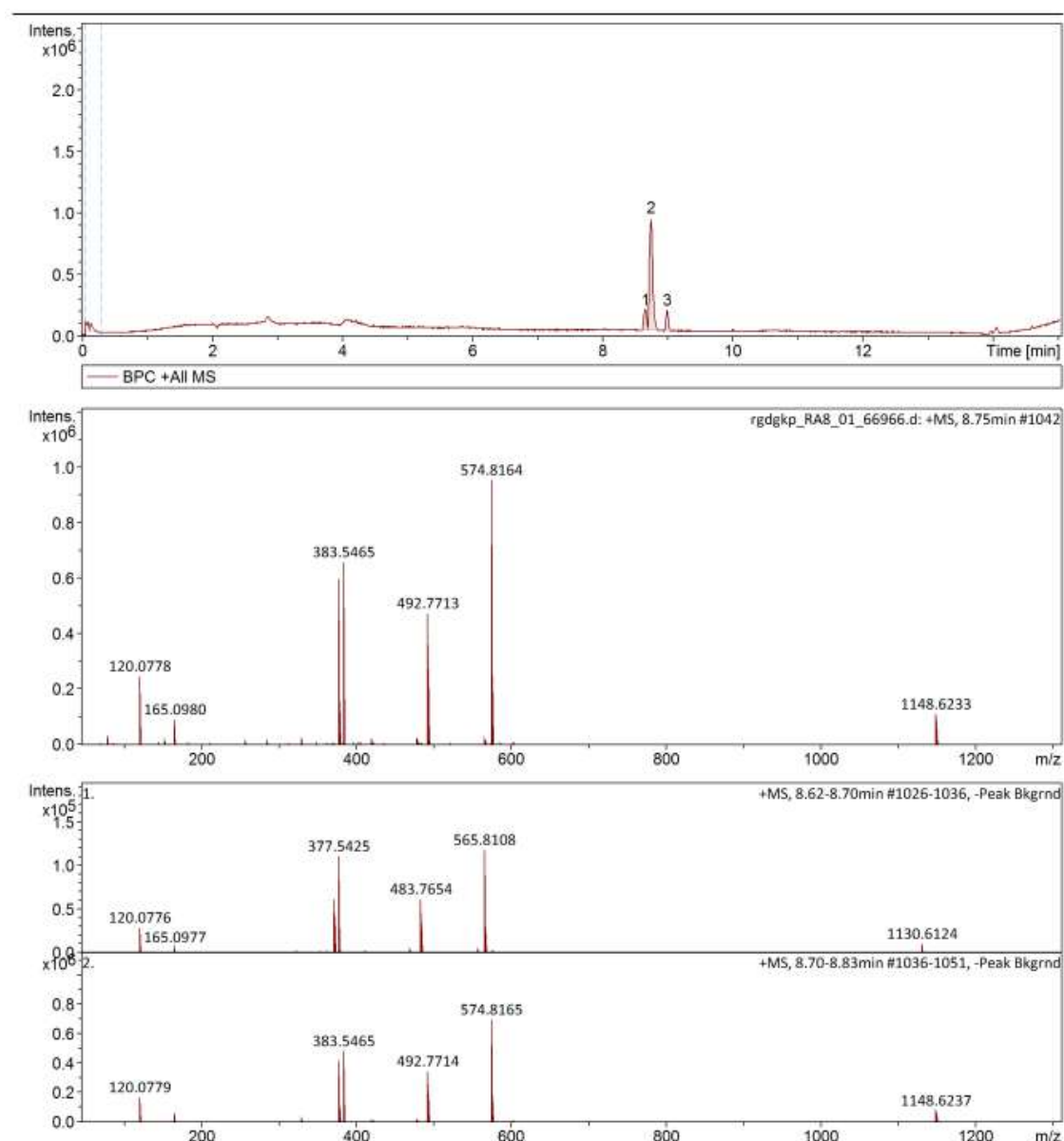


Figure 4.13 LCMS of purified RGDGKPILFF peptide

4.5.2 Synthesis of the BP peptide derivatives

4.5.2.1 Synthesis of the BP peptide

The same optimized procedure outlined in **section 4.4.3** was utilized in the synthesis of BP peptide and its derivatives. Purification on prep HPLC using the optimized procedure presented in **section 4.4.2**, yielded 54 mg (32 %) of the BP peptide in **Figure 4.14**, with a purity of 93%. The HPLC-MS retention time was found to be 6.7 minutes, and the mass found was $[M+3H]^{3+}$ 398.9391 m/z. This corresponded with the expected mass calculated

using ChemDraw and gave a value of 1193.8132 g/mol and molecular formula of $C_{50}H_{99}N_{25}O_9$.

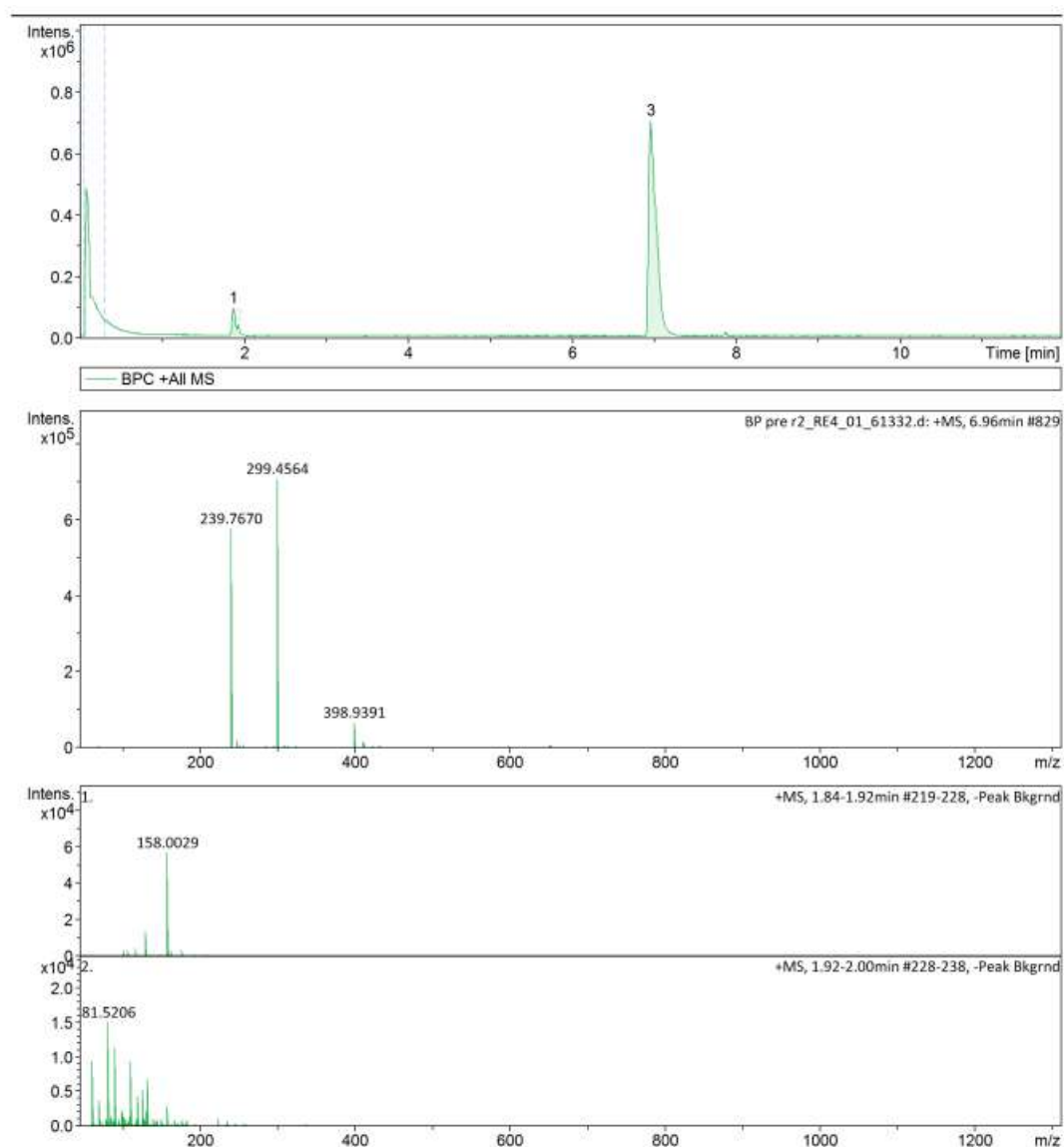


Figure 4.14 LCMS of purified RLRLRIGRR peptide sequence

4.5.3 Summary of all synthesized analogues

In summary, all the derivatives were successfully synthesized, and the yields were reasonable, as seen in **Table 4.4**. The different peptide derivatives resulted in different retention times due to the addition of lipophilic moieties, and the chromatograms are presented in the appendices.

Table 4.4 Summary of peptides derived from GKPIILFF and RLRLRIGRR sequences.

PEPTIDE	EXPECTED MASS (G/MOL)	MASS FOUND [M+H] ⁺	RETENTION TIME (MIN)	MASS (MG)	YIELD %
NH ₂ -GKPIILFF- COOH	819,5007 C ₄₃ H ₆₅ N ₉ O ₇	820,5070 [M+H] ⁺	8,94	80	62
NH ₂ -GKPIILFF- ADA	981,6052 C ₅₄ H ₇₉ N ₉ O ₈	982,6034 [M+H] ⁺	8,10	15	38
NH ₂ -GKPIILFF- PAL	1057,7304 C ₅₉ H ₉₅ N ₉ O ₈	1058,7377 [M+H] ⁺	14,20	21	42
D-NH ₂ -GKPIILFF	819,5007 C ₄₃ H ₆₅ N ₉ O ₇	820,5030 [M+H] ⁺	5,82	34	56
D-NH ₂ -GKPIILFF- ADA	981,6052 C ₅₄ H ₇₉ N ₉ O ₈	982,6044 [M+H] ⁺	7,08	16	39
D-NH ₂ -GKPIILFF- PAL	1057,7304 C ₅₉ H ₉₅ N ₉ O ₈	1058,7324 [M+H] ⁺	9,55	18	40
NH₂ - RGDGKPIILFF CONH₂	1147.6292 C ₅₅ H ₈₅ N ₁₅ O ₁₂	1148.6233 [M+H] ⁺	8.75	21	43
NH₂ - RLRLRIGRR CONH₂	1193.8132 C ₅₀ H ₉₉ N ₂₅ O ₉	398.9190 [M+3H] ³⁺	2.75	54	32
NH₂ - RLRLRIGRR - ADA	1355.9144 C ₆₁ H ₁₁₃ N ₂₅ O ₁₀	339.9728 [M+4H] ⁴⁺	9.53	52	25

PEPTIDE	EXPECTED MASS (G/MOL)	MASS FOUND [M+H] ⁺	RETENTION TIME (MIN)	MASS (MG)	YIELD %
NH₂- RLRLRIGRR - PAL	1432.0475 C ₆₆ H ₁₂₉ N ₂₅ O ₁₀	359.0077 [M+4H] ⁴⁺	12.07	49	62
D-NH₂- RLRLRIGRR CONH₂	1193.8147 C ₅₀ H ₉₉ N ₂₅ O ₉	597.8994 [M+2H] ²⁺	2.66	58	20
D-NH₂- RLRLRIGRR - ADA	1355.9174 C ₆₁ H ₁₁₃ N ₂₅ O ₁₀	452.9627 [M+3H] ³⁺	12.01	30	34
D-NH₂- RLRLRIGRR - PAL	1432.0498 C ₆₆ H ₁₂₉ N ₂₅ O ₁₀	359.0095 [M+4H] ⁴⁺	9.40	54	48

D-NH₂- represents the peptides with D-configured amino acids.

4.6 Conclusion

The peptides derived from the peptide accelerating sequence GKPIILFF and the cancer-selective peptide RLRLRIGRR were successfully synthesized. Furthermore, an optimized synthesis procedure was developed, which reduced the coupling time and increased the purity. The incorporation of adamantane and palmitic acid appeared to increase the retention time of the peptides. This is due to the hydrophobic nature of the moieties and the overall possible change in the secondary structure. The RGDGKP peptide appeared at a lower retention time of 8.75 minutes compared to the GKP peptide at 8.95, owing to the additional hydrophilic residues, arginine, and aspartic acid. The yields varied from 20% to 62%, which suggests that the cleavage and purification step significantly affected the final yield. The cleavage step may lead to side reactions that can potentially affect the peptides. Moreover, the impurities found in the samples varied, which affected how each peptide was purified. This challenge may be investigated in the future by varying the cleavage conditions for optimum results. These peptides will be sent to permeability studies to investigate how the incorporation of palmitic acid, adamantane and D-amino acids will affect the activity.

4.7 References

1. Apostolopoulos, V., Bojarska, J., Chai, T.-T., Elnagdy, S., Kaczmarek, K., Matsoukas, J., New, R., Parang, K., Lopez, O. P., Parhiz, H., Perera, C. O., Pickholz, M., Remko, M., Saviano, M., Skwarczynski, M., Tang, Y., Wolf, W. M., Yoshiya, T., Zabrocki, J., Zielenkiewicz, P., AlKhazindar, M., Barriga, V., Kelaidonis, K., Sarasia, E. M. & Toth, I. A Global Review on Short Peptides: Frontiers and Perspectives. *Molecules* **26**, 430 (2021).
2. Takayama, K., Nakase, I., Michiue, H., Takeuchi, T., Tomizawa, K., Matsui, H. & Futaki, S. Enhanced intracellular delivery using arginine-rich peptides by the addition of penetration accelerating sequences (Pas). *J. Controlled Release* **138**, 128–133 (2009).
3. Takayama, K., Hirose, H., Tanaka, G., Pujals, S., Katayama, S., Nakase, I. & Futaki, S. Effect of the Attachment of a Penetration Accelerating Sequence and the Influence of Hydrophobicity on Octaarginine-Mediated Intracellular Delivery. *Mol. Pharm.* **9**, 1222–1230 (2012).
4. Varkouhi, A. K., Scholte, M., Storm, G. & Haisma, H. J. Endosomal escape pathways for delivery of biologicals. *J. Controlled Release* **151**, 220–228 (2011).
5. Erazo-Oliveras, A., Muthukrishnan, N., Baker, R., Wang, T.-Y. & Pellois, J.-P. Improving the Endosomal Escape of Cell-Penetrating Peptides and Their Cargos: Strategies and Challenges. *Pharmaceuticals* **5**, 1177–1209 (2012).
6. Hao, X., Li, Q., Ali, H., Ali Zaidi, S. S., Guo, J., Ren, X., Shi, C., Xia, S., Zhang, W. & Feng, Y. POSS-cored and peptide functionalized ternary gene delivery systems with enhanced endosomal escape ability for efficient intracellular delivery of plasmid DNA. *J. Mater. Chem. B* **6**, 4251–4263 (2018).
7. Khodabakhsh, F., Salimian, M., Hedayati, M. H., Ahangari Cohan, R. & Norouzian, D. Challenges and advancements in the pharmacokinetic enhancement of therapeutic proteins. *Prep. Biochem. Biotechnol.* **51**, 519–529 (2021).
8. Soleymani-Goloujeh, M., Nokhodchi, A., Niazi, M., Najafi-Hajivar, S., Shahbazi-Mojarrad, J., Zarghami, N., Zakeri-Milani, P., Mohammadi, A., Karimi, M. & Valizadeh, H. Effects of N-terminal and C-terminal modification on cytotoxicity and cellular uptake of amphiphilic cell penetrating peptides. *Artif. Cells Nanomedicine Biotechnol.* **46**, 91–103 (2018).

9. Sui, Q., Borchardt, D. & Rabenstein, D. L. Kinetics and Equilibria of Cis/Trans Isomerization of Backbone Amide Bonds in Peptoids. *J. Am. Chem. Soc.* **129**, 12042–12048 (2007).
10. Dathe, M., Nikolenko, H., Meyer, J., Beyermann, M. & Bienert, M. Optimization of the antimicrobial activity of magainin peptides by modification of charge. *FEBS Lett.* **501**, 146–150 (2001).
11. Avrahami, D. & Shai, Y. A New Group of Antifungal and Antibacterial Lipopeptides Derived from Non-membrane Active Peptides Conjugated to Palmitic Acid *. *J. Biol. Chem.* **279**, 12277–12285 (2004).
12. Iwasaki, T., Ishibashi, J., Kubo, M., Taylor, D. & Yamakawa, M. Multiple Functions of Short Synthetic Enantiomeric Peptides Based on Beetle Defensins. *Biosci. Biotechnol. Biochem.* **73**, 683–687 (2009).
13. Saido-Sakanaka, H., Ishibashi, J., Momotani, E., Amano, F. & Yamakawa, M. In vitro and *in vivo* activity of antimicrobial peptides synthesized based on the insect defensin. *Peptides* **25**, 19–27 (2004).
14. Iwasaki, T., Ishibashi, J., Tanaka, H., Sato, M., Asaoka, A., Taylor, D. & Yamakawa, M. Selective cancer cell cytotoxicity of enantiomeric 9-mer peptides derived from beetle defensins depends on negatively charged phosphatidylserine on the cell surface. *Peptides* **30**, 660–668 (2009).
15. Feng, Z. & Xu, B. Inspiration from the mirror: D-amino acid containing peptides in biomedical approaches. *Biomol. Concepts* **7**, 179–187 (2016).
16. Liang, G., Yang, Z., Zhang, R., Li, L., Fan, Y., Kuang, Y., Gao, Y., Wang, T., Lu, W. W. & Xu, B. Supramolecular Hydrogel of a d-Amino Acid Dipeptide for Controlled Drug Release *in Vivo*. *Langmuir* **25**, 8419–8422 (2009).
17. Gopalan, B., Ponpandian, T., Kachhadia, V., Bharathimohan, K., Vignesh, R., Sivasudar, V., Narayanan, S., Mandar, B., Praveen, R., Saranya, N., Rajagopal, S. & Rajagopal, S. Discovery of adamantane based highly potent HDAC inhibitors. *Bioorg. Med. Chem. Lett.* **23**, 2532–2537 (2013).
18. Grillaud, M. & Bianco, A. Multifunctional adamantane derivatives as new scaffolds for the multipresentation of bioactive peptides. *J. Pept. Sci.* **21**, 330–345 (2015).
19. Carta, G., Murru, E., Banni, S. & Manca, C. Palmitic Acid: Physiological Role, Metabolism and Nutritional Implications. *Front. Physiol.* **8**, 902 (2017).
20. Carta, G., Murru, E., Banni, S. & Manca, C. Palmitic Acid: Physiological Role, Metabolism and Nutritional Implications. *Front. Physiol.* **8**, (2017).

21. Ledwoń, P., Errante, F., Papini, A. M., Rovero, P. & Latajka, R. Peptides as Active Ingredients: A Challenge for Cosmeceutical Industry. *Chem. Biodivers.* **18**, e2000833 (2021).
22. Zhao, X., Zhang, X. & Liu, D. Collagen peptides and the related synthetic peptides: A review on improving skin health. *J. Funct. Foods* **86**, 104680 (2021).
23. Yang, G., Gao, H., Luo, C., Zhao, X., Luo, Q., Shi, J. & Wang, F. Palmitic Acid-Conjugated Radiopharmaceutical for Integrin $\alpha\beta 3$ -Targeted Radionuclide Therapy. *Pharmaceutics* **14**, 1327 (2022).
24. Le Joncour, V. & Laakkonen, P. Seek & Destroy, use of targeting peptides for cancer detection and drug delivery. *Bioorg. Med. Chem.* **26**, 2797–2806 (2018).
25. Notni, J., Pohle, K. & Wester, H.-J. Be spoilt for choice with radiolabelled RGD peptides: Preclinical evaluation of ^{68}Ga -TRAP(RGD) $_3$. *Nucl. Med. Biol.* **40**, 33–41 (2013).
26. Temming, K., Schiffelers, R. M., Molema, G. & Kok, R. J. RGD-based strategies for selective delivery of therapeutics and imaging agents to the tumour vasculature. *Drug Resist. Updat.* **8**, 381–402 (2005).
27. van den Kerkhof, D. L., Nagy, M., Wichapong, K., Brouns, S. L. N., Suylen, D. P. L., Hackeng, T. M. & Dijkgraaf, I. Unraveling the role of the homoarginine residue in antiplatelet drug eptifibatide in binding to the $\alpha\text{IIb}\beta 3$ integrin receptor. *Thromb. Res.* **217**, 96–103 (2022).
28. Jadhav, K. B., Woolcock, K. J. & Muttenthaler, M. Anhydrous hydrogen fluoride cleavage in Boc solid phase peptide synthesis. *Methods Mol. Biol. Clifton NJ* **2103**, 41–57 (2020).
29. Musaimi, O. A., Torre, B. G. de la & Albericio, F. Greening Fmoc/ t Bu solid-phase peptide synthesis. *Green Chem.* **22**, 996–1018 (2020).
30. Condron, M. M., Monien, B. H. & Bitan, G. Synthesis and Purification of Highly Hydrophobic Peptides Derived from the C-Terminus of Amyloid β -Protein. *Open Biotechnol. J.* **2**, 87–93 (2008).
31. Yang, Y. & Hansen, L. Optimized Fmoc-Removal Strategy to Suppress the Traceless and Conventional Diketopiperazine Formation in Solid-Phase Peptide Synthesis. *ACS Omega* **7**, 12015–12020 (2022).

5. The total structure of the penetration accelerating sequences (Pas) by two-dimensional nuclear magnetic resonance spectroscopy.

Machumele, KP., Ntombela, T., Peme, T., Maubane-Nkadimeng, M.S. and Makatini, M. 2023. Title: The total structure of the penetration accelerating sequences (Pas) by two-dimensional nuclear magnetic resonance spectroscopy. Manuscript ID: MRC-23-0073. (Submitted-28 July 2023)

5.1 Abstract

Researchers are increasingly drawn to the short penetration accelerating sequence (Pas) because of its capacity to improve cellular absorption. The seven-amino acid sequence recognized by lysosomal protease cathepsin D promotes the endosomal escape of cell-penetrating peptides (CPPs) and improves the cytosolic release of the delivered cargo. To date, the peptide's mechanism is still unknown, and there is no available NMR data or other structural information. This study describes the determination of L and D- peptide secondary structures using two-dimensional (2D) NMR. Characterization of the peptides was confirmed using Ultra-high performance liquid chromatography Mass Spectrometry (UHPLC-MS), and preparative High-Pressure Liquid Chromatography (prep-HPLC) was used for purification. The 2-dimensional structure was elucidated using total correlation spectroscopy (TOCSY) and rotating-frame nuclear Over-Hauser effect correlation spectroscopy (ROESY). The L-GKPILFF peptide demonstrated the preferred helical structural conformation for membrane permeation, while the D peptides adopted a random coil structure which was confirmed using NMR, Circular dichroism (CD), and computational studies.

Keywords: Nuclear magnetic resonance, circular dichroism, molecular dynamics, TOCSY NMR; ROESY NMR, intracellular delivery peptides; penetration accelerating sequence;

5.2 Introduction

The GKPILFF fragment is a penetration-accelerating sequence (Pas) derived from the cathepsin D enzyme, a lysosomal aspartyl protease.¹ Literature has shown that when the Pas peptide is bonded to a bioactive agent, it can substantially enhance the cellular uptake of the agent.² In a study by Woldetsadik, A. D, the GKPILFF peptide was covalently coupled to an apoptosis-inducing pHK peptide, and as a result, an enhancement in cellular uptake and cell death efficacy was observed.³ In similar studies, the Pas has been reported to promote the endosomal escape of cell-penetrating peptides (CPPs) and thus improve the cytosolic release of the delivered cargos.⁴ Though the peptide is found in literature and used to enhance cell penetration, the structure-activity relationship is not fully understood, and elucidating the peptide's 3-dimensional structure may provide insight. In this study, 2-dimensional NMR experiments were conducted to fully elucidate the structural conformation of the L and D isomers of the seven amino acid sequence, GKPILFF. The D amino acid peptide was synthesized to explore the structure-activity relationship and to improve protease stability.⁵

According to the literature, peptides containing D-amino acids are resistant to degradation owing to the preference of proteases binding to L amino acids to hydrolyse the peptide bond.⁶ This is because D-amino acids are stereo-chemically unfavourable to peptidases.^{7,8} Additionally, D-amino acids can introduce a degree of structural rigidity to the peptide, which can further increase its stability.⁹ However, the structural changes induced by the use of D-amino acid can cause a loss of activity due to the structure-activity relationship.¹⁰ Other modification strategies, such as the stapled hydrogen bond surrogates (SHBs) approach, can also be utilized to reduce enzyme susceptibility.¹¹ SHBs consist of a peptide sequence that is covalently linked by a hydrocarbon staple. This rigidifies the molecule's structure and promotes hydrogen bond-like interactions enforcing a specific or preferred secondary structure, typically an alpha helix.¹²

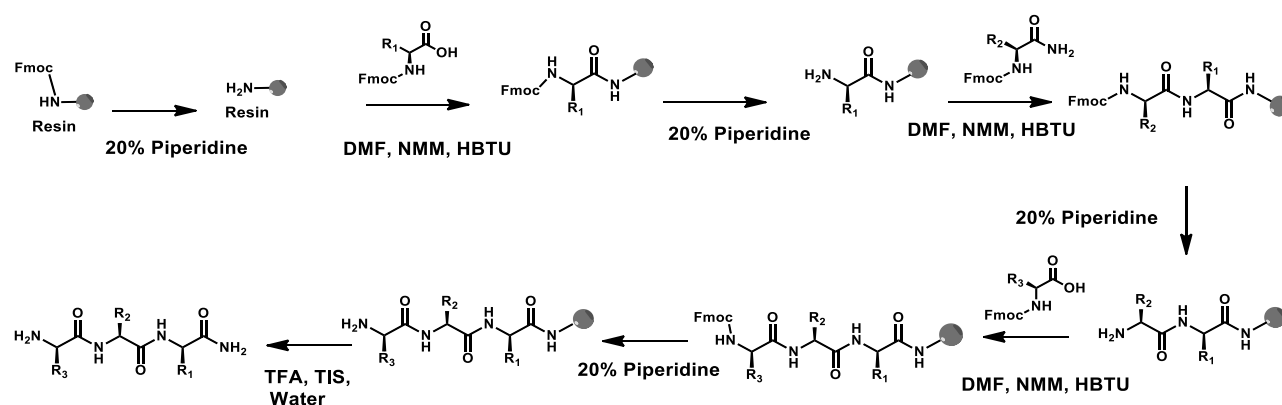
The peptide conformation can affect its ability to interact with receptors, enzymes, and antibodies¹³; hence, it significantly impacts the peptide's therapeutic potential.¹⁴ The peptide conformation is determined by its amino acid sequence and can be influenced by the surrounding environment, such as pH, temperature, and the presence of other molecules.^{15,16} Herein, the secondary structure of the GKPILFF peptide is elucidated using NMR techniques and molecular dynamics.

5.3 Materials

Chlorotriptyl resin, triisopropylsilane (TIPS), formic acid, trifluoroacetic acid (TFA), piperidine, *N, N, N', N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), paraformaldehyde, *p*-toluenesulfonic acid, toluene, diisopropylethylamine (DIPEA), *N*-Methylmorpholine (NMM) were purchased from Dld Scientific. Dichloromethane (DCM) was purchased from Sigma Aldrich, and dried upon distillation over calcium hydride. Dimethylformamide (DMF), acetonitrile, dimethyl sulfoxide, and ether were purchased from Radchem.

5.4 Synthesis of peptides

The synthesis of the L-amino acid and D-amino acid GKPIILFF peptides followed the general Fmoc-based solid phase peptide synthesis protocol.⁶ In a 15 ml centrifuge tube, the rink amide resin (300 mg) was swollen in DMF (10 ml). The resin was then transferred into a fritted reaction vessel, and a suction vacuum was used to remove the solvent. To deprotect the resin, 15 ml of 20% piperidine in DMF was added twice for 5 minutes each time. The reaction was filtered, and the resin was washed with DMF (3 X 10 ml) and DCM (3 X 10 ml), as shown in **Scheme 5.1**.



Scheme 5.1 Synthetic scheme of peptide formation

Subsequently, a solution of NMM (1.0 M, 12.0 ml), HBTU (1.9 M), and the first amino acid phenylalanine (2.0 M) were added to the reaction vessel, and the reaction was mixed by bubbling a gentle stream of nitrogen gas for 15 minutes. Upon filtration, the resin was washed with DMF (3 x 15 ml) and DCM (3 x 15 ml). A 20% piperidine solution (15 ml) was

added to the resin to remove the protecting group for ± 5 minutes twice. The resin was then washed with DMF (3 $\times$ 15 ml) followed by DCM (3 $\times$ 15 ml). This method of deprotection and coupling was repeated for each amino acid added to the growing peptide chain on the resin. Once the full peptide was synthesized, it was then washed with (3 x 15 ml) DMF, and then (3 x 15 ml) DCM dried under suction. A cleavage mixture was prepared by mixing 9.6:2.5:1.5 % (v/v) TFA: H₂O: TIPS in a 15 ml centrifuge tube. The dry resin was then transferred into the 50 ml centrifuge tube containing 20 ml of the cleavage mixture and placed in a shaker for 3 hours. The resin was filtered and washed with the cleavage mixture, and 50 ml of cold diethyl ether was added to the filtrate, which caused the peptide to precipitate. The samples were centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded, and the precipitate was washed with cold diethyl ether three times. The white powder was then dissolved and purified using prep-HPLC. The sample was analyzed on the UHPLC-MS, and the mass found was $[M+H]^+$ 820,5030 m/z with 97% purity **Figure S5.1 (supplementary information)**. The loading capacity was found to be 0.065 mmol/g, and the final yield was 62%.

5.5 Results and discussions

5.5.1 NMR elucidation of L-GKPILFF peptide

The structure of the L-GKPILFF peptide was characterized using ¹H-/¹³C-, TOCSY, and ROESY NMR spectroscopy to determine the primary structure shown in **Figure 5.1**, and to also evaluate the possible secondary structure formation.

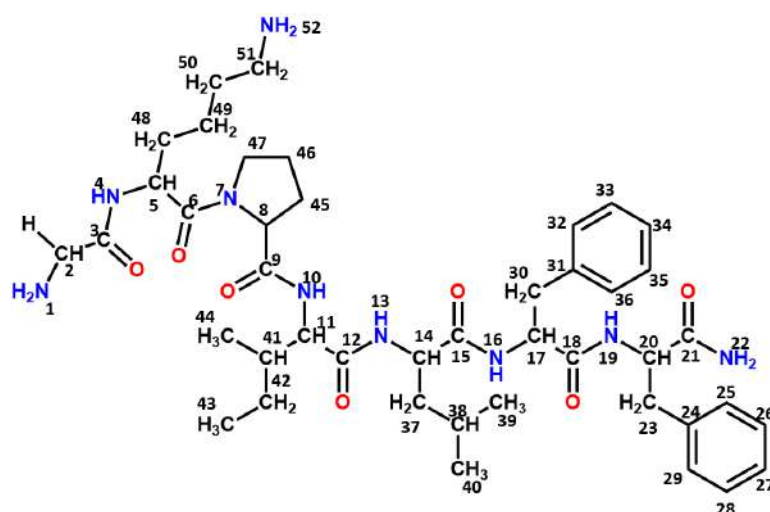


Figure 5.1 An illustration of the primary structure of the GKPILFF penetration acceleration sequence

For elucidating the structure, conformation, dynamics, and connections of peptides and proteins, amide peaks in NMR spectra are fundamental. Due to the well-established and distinct spin patterns they produce, amide protons are an important starting point because they provide significant data about the surrounding environment. Once the amide protons were assigned, other protons such as the alpha and beta protons could easily be identified.

The ^1H 1D NMR experiment was obtained at the optimal temperature of 293.15 K, and the spectrum is shown in **Figure 5.2**. The figure reveals the presence of amide peaks from the backbone of the L-GKP peptide between 7.5 ppm and 8.5 ppm, which are very crucial in the identification of atom-specific chemical shifts. The region between 7.25 ppm and 6.75 ppm contains the distinct aromatic protons from the two phenylalanines found in the L-GKPILFF sequence, which makes it easier to assign the sequence and determine correlations.

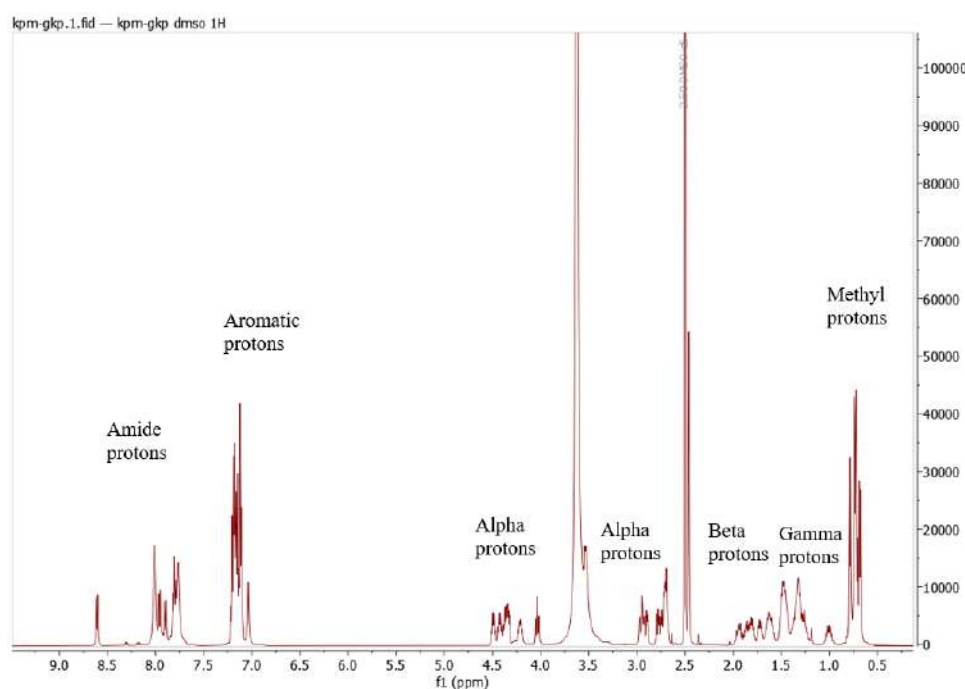


Figure 5.2 ^1H NMR spectrum of the L-GKPILFF peptide.

Illustrated in **Figure 5.3a** and **Figure 5.3b**, the ^1H NMR between 8.7 ppm and 7.6 ppm for the L- and D-GKPILFF peptide shows six spin patterns in the amide region. The seventh spin system, which was attributed to the proline, appears in a different region owing to the distinguishable lack of the amide proton in the proline residue seen in **Figure S5.2 (supplementary information)**; however, further characterization was conducted in the

section below for confirmation. There is a shift upfield of approximately 0.1 ppm in the peaks that are attributable to the conformational differences in the secondary structure between the L and D-peptides. A trend towards lower ppm levels is referred to as a "upfield shift" and suggests a more shielded proton environment in the peptide backbone. Moreover, the peak appearing at 7.71 ppm in the L peptide shifted to 7.95 ppm in the D peptide.

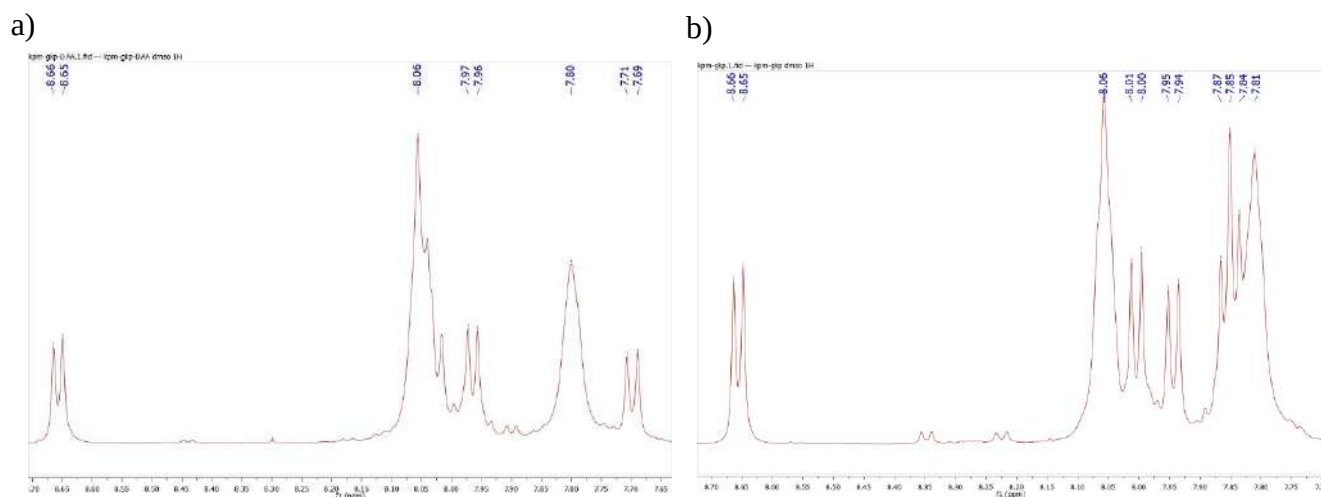


Figure 5.3 An amide region in the ^1H NMR spectra of the GKPILFF peptides a) L peptide and b) D peptide.

During purification, the GKP peptide coeluted with a truncated peptide sequence, and its low-intensity peaks are observed at chemical shifts, such as 8.35 ppm and 8.25 ppm. The GKP peptide was obtained with 97% purity (62% yield), and the truncated peptides made up 3% of the analysed sample. The structure of the truncated peptide was also elucidated and was discussed further later in this chapter.

The TOCSY 2D ^1H NMR experiment was used for the elucidation of the peptide's spin systems. In the spectra of the L-, and D peptides in **Figure 5.4**, the amide protons are between 7.75 ppm and 8.70 ppm, as shown with six dashed lines representing each spin system. The area between 8.10 and 7.95 ppm in the ^1H NMR are three amide protons in very close proximity, which can be distinguished from each other in TOCSY and ROESY NMR. Interestingly, the spin systems of the truncated peptide match the spin pattern of some of the GKP peptide amino acid residues and appear close to the peptide of interest. This further validates the suggestion that the impurity may be a truncated peptide. The UHPLC-MS spectrum showed the mass of the coeluted side product to be 656.4117 $[\text{M}+\text{H}]^+$ which correlates to the GKPILF sequence seen in **Figure S5.1 (supplementary information)**.

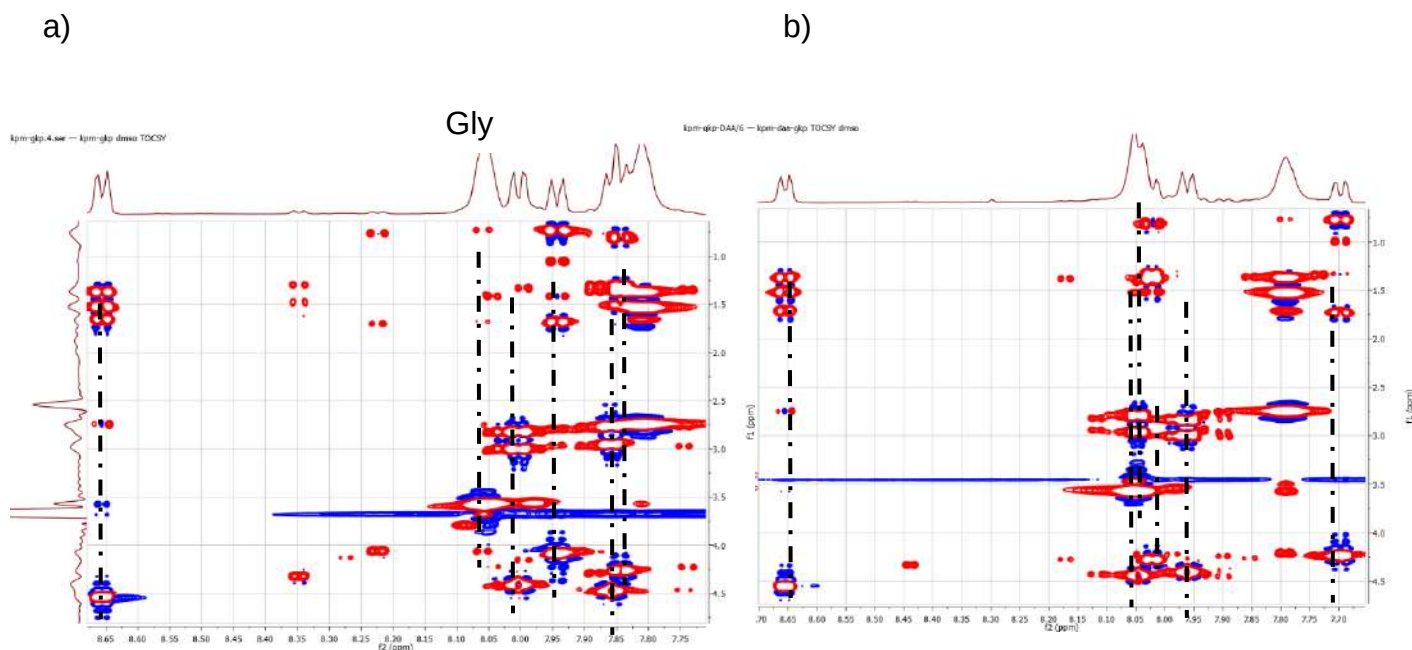


Figure 5.4 A TOCSY NMR spectra of the GKPIILFF peptide in the amide region with dashed lines representing each spin system between 7.75 ppm and 8.70 ppm for a) the L peptide and b) D-peptide.

The identification and sequential assignment of the amino acids in the peptide were assigned using a combination of TOCSY, HSQC, COSY, and ROESY NMR due to the complex assignment of the peptide. The first amino acid that was readily recognized was the glycine residue which is widely known to exhibit a single peak and has only two α -protons whose chemical shift appears around 3.5-4 ppm.¹⁷ In this instance, the two alpha protons' have nearly equal chemical shifts, which caused an overlap. The HN-H coupling is detected for protons on the same amide H line in the COSY or TOCSY spectra at NH α 8.06 ppm. Furthermore, this showed a H_i^α - $H^{N_{i+1}}$ correlation to the lysine spin system at (8.65,3.57) ppm, as seen in **Figure 5.5**.

The Lys2 has a distinct feature which is the ϵ -protons of lysine's side chain, which resonate at around 2.6-3.0 ppm, while the α -protons resonate at around 3.4-4.5 ppm, which is observed in **Figure 5.4**. The spin system with NH at 8.65 ppm attributed to Lys correlated to the α -proton at 4.56 ppm, β -protons at 1.54 and 1.66 ppm, δ - protons at 1.37, and finally ϵ - protons at 2.75 ppm.¹⁸ The Lys correlation to 3.58 glycine and proline $^1H\beta$ 3.67 on the ROESY at

8.65 ppm was observed, as shown in **Figure 5.5**. The proline spin system was observed in the TOCSY NMR spectra in the region between 4.56 and 4.06 ppm in **Figure S5.2 (supplementary)**. The proline was attributed to the spin system with the α -proton at 4.38 ppm, β -protons at 3.67 ppm, γ - protons at 1.74 and 1.94 ppm. Since there is a lack of NH in Pro3, the sequence continues at (7.94,4.38), which is the $H_i^a - H^{N_{i+1}}$ connectivity from the α -proton of proline and the NH of Ile4.

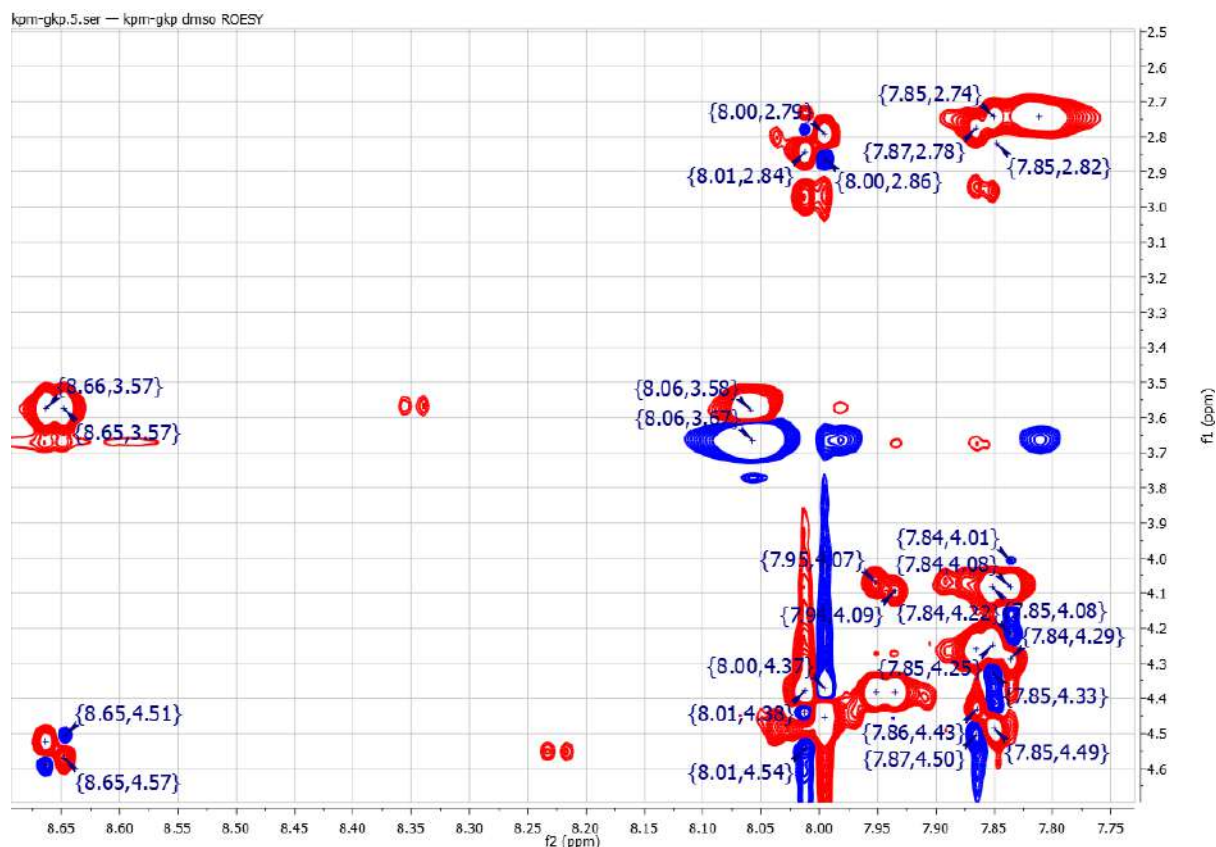


Figure 5.5 A Sequential walk of the ROESY spectra in the region between 8.85 and 7.55 ppm connecting Gly-1, Lys-2, and Pro-3.

The methyl resonances with chemical shifts of 0.81 and 0.73 ppm were exploited to identify leucine and isoleucine.¹⁹ The leu5 was attributed to the spin system with NH at 7.85 ppm correlating to the α -proton at 4.26 ppm, β -protons at 1.34 ppm, γ - protons at 1.50ppm and δ -protons at 0.83 ppm which are the methyl groups. The HSQC correlation showed that the carbon at 41.14 correlates to two protons at 1.31-1.34 ppm, which are in proximity, as seen in **Figure S5.3 (supplementary information)**. This is distinct from the Ile4, which was assigned to the spin system with NH at 7.94 correlating to the α -proton at 4.08 ppm, β -proton

at 1.68, γ - protons at 1.05 ppm and 1.40 ppm and δ - protons at 0.73 ppm of the two methyl groups. The distinct feature of the Ile is the CH₂ at 1.05 ppm and 1.40 ppm, which both correlate with the methyl groups 0.73-0.78 ppm seen in the COSY NMR in **Figure S5.4 (supplementary information)**. Additionally, the α -H of Ile4 at 4.08 ppm correlates with the NH of Leu5 at 7.85 ppm, which is Hi ^{α} -H^N_{i+1} on the ROESY NMR in **Figure 5.6**.

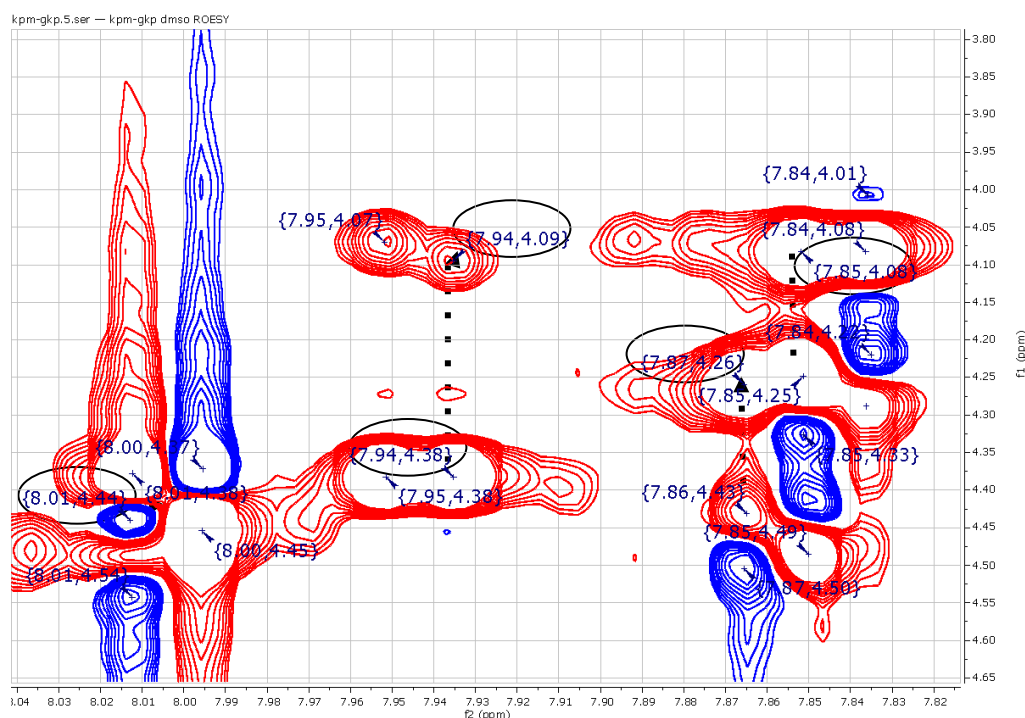


Figure 5.6 A magnification of the sequential walk on the ROESY spectra in the region between 8.02 and 7.85 ppm connecting Ile-4, leu-5, Phe-6, and Phe-7.

The only repeating unit found in the peptide sequence is phenylalanine, and we expect the spin systems to be equivalent in the TOCSY spectrum.²⁰ It was facile to assign the short spin systems for Phe (consisting of only an α -H, β , and γ protons) using COSY or TOCSY spectra. Two equivalent spin systems were observed at 8.01 and 7.86 ppm in the TOCSY spectrum, and using the ROESY experiment, the spin systems were distinguishable. This was accomplished by identifying the Hi ^{α} -H^N_{i+1} connectivity of the α -proton of Leu at 4.26 ppm and the NH of Phe 6 at 7.87 ppm. The protons of the spin system of Phe 6 were observed at NH 7.86 ppm, α -proton at 4.48 ppm, and β -protons at 2.76, 2.95 ppm. Finally, the spin system of Phe7 was observed at NH 8.01 ppm, α -proton at 4.40 ppm, and β -protons at 3.00, 2.82 ppm. The cross-correlation was observed at (7.68, 4.43) ppm for the Phe 6 NH and α -proton of Phe 7.

Therefore, the NMR data from the ROESY and TOSCY demonstrates the successful assignment of the amino acids in the peptide. Additionally, the sequential ROESY-walk further confirms the assignment of the amino acids in the Pas sequence, which is summarized in **Table 5.1**.

Table 5.1 Assignment of NMR chemical shifts (ppm) of long-range interaction in L-GKPILFF

Spin system no.	Residue	Residue correlation	H-NH	¹ H _α	C _α	¹ H _β	¹ H _γ	¹ H _δ	ROESY correlation
1	(Lys-2)	(Lys-2)–(Pro-3)	8.65	4.56	50.7	1.54,1.66	1.37	2.75	(8.65,3.67)
2	(Gly-1)	(Gly-1)–(Lys2)	8.06	3.57	40.4				(3.57, 8.65)
3	(Phe-7)	(Phe-7)	8.01	4.40	54.3	3.00,2.82			
4	(Leu-5)	(Ile-4)–(Leu-5)	7.85	4.26	57.6	1.34 *CH2 (C-41.82)	1.50	0.81	(7.85,4.08)
5	(Phe-6)	(Phe-6)–(Phe-7)	7.86	4.48	51.4	2.76, 2.95			(7.86,4.40)
6	(Ile-4)	(Leu-5)–(Phe-6)	7.94	4.08	59.8	1.68 *CH (C-36.6)	1.05, 1.40 *CH2 (C-24.8)	0.73	(7.94,4.38)
7	(Pro-3)	(Pro-3)–(Ile-4)		4.38	38.9	3.67	1.74,1.94		

As previously mentioned, the truncated peptide exhibited properties that were like the GKPILFF peptide, and now that the main peptide residues were assigned, the truncated peptide could be investigated. The peaks at 8.35, 8.23, 8.08, 7.97, and 7.75 ppm in the TOCSY NMR match lysine, isoleucine, glycine, leucine, and phenylalanine spin patterns. The spin system of the impurities indicates a truncated peptide; however, other impurities found in the spectra couldn't be assigned but are present in the sample.

The ROESY NMR spectrum of the D peptide in yellow was then compared to the ROESY NMR spectrum of the L peptide in red, as seen in **Figure 5.7**. The only difference seems to be the shift in the peaks which is a direct result of the conformational difference in the structure. Furthermore, the previously mentioned peak appearing at 7.71 ppm in the L peptide, which shifted to 7.95 ppm in the D peptide, is ascribed to isoleucine.

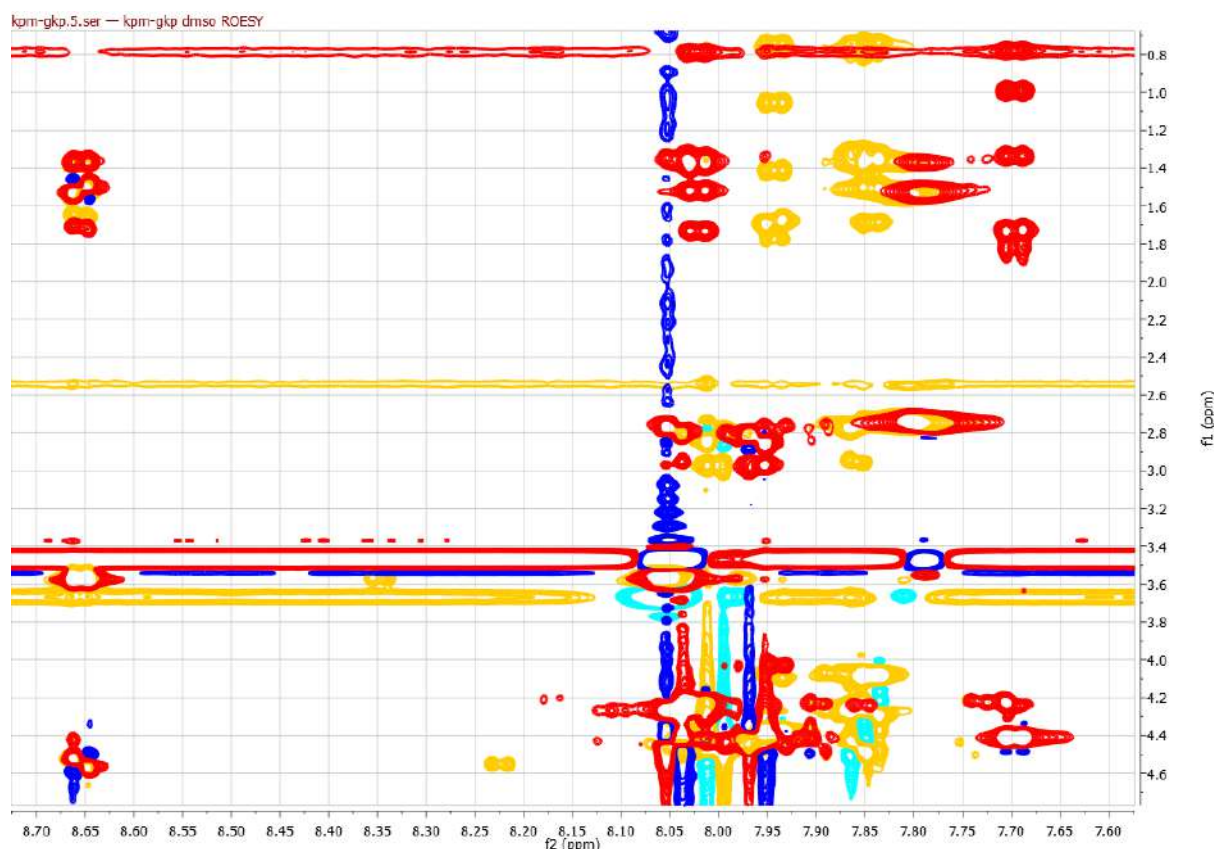


Figure 5.7 A ROESY overlapping spectra of the L- GKPILFF peptide in red and blue and D- GKPILFF peptide in yellow and turquoise in the region between 8.70 and 7.65 ppm.

5.5.2 Full structural elucidation using HSQC NMR

The identification of the amino acid residues was used as a foundation for fully assigning all of the other proton and carbon atoms present in the peptide. The HSQC (Heteronuclear Single Quantum Coherence) experiment was used to determine proton-carbon single bond correlations, where the protons lie along the observed F2 (X) axis, and the carbons are along the F1 (Y) axis. In **Figure 5.8**, HSQC spectra of the aromatic area are displayed between

130-124 ppm F1 (Y) axis and 7.10-7.05 ppm F2 (X) axis. The ten aromatic protons found on the ^1H NMR were used to assign the corresponding carbons. Five peaks were observed since the sixth carbon was quaternary. The carbonyl carbons were easily confirmed on the ^{13}C NMR, and all appeared above 165 ppm since they disappeared on DEPT NMR as shown in **Figure S5.5** and **Figure S5.6**, respectively (**supplementary information**). The aromatic protons were easily identified on the ^1H NMR, and the aromatic carbons were correlated using HSQC NMR. This provided all the information required for the full elucidation which was summarized in **Table 5.2**.

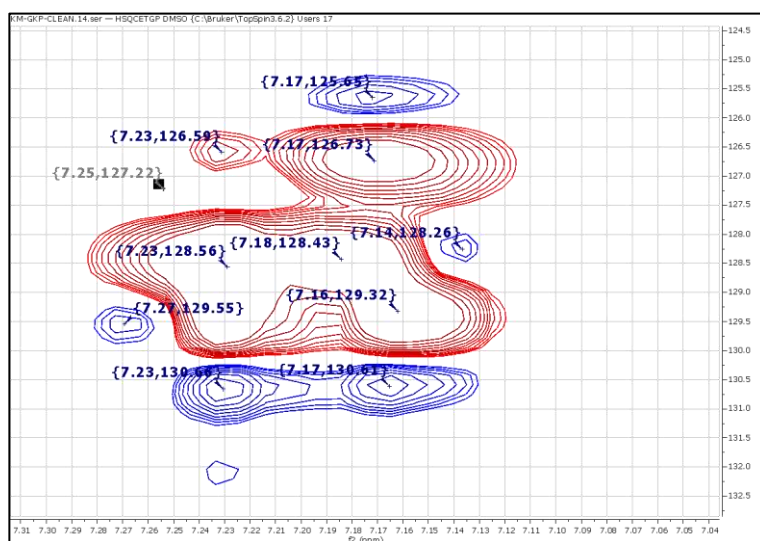


Figure 5.8 A magnification of the HSQC NMR spectra in the region between 7.31 and 7.04 ppm in the F2(X) axis and 132.5 and 124.5 ppm in the F1(Y) axis for the L-peptide

Table 5.2 Full assignment of all the protons and carbons in the L-peptide.

Atom	$^1\text{H}_{\text{a,b,c}}$	^{13}C	Atom	$^1\text{H}_{\text{a,b,c}}$	^{13}C
1	7,1	-	27	7,23	128,56
2	3,57	40,38	28	7,23	126,59
3	*no proton	166,18	29	7,17	126,73
4	8,06	*no carbon	30	3.00, 2,82	37,92
5	4,56	50,74	31	*no proton	138,16
6	*no proton	169,94	32	7,16	129,35
7	*no proton	*no carbon	33	7,18	128,43
8	4,38	59,77	34	7,23	128,56
9	*no proton	170,94	35	7,23	126,59
10	7,94	*no carbon	36	7,17	126,73
11	4,08	57,55	37	1,34	41,82
12	*no proton	171,34	38	1,50	41,82
13	7,85	*no carbon	39	0,77	23,42
14	4,26	51,36	40	0,83	21,79
15	*no proton	171,79	41	1,68	36,72
16	7,94	*no carbon	42	1,05,1,40	24,75
17	4,39	54,31	43	0,73	11,32
18	*no proton	172,15	44	0,76	15,63
19	8,01	*no carbon	45	3,67	47,42
20	4,48	50,71	46	1,74,1,94	29,34
21	*no proton	173,12	47	1,32	41,14
22	7,86	*no carbon	48	1,66,1,54	31,27
23	2,95, 2,76	37,99	49		27,15
24	*no proton	137,91	50	1,37	22,17
25	7,16	129,35	51	2,75	11,32
26	7,18	128,43	52	7,82	*no carbon

5.5.3 Secondary structure of GKPIILFF peptide

CD spectroscopy is an important technique for the characterization and analysis of peptide structures, and it can provide valuable insights into the structure-function relationship of peptides.²¹ The CD experimental were performed using acetonitrile as the solvent, and the results are shown in **Figure 5.9.**, The results of the D-GKP depict a random coil structure going to a helix conformation with a negative peak around 202 nm, while the results for L-GKP in acetonitrile display a minimum absorption with a negative peak around 204 nm, which is a characteristic of an α -helix structure.

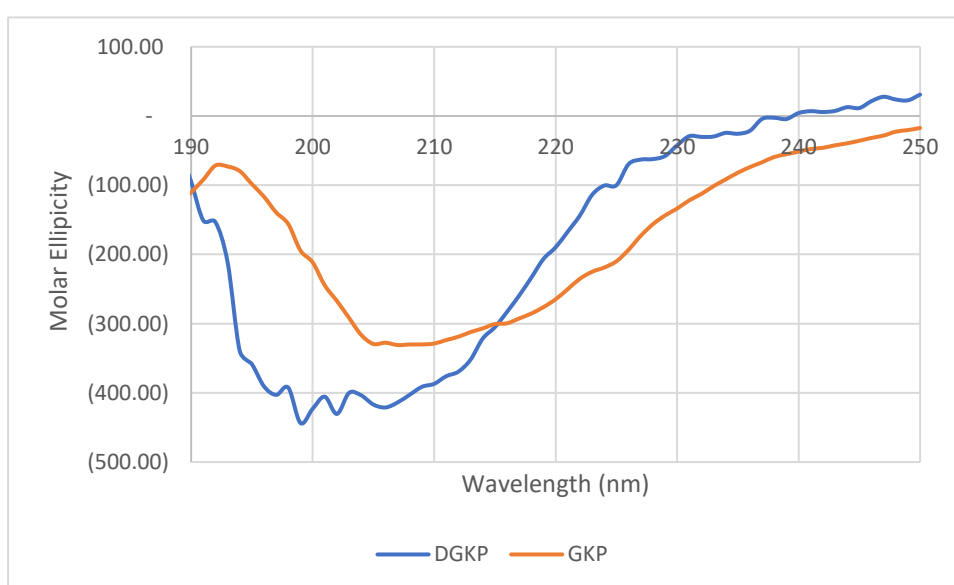


Figure 5.9 The CD spectra of α -helical peptides and the secondary structure of the GKPIILFF penetration acceleration sequence for a) the L peptide in orange and b) the D-peptide in blue.

Table 5.3 Peptide fold occurrence in the dataset

	DGKP	GKP
Helix	30.7	21,8
Antiparallel	6.9	15,9
Parallel	0.0	0
Turn	8.3	25,7
Random coil	54.2	36,7
Spectral deviation		
=====		
==		
RMSD	3.2703	2,1532
NRMSD	0.1174	0,1094

In **Table 5.3**, it appears that most of the structural elements of both peptides were random coils; however, the structure of D-GKP appears to be a more random coiled (54.2%) than L-GKP (36.7%). This suggests that the helical structure of the peptides is distorted due to the use of D amino acids.²²

Interestingly, proline also has been shown to result in proline-mediated helix kinks, which may contribute to the distortion; however, literature shows that it is often crucial for activity.²³ The conflicting steric geometry with the preceding residue and interruption of the backbone H bond results in helix deformation. 60 % of transmembrane helix deformations are caused by proline; however, proline-induced distortion is often essential in the function of the peptide.²⁴

A helical wheel is a type of plot or visual representation used to illustrate α -helices properties in proteins.²⁵ The sequence of amino acids that make up a helical region of the protein's secondary structure is plotted rotating where the rotation angle between consecutive amino acids is 100° so that the final representation looks down the helical axis. The helical wheel projection of the L- and D- GKP peptide seen in **Figure 5.10** demonstrates possible interactions that can be expected, and the interaction between proline and phenylalanine

found in the ROESY NMR spectra investigated in the next section corresponds with what is predicted in the helical wheel projection.

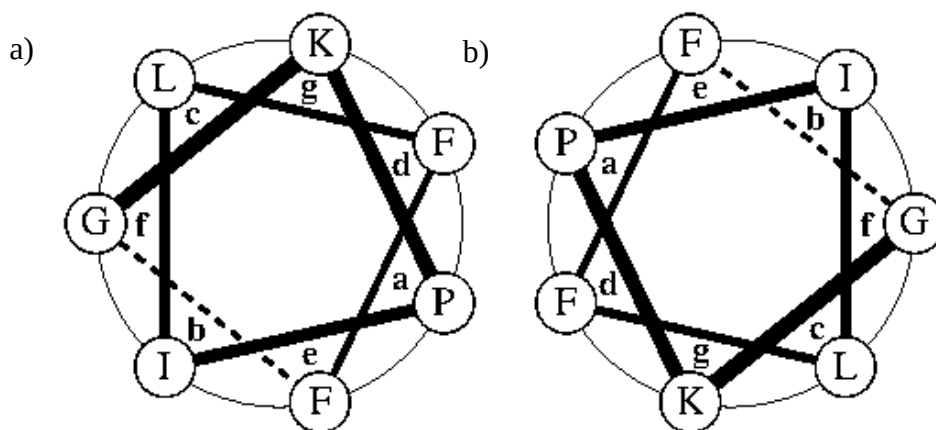


Figure 5.10 The helical wheel projection of the GKPILFF penetration acceleration sequence for a) the L peptide and b) the D-peptide

The folding properties of peptides can also be investigated through ROESY experiments and molecular dynamics simulations.²⁶ These techniques can provide accurate information about the three-dimensional spatial structure of peptides. Furthermore, crucial insights, especially potential chemical reactions and interactions, are achieved with these techniques.

Since the chemical shift of protons from different amino acids has been demonstrated using TOCSY and ROESY spectra, long-range interactions between peptide residues that are two or more amino acids away provide insight regarding the potential folding properties that the peptide possesses.²⁷ In **Figure 5.11**, the ROESY spectra of the GKP peptide are displayed in the range between 4.15 ppm and 5.0 ppm in the frequency 1 (f1) axis and range between 2.60 ppm and 3.10 ppm in the frequency 2 (f2). Long-range interactions were found between phe-7 (CH_2 , 2.82 ppm) and pro-3 (NH, 4.38) for the L peptide in **Figure 5.11a**), which corresponds with the helical wheel projection structure in **Figure 5.10a**) labelled as **(a)** and **(e)**. The other peaks which appear in this region are ascribed to the α protons of the Phe-6 and Phe-7 interacting with their respective beta protons. On the contrary, the D-amino acid seen in **Figure 5.11b**) only correlates with their respective beta protons of the phenylalanine groups. Therefore, the side-by-side ROESY comparison demonstrates that the phenylalanine interacts with proline that is four amino acids away from the L-peptide due to its alpha-

helical structure. The lack of this long-range interaction in the D-peptide suggests that the 2D structure does not possess a helical structure.

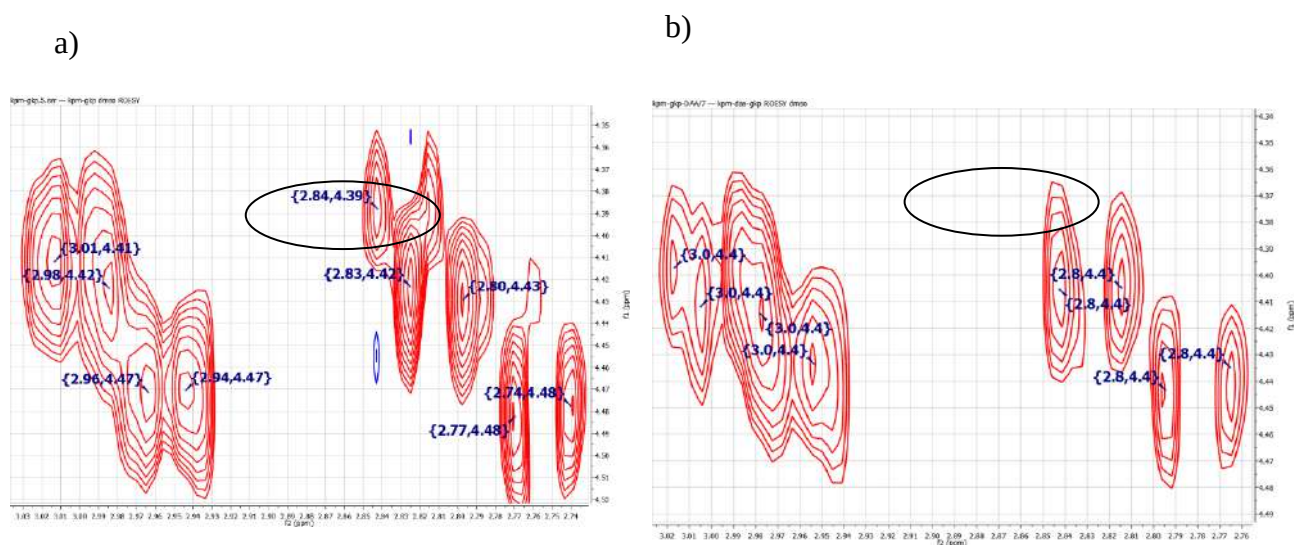


Figure 5.11 A magnification of the ROESY spectra in the region between 3.10 and 2.60 ppm in the F2(X) axis and 5.50 and 4.10 ppm in the F1(Y) axis for a) the L peptide and b) D-peptide showing the difference in the long-range interactions.

5.5.4 Structural elucidation using computational techniques

Molecular modelling is a useful tool in protein/peptide structural predictions and assists in elucidating their folding properties in a three-dimensional structure.²⁸ Molecular dynamics (MD) is one of the prominent molecular mechanic techniques used to study the dynamic behaviour of molecules using the fundamental Newton's laws of motion. MD simulation calculations were performed using Amber18²⁹, one of the most frequently used software programs, to predict the peptides' structural properties relative to their energies.³⁰ Using the sequence command in Amber, a linear peptide was constructed from the amino acid sequences (GKPILFF) and both the L- and D-conformations.

The peptide's energy is minimized to remove any possible clashes between amino acid residues. Subsequently, the peptide was gradually heated from 0 to 325 K before the MD production simulation, as seen in **Figure 5.12**. Upon heating, the peptide adopted a semi-folded state and was subjected to MD simulation at a constant temperature of 325 K in the simulation of the D peptide. The temperature had to be adjusted to prevent the structure from being trapped in local minima.

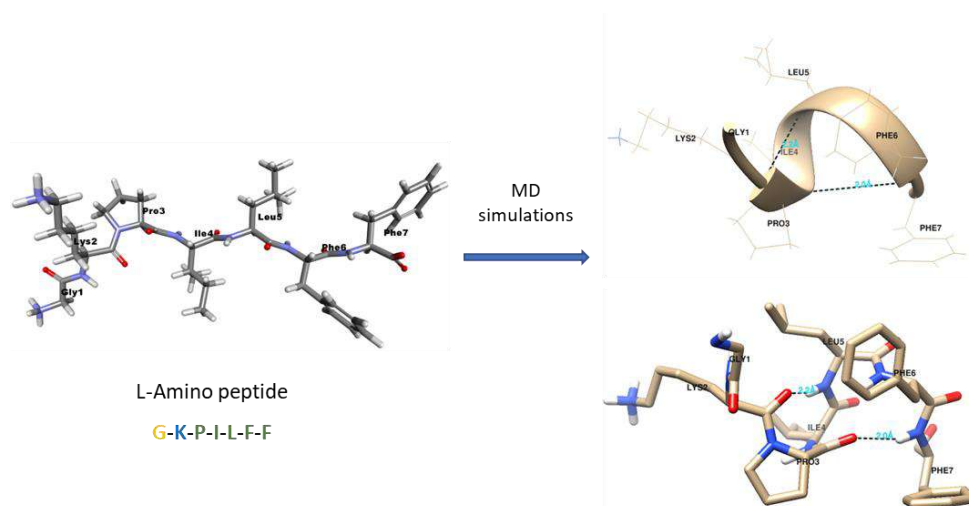


Figure 5.12 The structure of the linear L-GKPILFF peptide and its folded state after MD simulations at 325 K.

The L-peptide adopted a left-hand α -helical folded structure after a 150 ns MD simulations. The folded peptide, especially peptide 1, shows intramolecular hydrogen bond interactions, and the amino acid residues involved are Lys2—Leu5 and Pro3—Phe7. The folding conformation of the D-peptide is somewhat different from the L-peptide, as seen in **Figure 5.13**. The hydrogen bonds observed in the D-peptide are from Lys2—Leu5, Lys2—Phe7, and Ile4—Phe6.

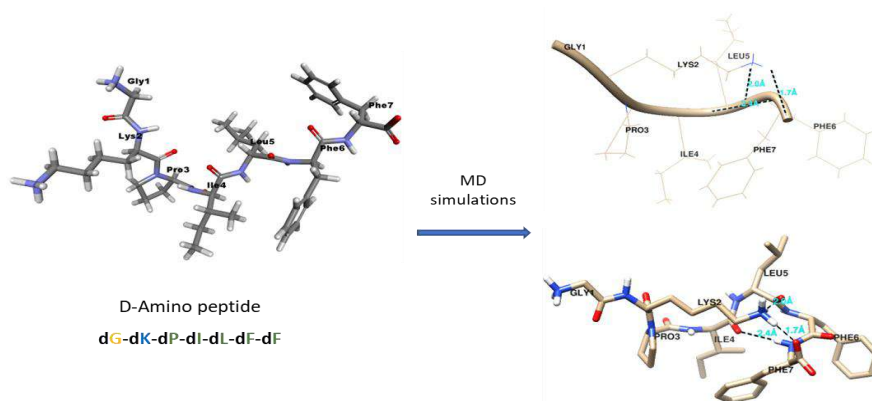


Figure 5.13 The structure of the linear D-GKPILFF peptide and its folded state after MD simulations at 325 K.

The potential energy and root-mean-square deviation (RMSD) analyses were performed to assess the atoms' positions upon peptide folding and measure their stability, respectively, during the simulation. The potential energies of both peptides are similar, thus indicating their folding favourability, as shown in **Figure 5.14 (a)**.³¹ On the other hand, the peptides' RMSD in **Figure 5.14 (b)** shows more conformational dynamics, which means the peptides

are stable at certain points in time. This observation entails prolonging the simulation time frame may result in a better overview of the peptide behaviour.

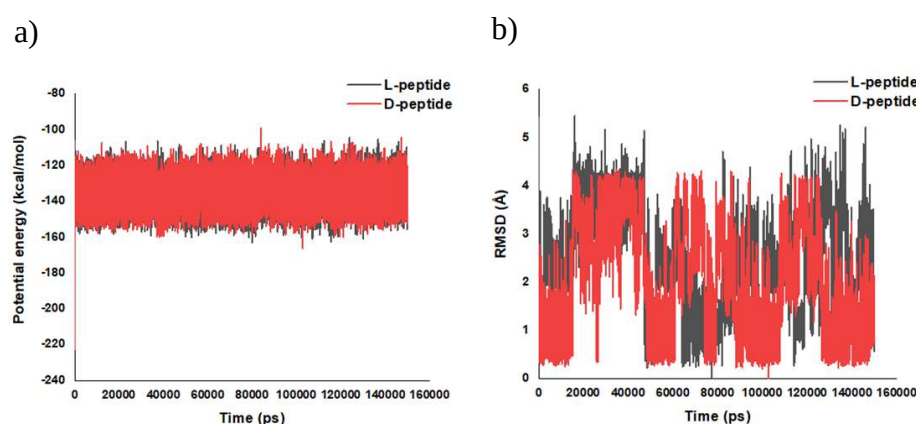


Figure 5.14 The potential energy and root-mean-square deviation (RMSD) analyses of the L- and D- GKPILFF peptide.

To affirm the observed helicity on the peptides, a Ramachandran plot was used to examine the spatial arrangement of atoms in the peptides, as seen in **Figure 5.15**. The backbone psi (Φ) – phi (Ψ) dihedral angles of the peptides are measured based on the positions of amino acid residues in the allowed, favoured, disallowed, and unfavoured regions.³²

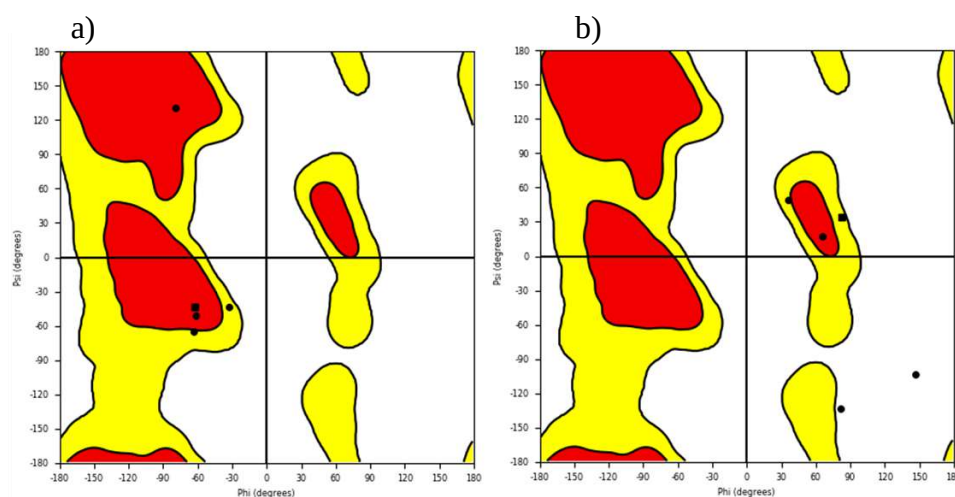


Figure 5.15 The Ramachandran plots show the stereochemical analysis of the peptides' psi (Φ) – phi (Ψ) dihedral angles. (a) L-peptide and (b) D-peptide. The red regions represent the favoured region, the yellow represents the allowed regions, and the white represents the disallowed/unfavoured regions.

These properties provide information on amino acid positioning associated with the secondary structure assignment. Typically, amino acid residues whose psi (Φ) – phi (Ψ) dihedral angles lie between -95° and -35° for psi (Φ) and between -15 and -70 for phi (Ψ), respectively, are classified as helical in shape or structure.³³ The psi (Φ) – phi (Ψ) dihedral angles of each amino acid residue in the peptides were provided in **Table 5.4** below and demonstrated that the L-GKPILFF peptide adopted a helical structure and the D-GKPILFF peptide did not.

Table 5.4 Stereochemical analysis of the peptides' backbone psi (ϕ) – phi (ψ) parameters of each amino acid residue

L-Amino peptide	ϕ (degrees)	Ψ (degrees)	D-Amino peptide	ϕ (degrees)	Ψ (degrees)
GLY 1	–	–	GLY 1	–	–
LYS 2	130.8	-78.8	LYS 2	132.9	81.5
PRO 3	-43.7	-62.3	PRO 3	34.2	82.4
ILE 4	-43.0	-32.2	ILE 4	103.0	146.8
LEU 5	-50.9	-61.4	LEU 5	48.9	36.1
PHE 6	-65.0	-63.0	PHE 6	17.5	65.7
PHE 7	–	–	PHE 7	–	–

5.6 Conclusion

In summary, the two peptides were successfully elucidated using NMR. Major overlapping seen with one-dimensional NMR spectroscopy observed in the ^1H NMR spectra posed a challenge for assigning the peaks; however, this was overcome with the use of two-dimensional NMR spectroscopy such as TOCSY and ROESY NMR. It has been shown that the L-GKPILFF penetration acceleration peptide sequence (Pas) conformation adopts a helical secondary structure, which has been verified by TOCSY and ROESY NMR method through-space interaction between the Pro 3 and Phe 7 amino acid residues. The data from the helical wheel projection, CD, and molecular dynamic simulations further confirmed the alpha-helical properties of the L-peptide and the random coil structure of the D-peptide. Furthermore, we propose that the α -helix peptide structure may facilitate the pore formation and binding to the phospholipids, thus accelerating the penetration of its cargo.³⁴ Therefore, for future work, permeability studies will be conducted to compare the biological activity of the structural conformers. Additionally, the stapled hydrogen bond surrogates (SHBs) approach will be worth investigating to improve the peptide structure activity.

5.7 References

1. Takayama, K., Nakase, I., Michiue, H., Takeuchi, T., Tomizawa, K., Matsui, H. & Futaki, S. Enhanced intracellular delivery using arginine-rich peptides by the addition of penetration accelerating sequences (Pas). *Journal of Controlled Release* **138**, 128–133 (2009).
2. Akita, T., Kimura, R., Akaguma, S., Nagai, M., Nakao, Y., Tsugane, M., Suzuki, H., Oka, J. & Yamashita, C. Usefulness of cell-penetrating peptides and penetration accelerating sequence for nose-to-brain delivery of glucagon-like peptide-2. *Journal of Controlled Release* **335**, 575–583 (2021).
3. Woldetsadik, A. D., Vogel, M. C., Rabeh, W. M. & Magzoub, M. Hexokinase II–derived cell-penetrating peptide targets mitochondria and triggers apoptosis in cancer cells. *FASEB J* **31**, 2168–2184 (2017).
4. Liu, B. R., Lo, S.-Y., Liu, C.-C., Chyan, C.-L., Huang, Y.-W., Aronstam, R. S. & Lee, H.-J. Endocytic Trafficking of Nanoparticles Delivered by Cell-penetrating Peptides Comprised of Nona-arginine and a Penetration Accelerating Sequence. *PLOS ONE* **8**, e67100 (2013).
5. Molhoek, E. M., van Dijk, A., Veldhuizen, E. J. A., Haagsman, H. P. & Bikker, F. J. Improved proteolytic stability of chicken cathelicidin-2 derived peptides by d-amino acid substitutions and cyclization. *Peptides* **32**, 875–880 (2011).
6. Carmona, G., Rodriguez, A., Juarez, D., Corzo, G. & Villegas, E. Improved Protease Stability of the Antimicrobial Peptide Pin2 Substituted with d-Amino Acids. *Protein J* **32**, 456–466 (2013).
7. Lu, J., Xu, H., Xia, J., Ma, J., Xu, J., Li, Y. & Feng, J. D- and Unnatural Amino Acid Substituted Antimicrobial Peptides With Improved Proteolytic Resistance and Their Proteolytic Degradation Characteristics. *Frontiers in Microbiology* **11**, (2020).
8. Jia, F., Wang, J., Peng, J., Zhao, P., Kong, Z., Wang, K., Yan, W. & Wang, R. D-amino acid substitution enhances the stability of antimicrobial peptide polybia-CP. *Acta Biochimica et Biophysica Sinica* **49**, 916–925 (2017).
9. Jia, F., Wang, J., Peng, J., Zhao, P., Kong, Z., Wang, K., Yan, W. & Wang, R. D-amino acid substitution enhances the stability of antimicrobial peptide polybia-CP. *Acta Biochimica et Biophysica Sinica* **49**, 916–925 (2017).

10. Hong, S. Y., Oh, J. E. & Lee, K.-H. Effect of d-amino acid substitution on the stability, the secondary structure, and the activity of membrane-active peptide. *Biochemical Pharmacology* **58**, 1775–1780 (1999).
11. Douse, C. H., Maas, S. J., Thomas, J. C., Garnett, J. A., Sun, Y., Cota, E. & Tate, E. W. Crystal Structures of Stapled and Hydrogen Bond Surrogate Peptides Targeting a Fully Buried Protein–Helix Interaction. *ACS Chem. Biol.* **9**, 2204–2209 (2014).
12. Henchey, L. K., Jochim, A. L. & Arora, P. S. Contemporary strategies for the stabilization of peptides in the α -helical conformation. *Current Opinion in Chemical Biology* **12**, 692–697 (2008).
13. Pavan, S. & Berti, F. Short peptides as biosensor transducers. *Anal Bioanal Chem* **402**, 3055–3070 (2012).
14. Dentino, A. R., Raj, P. A., Bhandary, K. K., Wilson, M. E. & Levine, M. J. Role of peptide backbone conformation on biological activity of chemotactic peptides. *Journal of Biological Chemistry* **266**, 18460–18468 (1991).
15. Kumaran, S. & Roy, R. p. Helix-enhancing propensity of fluoro and alkyl alcohols: influence of pH, temperature and cosolvent concentration on the helical conformation of peptides. *The Journal of Peptide Research* **53**, 284–293 (1999).
16. Morris, C. J., Beck, K., Fox, M. A., Ulaeto, D., Clark, G. C. & Gumbleton, M. Pegylation of Antimicrobial Peptides Maintains the Active Peptide Conformation, Model Membrane Interactions, and Antimicrobial Activity while Improving Lung Tissue Biocompatibility following Airway Delivery. *Antimicrobial Agents and Chemotherapy* **56**, 3298–3308 (2012).
17. Kamiya, M., Kumaki, Y., Nitta, K., Matsumoto, T., Hikichi, K. & Matsushima, N. The binding of copper ions to glycine-rich proteins (GRPs) from *Cicer arietinum*. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1722**, 69–76 (2005).
18. Smet-Nocca, C., Wieruszeski, J.-M., Melnyk, O. & Benecke, A. NMR-based detection of acetylation sites in peptides. *Journal of Peptide Science* **16**, 414–423 (2010).
19. Gayen, A. & Mukhopadhyay, C. Evidence for Effect of GM1 on Opioid Peptide Conformation: NMR Study on Leucine Enkephalin in Ganglioside-Containing Isotropic Phospholipid Bicelles. *Langmuir* **24**, 5422–5432 (2008).
20. Avilés, E. & Rodríguez, A. D. Euryjanicins E–G, poly-phenylalanine, and poly-proline cyclic heptapeptides from the Caribbean sponge *Prosuberites laughlini*. *Tetrahedron* **69**, 10797–10804 (2013).

21. Ladokhin, A. S., Fernández-Vidal, M. & White, S. H. CD Spectroscopy of Peptides and Proteins Bound to Large Unilamellar Vesicles. *J Membrane Biol* **236**, 247–253 (2010).
22. Yang, S.-T., Lee, J. Y., Kim, H.-J., Eu, Y.-J., Shin, S. Y., Hahm, K.-S. & Kim, J. I. Contribution of a central proline in model amphipathic α -helical peptides to self-association, interaction with phospholipids, and antimicrobial mode of action. *The FEBS Journal* **273**, 4040–4054 (2006).
23. Schmidt, T., Situ, A. J. & Ulmer, T. S. Structural and thermodynamic basis of proline-induced transmembrane complex stabilization. *Sci Rep* **6**, 29809 (2016).
24. S.P. Sansom, M. & Weinstein, H. Hinges, swivels and switches: the role of prolines in signalling via transmembrane α -helices. *Trends in Pharmacological Sciences* **21**, 445–451 (2000).
25. Schiffer, M. & Edmundson, A. B. Use of Helical Wheels to Represent the Structures of Proteins and to Identify Segments with Helical Potential. *Biophysical Journal* **7**, 121–135 (1967).
26. Tang, Y., Rigotti, D. J., Fairman, R. & Raleigh, D. P. Peptide Models Provide Evidence for Significant Structure in the Denatured State of a Rapidly Folding Protein: The Villin Headpiece Subdomain. *Biochemistry* **43**, 3264–3272 (2004).
27. Demarest, S. J., Fairman, R. & Raleigh, D. P. Peptide models of local and long-range interactions in the molten globule state of human α -lactalbumin¹¹Edited by P. E. Wright. *Journal of Molecular Biology* **283**, 279–291 (1998).
28. Porto, W. F., Pires, A. S. & Franco, O. L. Computational tools for exploring sequence databases as a resource for antimicrobial peptides. *Biotechnology Advances* **35**, 337–349 (2017).
29. Lee, T.-S., Cerutti, D. S., Mermelstein, D., Lin, C., LeGrand, S., Giese, T. J., Roitberg, A., Case, D. A., Walker, R. C. & York, D. M. GPU-Accelerated Molecular Dynamics and Free Energy Methods in Amber18: Performance Enhancements and New Features. *J. Chem. Inf. Model.* **58**, 2043–2050 (2018).
30. Wang, J., Alekseenko, A., Kozakov, D. & Miao, Y. Improved Modeling of Peptide-Protein Binding Through Global Docking and Accelerated Molecular Dynamics Simulations. *Frontiers in Molecular Biosciences* **6**, (2019).
31. Simmerling, C., Strockbine, B. & Roitberg, A. E. All-Atom Structure Prediction and Folding Simulations of a Stable Protein. *J. Am. Chem. Soc.* **124**, 11258–11259 (2002).

32. Ramachandran.pdf. at
<https://millhauser.chemistry.ucsc.edu/courses/Chem_200B_papers_handouts_2008/Ramachandran.pdf> (Last accessed: 21 Oct. 2023)
33. Guo, Z., Mohanty, U., Noehre, J., Sawyer, T. K., Sherman, W. & Krilov, G. Probing the α -Helical Structural Stability of Stapled p53 Peptides: Molecular Dynamics Simulations and Analysis. *Chemical Biology & Drug Design* **75**, 348–359 (2010).
34. Tian, Y., Jiang, Y., Li, J., Wang, D., Zhao, H. & Li, Z. Effect of Stapling Architecture on Physiochemical Properties and Cell Permeability of Stapled α -Helical Peptides: A Comparative Study. *ChemBioChem* **18**, 2087–2093 (2017).

6. The use of NMR techniques for Carbon dots-GKP peptide conjugates

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6.1 Abstract

Nuclear magnetic resonance (NMR) is a powerful technique for identifying molecular species in carbon-based nanomaterials. It is essential to include NMR as a standard characterization technique to identify molecular species in the final material and impurities. The conjugates' characteristics were determined using photoluminescent and UV-Vis spectrometers, revealing successful conjugation. NMR spectroscopy was used to show long-range interactions between the peptide and the CDs. Additionally, unreacted starting material and impurities in the CDs that could interfere with the properties of the CDs or conjugates were also investigated. Dialysis reduced the amount of unreacted urea and other impurities, and the presence of urea and a small molecule was revealed. The 2-D NMR analysis of the conjugates suggested that the helical peptide structure was affected after conjugation, as indicated by significant peak shifts. The peptide region in NMR spectra of some of the conjugates were showing fewer shifts in the peptide which could be indicative of conjugates with better stability for bioactivity.

Keywords: carbon dots-peptide conjugates; photoluminescence; wavelength excitation-dependence; pH-dependence, NMR

6.2 Introduction

Carbon dots-peptide conjugates for bioimaging refer to hybrid materials that consist of carbon dots (CDs), which are fluorescent nanoparticles, and peptides. These conjugates are designed for the purpose of visualizing biological structures and processes. The conjugates incorporate CDs as imaging agents, utilizing their strong fluorescence properties. Upon excitation by an external light source, these nanodots emit light, enabling the observation of biomolecules or cellular components. By conjugating peptides to the carbon nanodots, the

conjugates gain enhanced permeability capabilities. The peptides used for fabricating the conjugates were the penetration accelerating sequence (Pas) GKPILFF and the cancer selective enantiomeric 9-mer peptide RLRLRIGRR. Since the *Iso-N-Ge*-CDs exhibited the highest photoluminescent properties, it was used to form conjugates with the synthesized peptides.

The combination of peptides and CDs leverages the unique advantages of both components. The CDs offer brightness, stability, and compatibility with biological systems, while the peptides provide target-specific recognition. This amalgamation results in highly valuable tools for bioimaging, facilitating the visualization and investigation of biological processes with enhanced precision and sensitivity.

6.3 Synthesis of carbon nanodot-GKP peptide conjugates

Water-soluble CDs were synthesized according to the procedure described in section 3.2.2. A mixture of iso-ascorbic acid, Mili-Q water, and urea was added to a microwave reaction vessel and then shaken vigorously to dissolve the reagents. Then the solution was subjected to microwave irradiation under closed vessel conditions at a maximum power and pressure output of 150 W and 250 psi, respectively (CEM Discovery SP microwave). The resulting solution was purified by centrifugation to separate the supernatant from the precipitate. The supernatant was then filtered, and the solvent was removed using a rotary evaporator. The product was further dried in an oven overnight.

The peptides (**Table 6.1**) conjugated to the CDs were synthesized using a Fmoc solid phase synthesis strategy. The synthesis and characterization are reported in chapters 4 and 5.

Table 6.1 List of GKP and BP peptide analogues and their respective sequences, which were conjugated to Iso-N-Ge-CDs

PEPTIDE ID.	PEPTIDE SEQUENCE	MOLECULAR MASS	MODIFICATIONS
Parent sequence	COOH -GKPILFF- NH₂	820.48	
GKP	NH ₂ -GKPILFF- CONH₂	819.50	The free carboxylic end is changed to an amide.
GKP-ada	ADA GKPILFF- NH₂	981.61	The N terminus has been modified with adamantane.
GKP-pal	PAL GKPILFF- NH₂	1057.73	The N terminus has been modified with palmitic acid.
D-GKP	D-amino acid CONH₂ -GKPILFF- NH₂	819.50	The free carboxylic end is changed to an amide, and D-amino acids replace L-amino acids.
D-GKP-ada	D-amino acid ADA -GKPILFF- NH₂	981.61	The N terminus has been modified with adamantane and D-amino acids to replace L-amino acids.
D-GKP-pal	D-amino acid PAL -GKPILFF- NH₂	1057.73	The N terminus has been modified with palmitic acid, and D-amino acids replace L-amino acids.
RGDGKP	CONH₂ -RGDGKPILFF- NH₂	1147.6292	
BP	CONH₂ -RLRLRIGRR- NH₂	1193.81	The free carboxylic end is changed to an amide.
BP-ada	ADA -RLRLRIGRR - NH₂	1355.91	The N terminus has been modified with adamantane.
BP-pal	PAL -RLRLRIGRR- NH₂	1432.04	The N terminus has been modified with palmitic acid.
D-BP	D-amino acid CONH₂ -RLRLRIGRR- NH₂	1193.81	The free carboxylic end is changed to an amide, and D-amino acids replace L-amino acids.
D-BP-ada	D-amino acid ADA -RLRLRIGRR- NH₂	1355.91	The N terminus has been modified with adamantane and D-amino acids to replace L-amino acids.
D-BP-pal	D-amino acid PAL -RLRLRIGRR- NH₂	1432.04	The N terminus has been modified with palmitic acid, and D-amino acids replace L-amino acids.

Peptide–CDs were fabricated by adding 20 mg of CDs and 2 mg of the peptide into a solution of phosphate-buffered saline (PBS pH 7.4) and 5% DMSO. The reaction was gently stirred at room temperature overnight, as seen in **Figure 6.1**. *Iso-N-Ge*-CDs were further explored in conjugation reactions as it demonstrated the highest photoluminescent properties (See section 3.4.4).

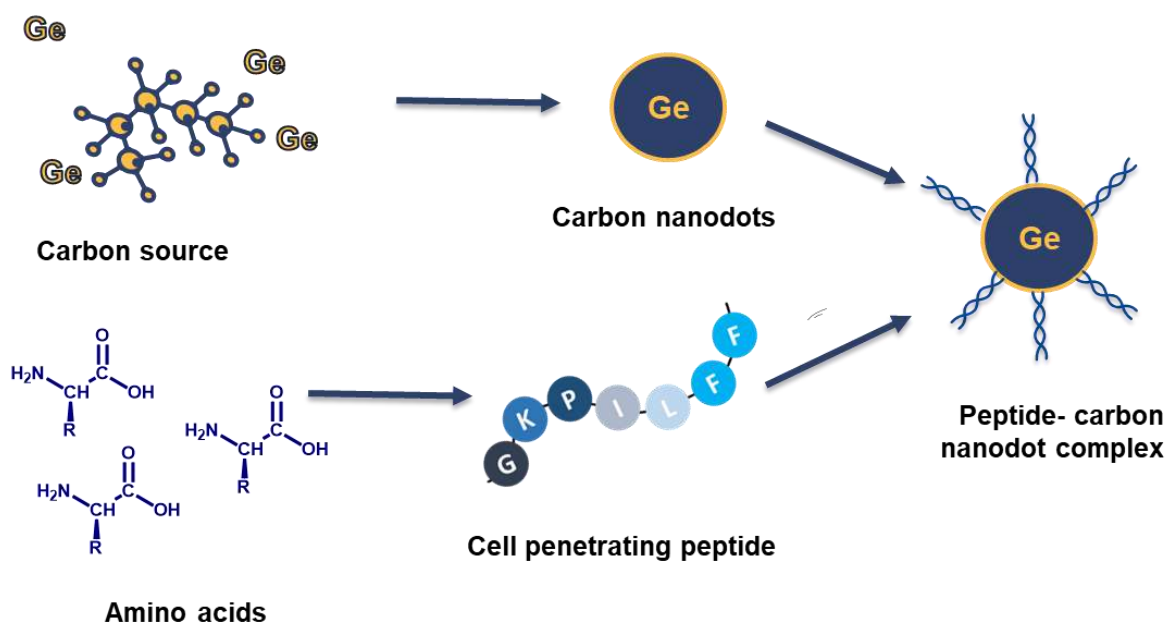


Figure 6.1 Illustration of the synthesized carbon dots conjugated to the peptide

Purification

Membrane dialysis is a method used in purification to divide molecules according to their size using a semipermeable membrane. It is a specific kind of diffusion-based procedure that depends on the osmosis and concentration gradient theories by which solvent molecules (usually water) move through a semipermeable membrane from an area of lower solute concentration to an area of higher solute concentration. This movement of water molecules continues until the concentration of solute molecules is equal on both sides of the membrane.

The purification of the conjugates was conducted using membrane dialysis since the unbound peptide and starting material have a smaller molecular size compared to the conjugated molecules. A seamless cellulose tubing with a partly permeable covering, a molecular weight cut-off of 12000 to 14000 Da, and pore sizes of 2.4 nanometers were used to remove the unreacted starting material.

6.4 Results and discussion

6.4.1 UV-Vis spectroscopy

The UV absorbance of peptides can vary depending on a number of factors, such as their sequence, structure, and environment. That being said, there are some general trends that can

be observed. Peptides typically have strong absorbance in the ultraviolet (UV) range due to the presence of aromatic amino acids such as tryptophan, tyrosine, and phenylalanine, which have absorption maxima in the range of 280-300 nm. However, a molecule's precise absorbance peaks will depend on both its electronic structure and the makeup of its chromophore, which is the component of the molecule that absorbs light in the UV-Visible range. The magnitude of the UV absorbance depends on the number and positioning of these aromatic amino acids in the peptide sequence. Peptides with more aromatic amino acids generally exhibit higher UV absorbance.

Due to their electronic transitions, side chains that include π -electrons can substantially absorb light in the UV-Vis spectrum. Examples of these groups include double bonds and aromatic rings. In aromatic rings, for instance, the π - π^* transition, which includes the excitation of an electron from the bonding orbital to the antibonding orbital, often takes place in the UV area and results in significant absorbance peaks. This is similar to the n - π^* transition, which includes the excitation of an electron from a non-bonding orbital to the π^* antibonding orbital, which can occur in molecules having carbonyl or nitrile functional groups. This transition often results in absorbance within the visible spectrum, which manifest as peaks in the photoluminescence spectra.

The absorption spectra retrieved from the instrument of GKP, GKP-*Iso-N-Ge*-CDs, and *Iso-N-Ge*-CDs are shown in **Figure 6.2** (any conjugating of the peptide and CDs will often be referred to as the conjugate). GKP showed absorption peaks at ~ 222 nm, which corresponds to the $\pi \rightarrow \pi^*$ transition of the conjugated double bonds or aromatic rings that absorb (C=C bonds).¹ The absorption peaks for GKP-*Iso-N-Ge*-CDs were observed at ~ 210 and 264 nm, corresponding to the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transition of C=C and C=O bonds, characteristic of the aromatic sp^2 domains and carbonyl moieties.² Finally, *Iso-N-Ge*-CDs absorbed UV-Vis radiation at ~ 207 and 260 nm, corresponding to the $\pi \rightarrow \pi^*$, characteristic of the aromatic sp^2 domains.³ The absorption edges of all three samples extend into the visible band, which is typically from 380 nm to 700 nm.

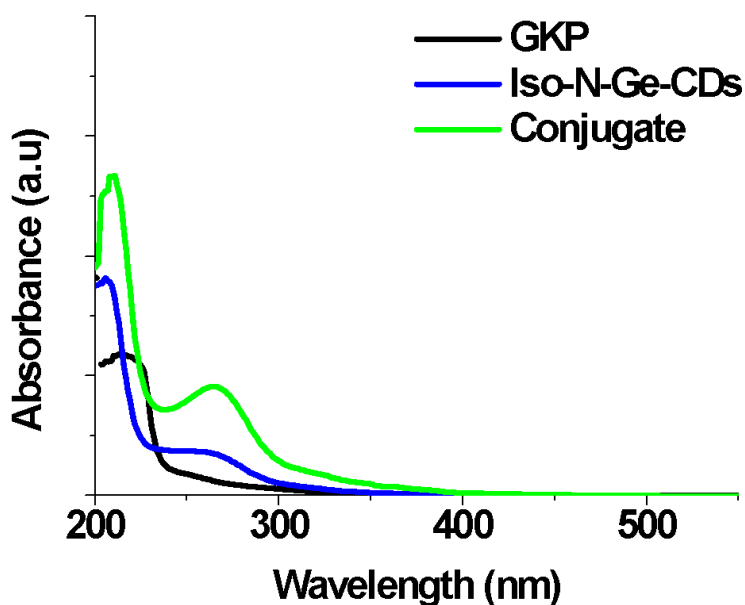


Figure 6.2 Absorption spectra of GKP, GKP-Iso-N-Ge-CDs, and Iso-N-Ge-CDs

The successful conjugation of GKP peptides derivatives to *Iso-N-Ge-CDs* was characterized using the UV-Vis spectrometer, as shown in **Figure 6.3**, and the data revealed that the various conjugates had slightly different absorbance peaks and different relative intensities, which was consistent with the literature.⁴

The different relative intensities may be attributed to the conformation and functional groups of the peptide. The two-dimensional arrangement of a peptide's atom, known as its conformation, can modify the electrical characteristics of these functional groups and have an impact on how they absorb light. The adamantane and palmitic moieties do not contain functional groups that absorb strongly in the UV-Vis region, which implies that they only contribute conformational changes.

The absorbance characteristics of the peptide bond, which comprises both a carbonyl group and an amide nitrogen, often dominate the UV-Vis spectra of peptides. The $n-\pi^*$ transition, which involves the excitation of an electron from the non-bonding lone pair on the nitrogen atom to the π^* antibonding orbital of the carbonyl group, is what causes the peptide bond absorption. For instance, a peptide bond adopts a planar shape in peptides with α -helical or β -sheet conformations, resulting in a significant absorbance peak at about 190-200 nm. This is due to the fact that the planar conformation enables maximum n and π^* orbital overlap, which raises the transition energy and absorbance intensity.

The degree of conjugation in the backbone is reduced in peptides with random coil or stretched conformations, which causes the peptide bond to adopt a twisted conformation, lowering the energy and intensity of the absorbance peak. Both peptides and carbon dots have distinctive UV-Vis absorption peaks, and when they are conjugated with one another, their spectra may overlap. However, a shift in the peaks is often observed, and the broadness of the peaks may vary, as seen in **Figure 6.3**.

The GKP-Iso-N-Ge-CDs conjugate seen in green in **Figure 6.3a)** and **b)** appeared to have a greater relative intensity of the peak at 210 nm than the peak at 261 nm compared to the other conjugates in **Figure 6.3c)-f)**. The loss of relative intensity in the other conjugates may indicate that the peptide is more stretched/linear and adopts a random coil characteristic. A summary shown UV-Vis spectra of the conjugates is shown in **Table 6.2** and a similar trend was observed in the other conjugates.

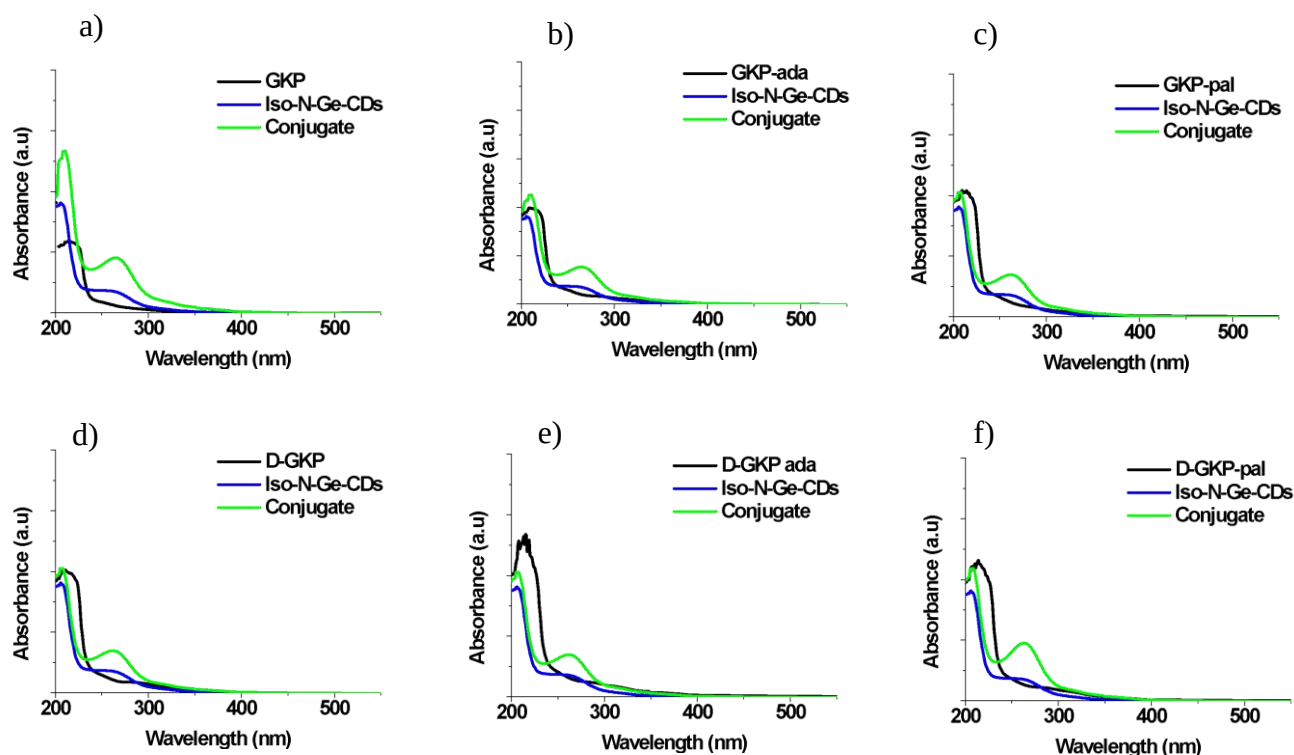


Figure 6.3 Side by side comparison of all the GKP peptide analogue-conjugates against the Iso-N-Ge-CDs. Black line represents the GKP peptide derivative, blue line represents the Iso-N-Ge-CDs and the green/lime line Conjugate.

Table 6.2 Summary of the peaks observed in the UV-Vis spectrometer of GKP conjugates.

Conjugate Name	Absorption peak 1 (nm)	Absorption peak 2 (nm)
1. <i>GKP-Iso-N-Ge-CDs</i>	210	264
2. <i>GKP-ada- Iso-N-Ge-CDs</i>	205	261
3. <i>GKP-pal-Iso-N-Ge-CDs</i>	208	265
4. <i>D-GKP-Iso-N-Ge-CDs</i>	207	266
5. <i>D-GKP-pal- Iso-N-Ge-CDs</i>	203	263
6. <i>D-GKP-ada-Iso-N-Ge-CDs</i>	204	262
7. <i>GKP</i>	221	-
8. <i>GKP-pal</i>	211	-
9. <i>GKP-ada</i>	213	-
10. <i>D-GKP</i>	211	-
11. <i>D-GKP-pal</i>	213	-
12. <i>D-GKP-ada</i>	214	-
13. <i>Iso-N-Ge-CDs</i>	204	267

A similar trend was observed for the BP peptide derivative and their conjugates. The UV-Vis spectra of peptides appeared dominated by the peptide bond's absorbance properties, which include both a carbonyl group and amide nitrogen which was observed for the GKP analogues as well.

Although the BP peptide derivatives lack aromatic groups, the arginine side chain contains guanidine groups ((NH₂)-C=NH) which can undergo various electronic transitions and shows a peak around 200-220 nm. The two peptides were expected to have spectra that appear different since the sequences are different, but the characteristic peak around 200 nm was observed in both peptide derivatives. Interestingly, for the conjugates, the 260 nm peak ascribed to the C=O appeared to be much lower than the GKP conjugates. This demonstrates that the peptide sequence affects the overall conjugate. Furthermore, the conjugates in **Figure 6.4a), b), and d)** appear to lose intensity in the peak around 260 nm more significantly compared to the other conjugates. This suggests that the conformation of the conjugates may

be different, and the lowered intensity may indicate that the peptide is wrapping around the dots and shielding the carbon nanodots.⁵ The effects of conjugation on the peptide structure were further investigated using NMR studies which will be discussed in a later section of this chapter. In **Table 6.3**, a summary of the absorbance peak in the UV-Vis spectra was shown.

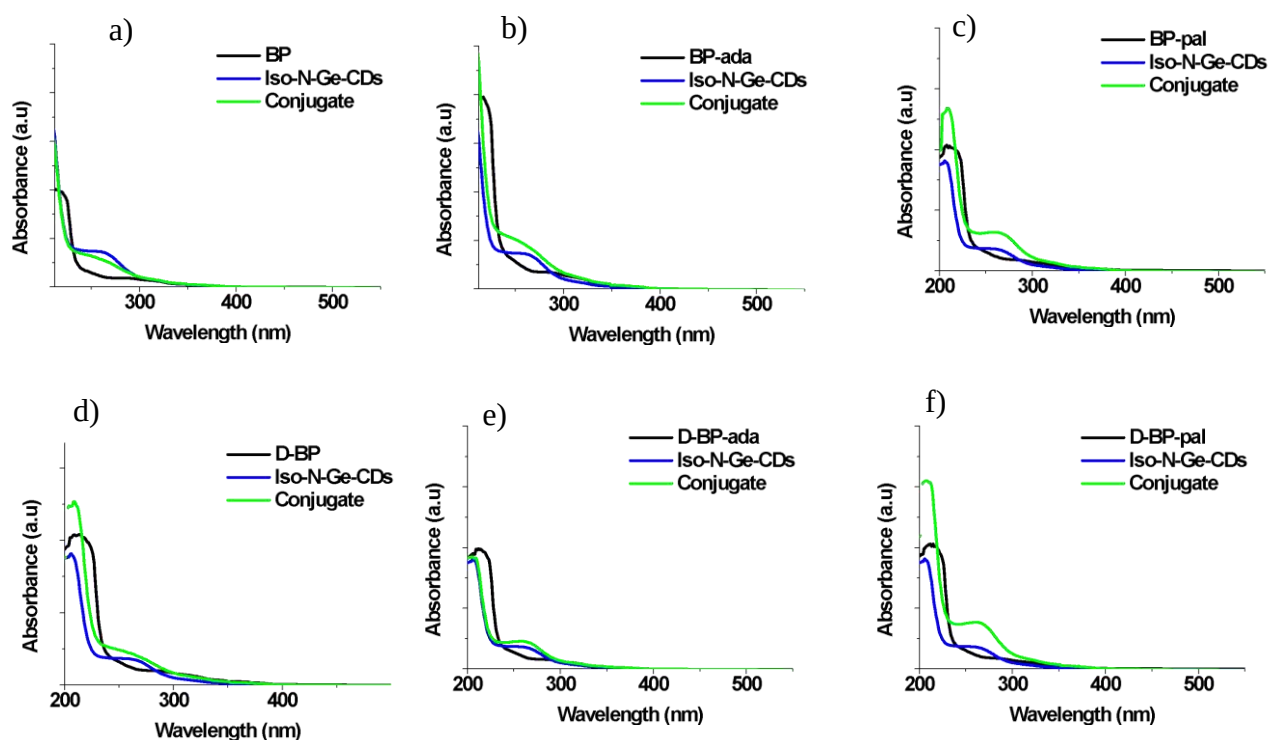


Figure 6.4 Side by side comparison of all the BP peptide analogue-conjugates against the Iso-N-Ge-CDs. Black line represents the BP peptide derivative, blue line represents the Iso-N-Ge-CDs and the green/lime line Conjugate.

Table 6.3 Summary of the peaks observed by the UV-Vis spectrometer of the BP conjugates.

Conjugate Name	UV-Vis data	
	Absorption peak 1(nm)	Absorption peak 2 (nm)
1. <i>BP-Iso-N-Ge-CDs</i>	211	259
2. <i>BP-ada-Iso-N-Ge-CDs</i>	209	262
3. <i>BP-pal-Iso-N-Ge-CDs</i>	208	258
4. <i>D-BP-Iso-N-Ge-CDs</i>	212	257
5. <i>D-BP-pal- Iso-N-Ge-CDs</i>	207	266
6. <i>D-BP-ada-Iso-N-Ge-CDs</i>	206	256
7. <i>BP</i>	214	-
8. <i>BP-pal</i>	212	-
9. <i>BP-ada</i>	210	-
10. <i>D-BP</i>	213	-
11. <i>D-BP-pal</i>	215	-
12. <i>D-BP-ada</i>	211	-
13. <i>Iso-N-Ge-CDs</i>	204	267

6.4.2 Photoluminescence spectroscopy

The CD-peptide conjugates show fascinating photoluminescent features ascribed to their ability to absorb and emit photons. The PL spectra of the freshly prepared CD-peptide conjugate in an aqueous 10 mM PBS solution are shown in **Figure 6.5**. The PL spectra of the *Iso-N-Ge-CDs*, *GKP-Iso-N-Ge-CDs*, and the *GKP* consists of almost similar peak broadening. The observed effects were attributed to the homogeneous broadening of the excitonic band due to similar distribution of structural makeup between the peptide, carbon nanodots, and conjugates.⁶

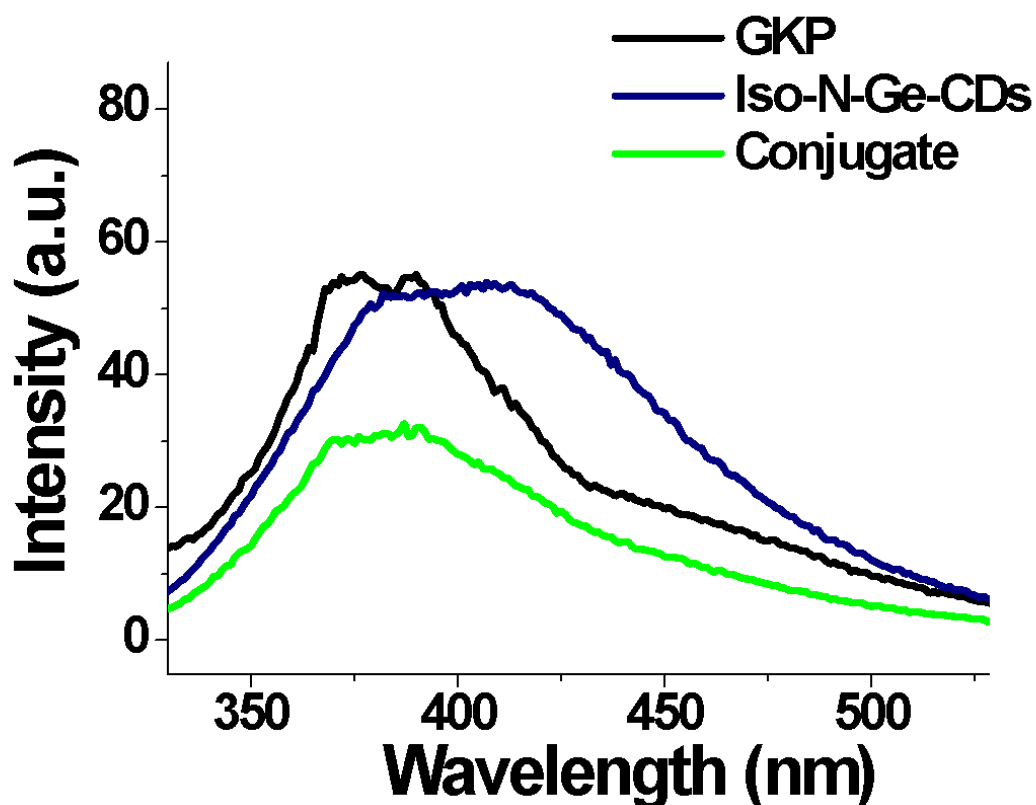


Figure 6.5 PL data for GKP in black, Iso-N-Ge-CDs in blue, and GKP-Iso-N-Ge-CDs in green

The emission (and full width at half maximum) was observed for GKP in black, Iso-N-Ge-CDs in blue, GKP-Iso-N-Ge-CDs in green with an excitation of 375 nm (60 nm), 398 nm (75 nm) and 383 nm (85 nm), respectively. The full width at half maximum (FWHM) of the peaks gives some insight into the effects of the defects that are present in the material and the inhomogeneity of the material.⁷ Literature has shown that when the material is doped, it then affects the maximum emission as well as the FWHM in PL spectra by introducing new energy levels, defects, or altering the energy landscape within the host material.⁸ Furthermore, a large FWHM value results in broad luminescence, which may affect the application of the material. This is characteristic of the effects on the width of the absorption line due to the lattice vibrations and lattice imperfections.⁹

A redshift is a change in the absorption peak from the unmodified molecule's absorption peak to longer wavelengths (i.e., lower energy). This indicates a decrease in the energy gap between the excited state and the molecule's or material's ground state, which is often referred to as the bandgap or the HOMO-LUMO gap. The redshift may be due to the extended conjugations that allow for more delocalization of the excited state electron. The

interaction between the carbon dots and the peptides has altered the electronic features of the carbon dots, affecting their absorption properties, as evidenced by a redshift in the UV-Vis spectra of carbon dot conjugates to peptides.¹⁰ Therefore, the redshift between the GKP peptide and its conjugate demonstrates the noncovalent interaction in the formation of the conjugates. This suggests that molecular planarity increases the electronic conjugation resulting in the redshift,¹¹ hence we hypothesize that the conjugates or conjugates peptide adopts a planar shape observed in peptides with α -helical or β -sheet conformations.

The shifts to lower energy seen in **Figure 6.5** indicate that the functional groups on carbon dots may interact with the molecular orbitals of the surface domain in the peptide sequences. The electrostatic forces between the carboxylic functional groups on the carbon dots and the amines from the peptide may facilitate this interaction.¹² The formation of molecular orbitals as a consequence of noncovalent interactions and orbital overlap between the π bonds of aromatic rings may also be taking place. Another possible interaction is π stacking (also called π - π stacking) through the aromatic ring present on the carbon dots, and the phenylalanine rings from the peptide.¹³ The surface chemistry of carbon dots appears to be affected by the presence of the peptide seen with the shift in the peak and narrowing of the peak, which changed the emission wavelength and can further affect the stability of their photoluminescences.¹⁴

When carbon dots have broad photoluminescence spectra, they produce light over a large range of wavelengths as opposed to an emission color purity.¹⁵ Wide photoluminescence spectra are frequently linked to the existence of various carbon dot sizes and configurations in the sample.¹⁶ This was observed in the TEM images and size distribution graphs in **section 3.5.1** resulting in a broad absorption peak for *Iso-N-Ge*-CDs in **Figure 6.5**. Larger carbon dots emit light at longer wavelengths than smaller carbon dots do on average, with smaller carbon dots emitting light at shorter wavelengths.¹⁷

For applications like biological imaging or sensing, broad photoluminescence spectra might be helpful since they can offer a greater variety of information about the sample. To acquire the requisite emission characteristics, it could be necessary to isolate or synthesize carbon dots with particular sizes or surface qualities for some applications where a light generated in the near-infrared (NIR) spectral range is desired.¹⁸

The benefits of a broad photoluminescence spectrum seen for the *Iso-N-Ge*-CDs are mainly versatility for a wide range of applications and sensitivity, such as detecting tiny levels of

changes. On the other hand, narrow photoluminescence (PL) spectra seen for the conjugate mean that they may be used in applications that require high precision and selectivity, which is useful for detecting certain analytes in a complicated matrix. Since bioimaging of cancerous cells may involve complex cell matrices and require selectivity towards cancer cells, narrow PL spectra are desirable for this application.¹⁹

In **Figures 6.6** and **6.7**, the conjugates appear to be narrow compared to the peptides and the CDs. This demonstrated that the conjugation of peptides to the CDs has a favorable effect on the PL spectra. Moreover, the conjugates in **Figure 6.6 d), e), and f)** have slightly narrower than the other conjugates. This shows that the conjugation of CDs to peptide can reduce the broadness of the peak and suggests that conformation and functional groups of the peptide can also affect the broadness of the peak.

Interestingly, each peptide before conjugation appeared to have distinct peaks appearing between 450-500 nm. Features such as different functional groups and ionization state of amino acid residues result in various peaks in the PL spectra.²⁰ Additionally, the secondary structure of the peptides affects the emission peaks.²¹ As a result, the PL spectra of the peptides vary significantly. Circular Dichroism Spectroscopy is previously mentioned spectroscopic technique (see **section 5.5.3**) that used the same optical activity for determining the secondary structure of peptides.

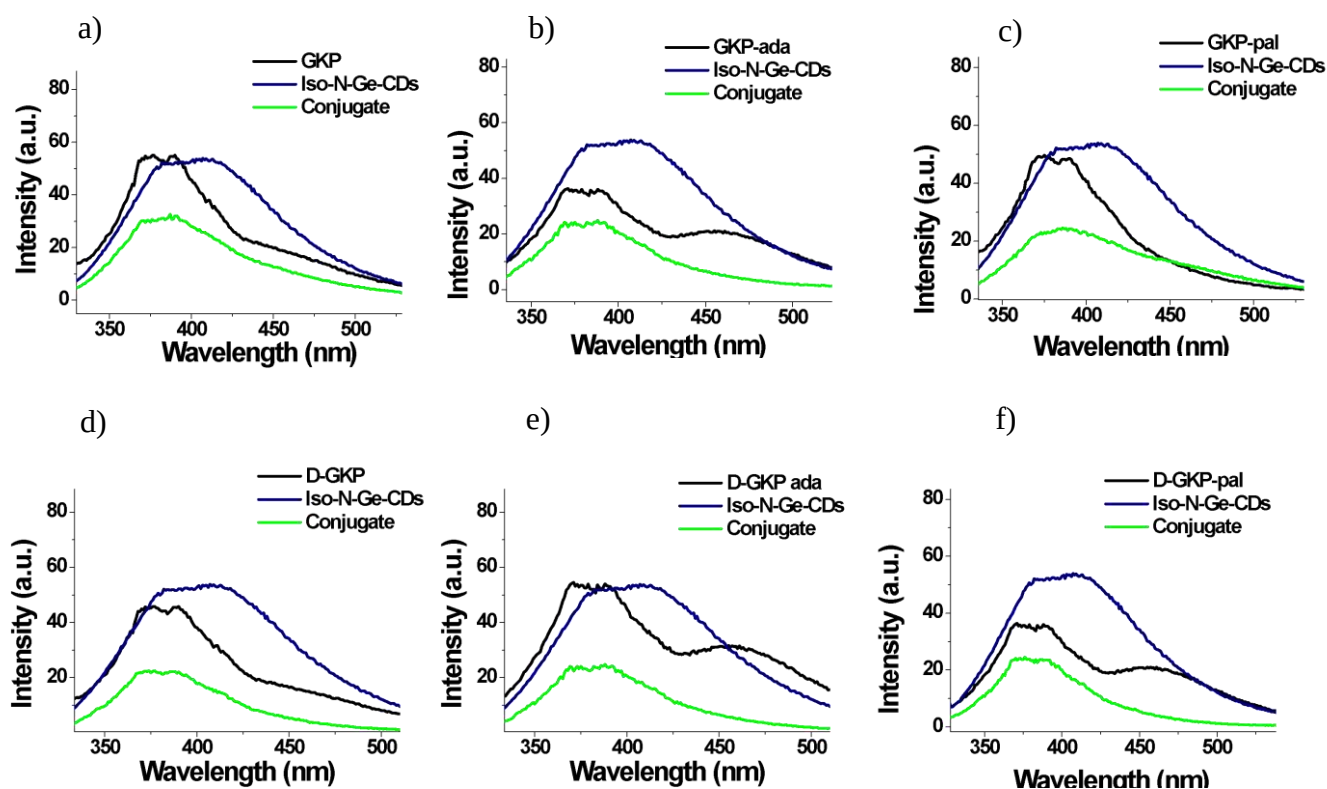


Figure 6.6 PL study on the effects of conjugation by different CDs. Black line represents the GKP peptide derivative, blue line represents the Iso-N-Ge carbon dots -and the green/lime line Conjugate.

In scientific and analytical applications, photoluminescence (PL) analysis using a single excitation wavelength is a commonly used technique. In this PL study the samples were analysed at a standard of excitation of 320 nm. In **Table 6.4**, the peptides, and conjugates were directly compared to spot distinctions, or similarities in their emission characteristics by maintaining a constant excitation wavelength. The similarity between the conjugates was the peak appearing between 375 and 425 nm. The difference in the PL spectra of the conjugates was the shift and broadness of the peaks.

Table 6.4 Summary of the peaks observed by the Photoluminescent spectrometer for GKP peptide derivatives and the conjugates.

Conjugate Name	PL data	
	Emission (nm) @ 320nm excitation	FWHM (nm)
1. <i>GKP-Iso-N-Ge-CDs</i>	382	68
2. <i>GKP-pal-Iso-N-Ge-CDs</i>	385	72
3. <i>GKP-ada-Iso-N-Ge-CDs</i>	383	75
4. <i>D-GKP-Iso-N-Ge-CDs</i>	379	65
5. <i>D-GKP-pal-Iso-N-Ge-CDs</i>	380	64
6. <i>D-GK-ada-Iso-N-Ge-CDs</i>	382	66
7. <i>GKP</i>	378	57
8. <i>GKP-pal</i>	379	59
9. <i>GKP-ada</i>	377	61
10. <i>D-GKP</i>	376	58
11. <i>D-GKP-pal</i>	379	62
12. <i>D-GKP-ada</i>	378	63
13. <i>Iso-N-Ge-CDs</i>	398	90

A similar trend was observed for the BP peptide derivative and their conjugates. The conjugation of CDs to the peptide can decrease the peak's width, indicating that both the conformation and functional groups of the peptide can influence the peak's broadness.

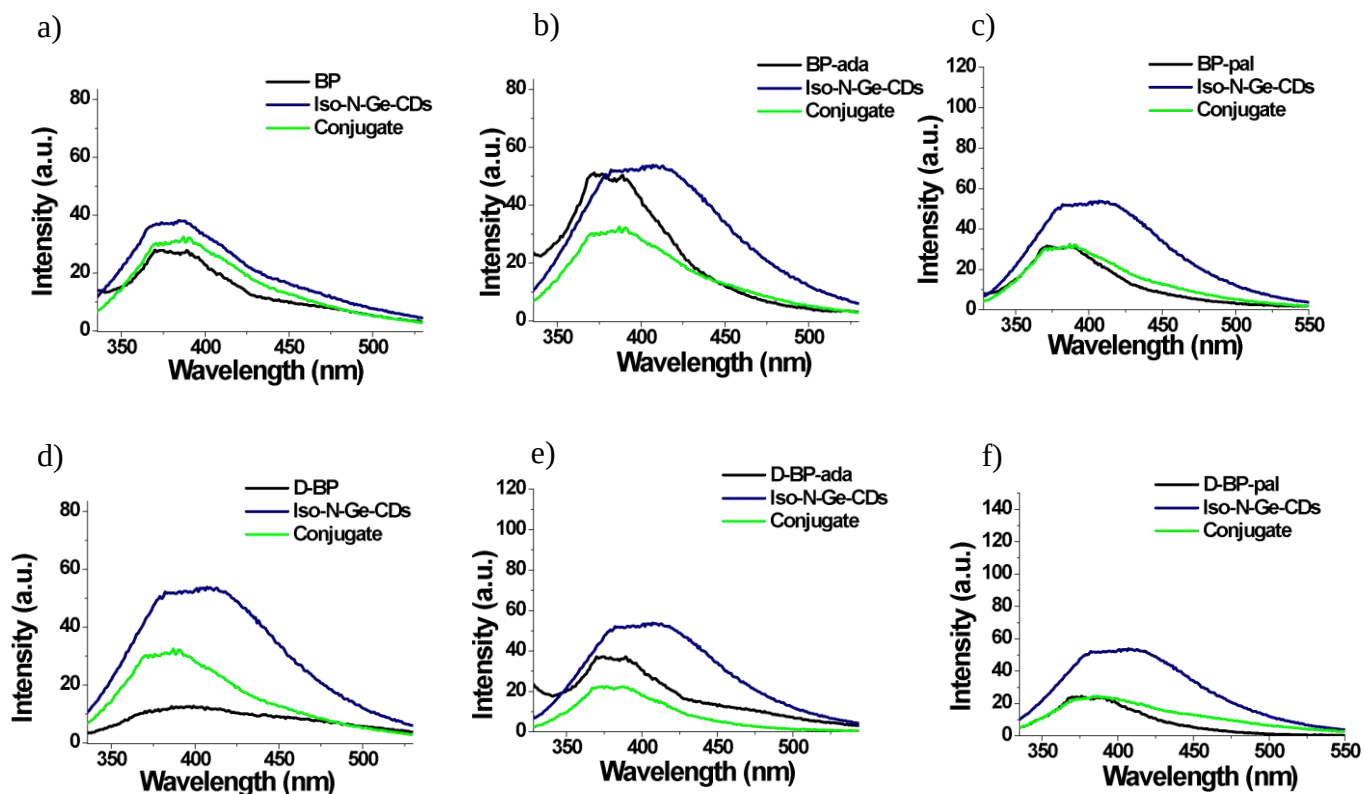


Figure 6.7 PL study on the effects of conjugation by different CDs. Black line represents the BP peptide derivative, blue line represents the Iso N Ge carbon dots -and the green/line line Conjugate.

A comparative analysis of the RLRLRIGRR sequence was conducted, enabling direct assessment of the PL characteristics across the different peptides. This approach was aimed to identify trends, differences, and similarities in emission properties among various materials. The same excitation wavelength of 320 nm was used in attempt to highlight their distinctive emission features. The notable difference was the absence of the peak between 425 and 500 nm seen in some of the peptides of the GKPIILF sequence. Additionally, the conjugates appeared to have a peak appearing in a similar region between 375 and 450 nm and a shift in the peaks was also observed as seen in **Table 6.5**.

Table 6.5 Summary of the peaks observed by the Photoluminescent spectrometer for BP peptide derivatives and the conjugates.

Conjugate Name	PL data	
	Emission (nm) @ 320nm excitation	FWHM (nm)
1. <i>BP-Iso-N-Ge-CDs</i>	387	68
2. <i>BP-pal- Iso-N-Ge-CDs</i>	385	58
3. <i>BP-ada-Iso-N-Ge-CDs</i>	383	66
4. <i>D-BP-Iso-N-Ge-CDs</i>	382	59
5. <i>D-BP-pal-Iso-N-Ge-CDs</i>	385	60
6. <i>D-BP-ada-Iso-N-Ge-CDs</i>	382	58
7. <i>BP</i>	377	62
8. <i>BP-pal</i>	375	61
9. <i>BP-ada</i>	378	60
10. <i>D-BP</i>	375	58
11. <i>D-BP-pal</i>	376	56
12. <i>D-BP-ada</i>	378	63
13. <i>Iso-N-Ge-CDs</i>	398	90

The interaction between electromagnetic radiation and the samples can also be investigated using an excitation study. These experiments use spectroscopic measurements whereby the frequency of the excitation light is varied, and the emission frequency of the sample is monitored in order to observe how it affects the luminescence.

Molecules are flexible entities capable of rotation and vibration. The molecule's energy landscape may be impacted by these vibrational and rotational transitions, leading to changes in energy levels that can produce a broad range of emission wavelengths. However,

The data in **Figure 6.8** shows that the GKP-Iso-N-Ge-CDs sample is excitation dependent, which means their fluorescence colour could be tuned simply by changing the excitation wavelength.²² The lowest emission is detected when the excitation is 400 nm, and the maximum emission is observed when the excitation is 340 nm. Most fluorophores can be selectively excited by a specific excitation wavelength.²³ Molecules, however, are flexible; they may spin and vibrate.²⁴ As a result, vibrational and rotational transitions are affected, leading to changes in energy levels that can produce range of emission wavelengths as seen in **Figure 6.8**.²⁵ Nevertheless, finding the highest emission wavelength is crucial for identifying a compound's electronic properties and analysing their electrical structure.²⁶ Additionally, in bio-imaging applications, knowledge of the maximum emission wavelengths can ensure compatibility with the biological sample or organism under investigation selectivity when analyzing cells.²⁷

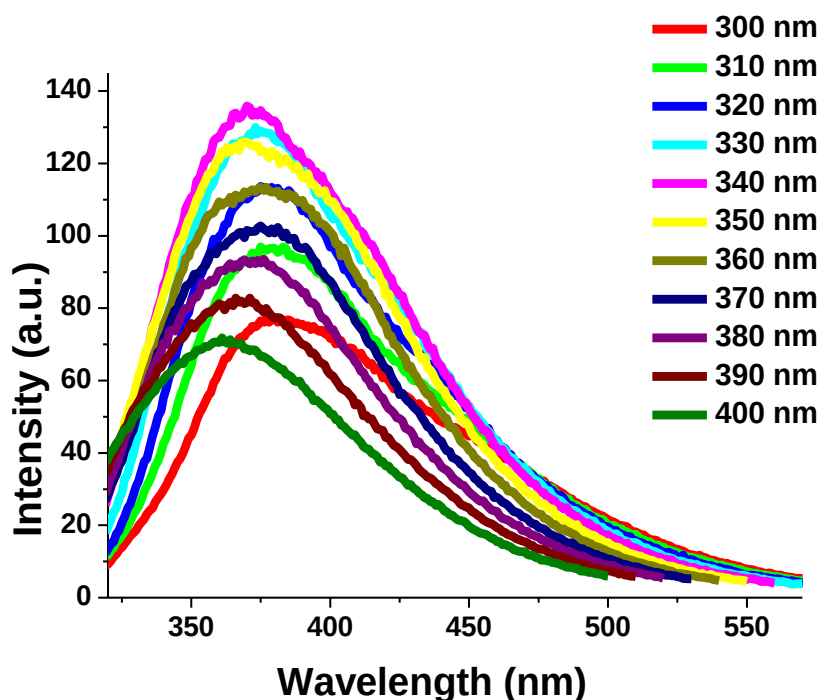


Figure 6.8 PL spectra of the GKP-Iso-N-Ge-CDs conjugate at various excitations

Another notable feature that most CDs can possess is their intrinsic pH-sensitive nature which can also be monitored using photoluminescence spectroscopy, which therefore demonstrates their great perspective for pH sensing applications, especially in pH sensors relating to living organisms, also known as biotic pH sensing.²⁸ The pH sensitivity of CDs is

considered to be ascribed to the reversible protonation and deprotonation of the functional groups on the surface of the CDs.²⁹ In addition, the favourable properties of CDs such as good biocompatibility and photostability, in conjunction with the great potential in pH sensitivity, make CDs a great candidate for bio-imaging tumours.³⁰ According to the literature tumours possess several pockets of acidity in their microenvironment, which can be exploited using the pH-sensitive CDs that can be hydrolysed at lower pH and result in superior drug delivery.³² The pH study of the GKP-Iso N Ge was conducted (Figure 6.9) on the photoluminescent spectrometer, and as the pH was increased from pH 1 to pH 10, the sensitivity of the conjugate fluctuated similarly to what is found in the literature.³² The lowest emission is detected when the pH is 7, which may be due to the fact that the least protonation and deprotonation can occur at this pH, and the maximum emission is observed when the pH is 3 and 8 attributed to the increased possibility of protonation and deprotonation which seems to increase the luminescent properties.

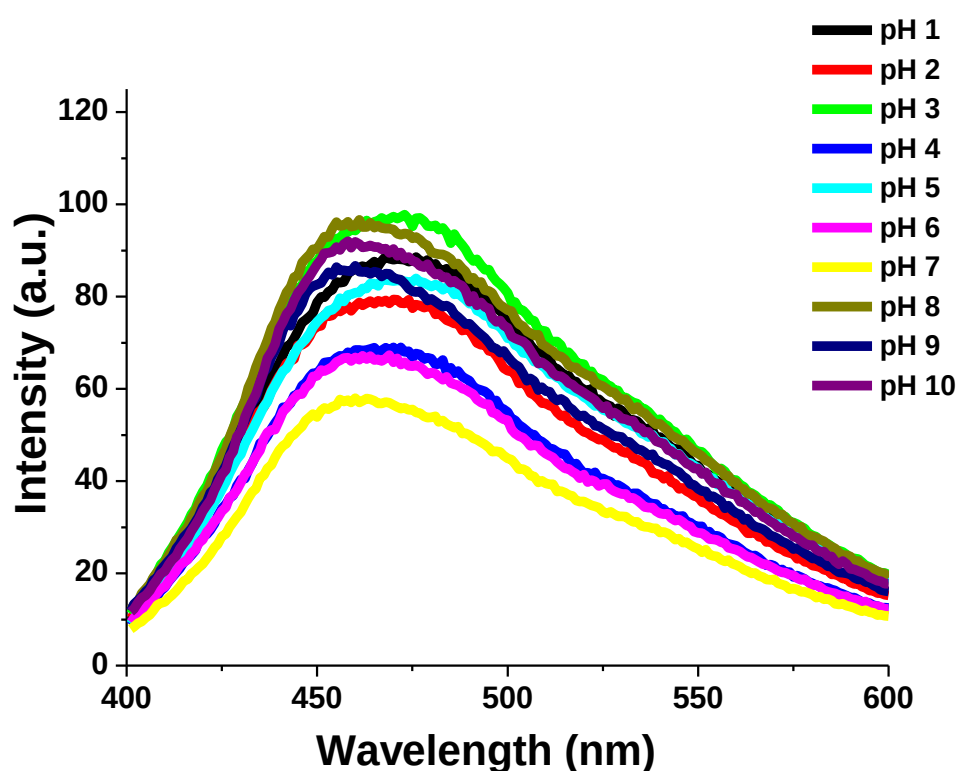


Figure 6.9 pH study on the GKP-Iso-N-Ge-CDs

6.4.3 NMR of CDs conjugates

There are several characterization techniques that provide information on the physical and chemical properties of carbon dots.³³ In some cases, the scanning electron microscope (SEM) coupled to energy-dispersive X-ray spectroscopy (EDX) (SEM/EDX) can be used to determine the size and elemental composition measurements.³⁴ Additionally, X-ray photoelectron spectroscopy (XPS) and infrared (IR) can supply valuable information about the nature of the composition, whether it has a high content of sp² hybridized carbon or a wide variety of functional groups.³⁵ NMR is a technique that is scarcely used for the characterization of carbon dots but can provide better clarity on the carbon species found in the material.³⁶ Moreover, we demonstrated that NMR could be used to analyse CDs-peptides conjugates. The NMR was used to understand the peptide's possible interactions with the CDs by monitoring regions in the NMR spectra that were affected by the noncovalent interactions. To get a better perspective on the NMR of the conjugates, the fully elucidated peptide and the NMR of the CDs were used for comparison.

In a study by Elena V Bichenkova *et al.*, the interactions of graphene with aromatic and aliphatic hydrocarbons in water were investigated.³⁷ The data revealed that changes in spin–spin coupling, increase in relaxation, line broadening, and decrease in NMR peak heights were a direct result of compounds bound to graphene. Moreover, when the concentration of the graphene was increased, the aromatic protons would vastly decrease.

The lack of magnetic field homogeneity results in the broadening of the peak. For example, in a study, by Simone C *et al.*, glucose (G), fructose (F), and ascorbic acid (A) were used to synthesize CDs.³⁸ The data showed that G-CDs and F-CDs were well-defined with lattice fringes with an interlayer spacing of 2.4 Å on HRTEM, but A-CDs were poorly defined. The ¹H NMR of the distinctly formed CDs possessed the characteristic broad peak between 3.0 and 4.5 ppm. Therefore, in addition to using UV-Vis and PL techniques to confirm conjugation, the shift and intensity of the peaks in the NMR of conjugates were examined to determine the success of the formation of noncovalent interaction between the peptide and CDs due to adsorption.

6.4.4 NMR of CDs before and after dialysis

The NMR spectra of CDs in deuterated DMSO before dialysis and after dialysis were analyzed, and revealed that the resolution of the peaks were improved after dialysis. This was

crucial for assigning the peaks found in the spectra of the CDs to determine the different functional groups present. The peak at 5.5 ppm was attributed to the urea molecule, which was doped into the CDs. Before dialysis of the CDs, the unreacted urea appears to be present in the sample. After dialysis, a significant reduction in the peak is observed in the urea peak at 5.5 ppm, as seen with asterisks in **Figure 6.10**. Since membrane dialysis relies on osmosis, most small molecules pass through the membrane, but some of the small molecules and unreacted reagents remain. The ^1H NMR of the CDs after dialysis (**Figure 6.10c**) appears to have more prominent peaks than the CDs before dialysis (**Figure 6.10b**). These are detected around 4.0 ppm, 3.5–3.1 ppm and 1.2 ppm. The unreacted precursors may have masked the peaks of the CDs, and upon removal, through membrane dialysis, the peaks appear more prominent.

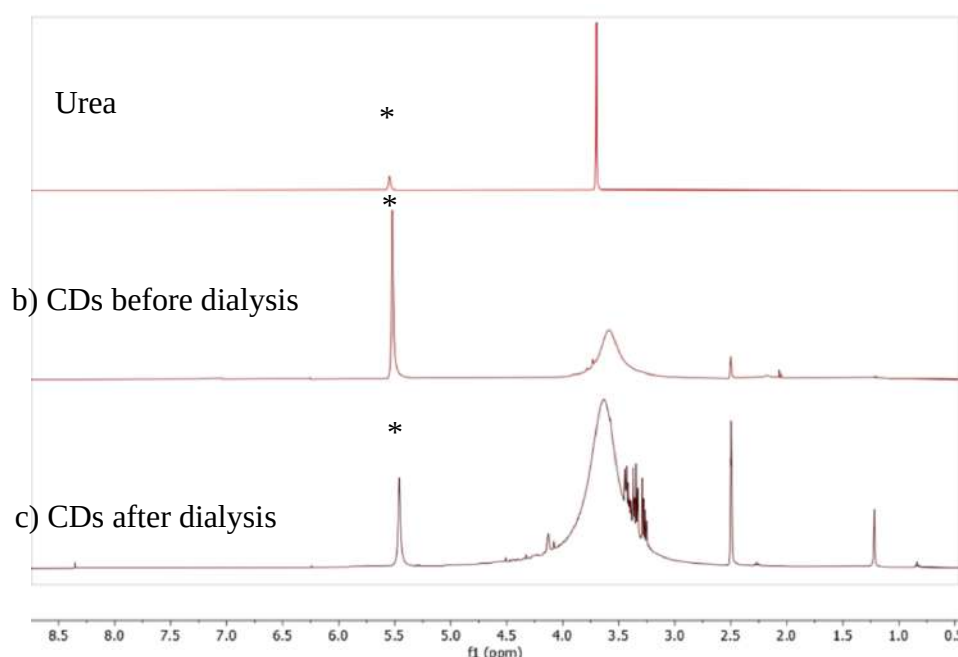


Figure 6.10 Stacked ^1H NMR spectra of urea (pink), CDs before (red), and after dialysis (brown) in deuterated DMSO.

A slight shift was detected in the peak ascribed to unreacted urea at around 5.5 ppm, which initially appeared at 5.45 ppm before carbonization and 5.54 ppm after, as seen in **Figure 6.11**, indicating physisorption interactions facilitated by the carboxylic group from the CDs and amine groups from urea.

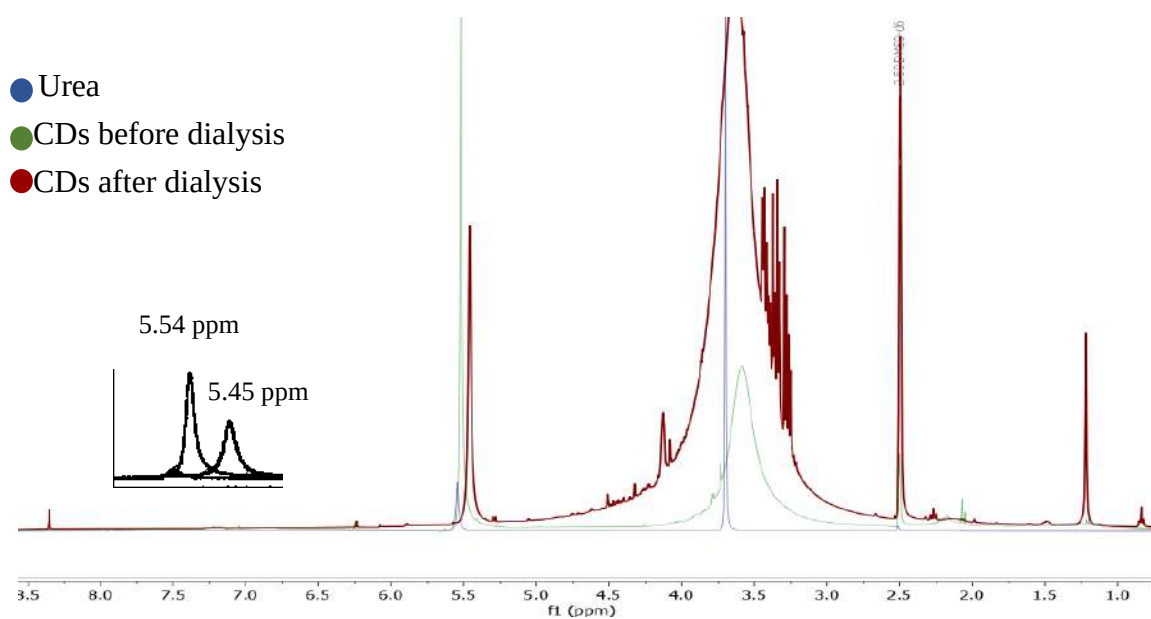


Figure 6.11 Overlapping ^1H NMR spectra of urea (pink), CDs before (red), and after dialysis (brown) in deuterated DMSO.

Cross peaks in the COSY experiment are between protons that are coupled two or three bonds away. These cross-peaks can reveal details on the connectivity of the carbon atoms in the molecule as well as the various functional groups present. **Figure 6.12a** shows correlations connecting 4 different chemical shifts in the COSY NMR spectrum. The methyl group at 0.85 ppm (methyl) correlates with the CH₂ at 1.25 ppm, which in turn further correlates to the CH₂ at 1.48 ppm and another CH₂ at 2.28 ppm. This CH₃CH₂CH₂CH₂ chain does not show a COSY correlation to other protons in the spectra. This suggests that the peak at 8.3 ppm might be an amide or aldehyde. The HSQC was then used to determine the carbon chemical shifts of CH₃CH₂CH₂CH₂ since it shows ¹H-¹³C direct correlations.

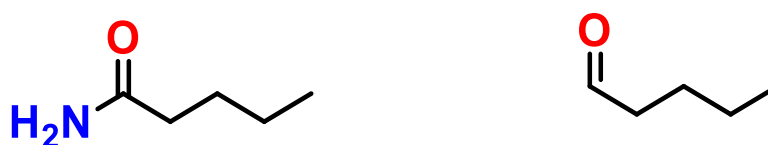


Figure 6.12 The COSY NMR of the CDs after dialysis and an insert of the allylic region between 2.5 and 0.4 ppm a) COSY and HSQC NMR.

In **Figure 6.13a** of the HMBC NMR, the region between 8.5 and 7.5 ppm does not show any correlations to the 8.3 ppm proton peak. However, other peaks that appeared in the ¹H NMR correlated to other carbons in the spectra. Additionally, the correlation at 2.28,173.68 ppm in **Figure 6.13a** of the HMBC shows that the CH₂ is correlating to a carbonyl carbon.

Carbon dots are known to produce a wide variety of functional groups, and the other peak may be attributed to that. However, the CH₃CH₂CH₂CH₂ chain was clearly connecting on the COSY and appeared to be a small side molecule that formed during synthesis, and the HMBC correlation H-2.28, C-173.68 ppm confirms the presence of pentanal. It is more likely

that the chemical shift of 173 ppm is an aldehyde due to its large ppm value. It is more common for an amide to show much lower chemical shift, perhaps 120–160 ppm. The NMR summary of the correlations is seen in **Table 6.6**.

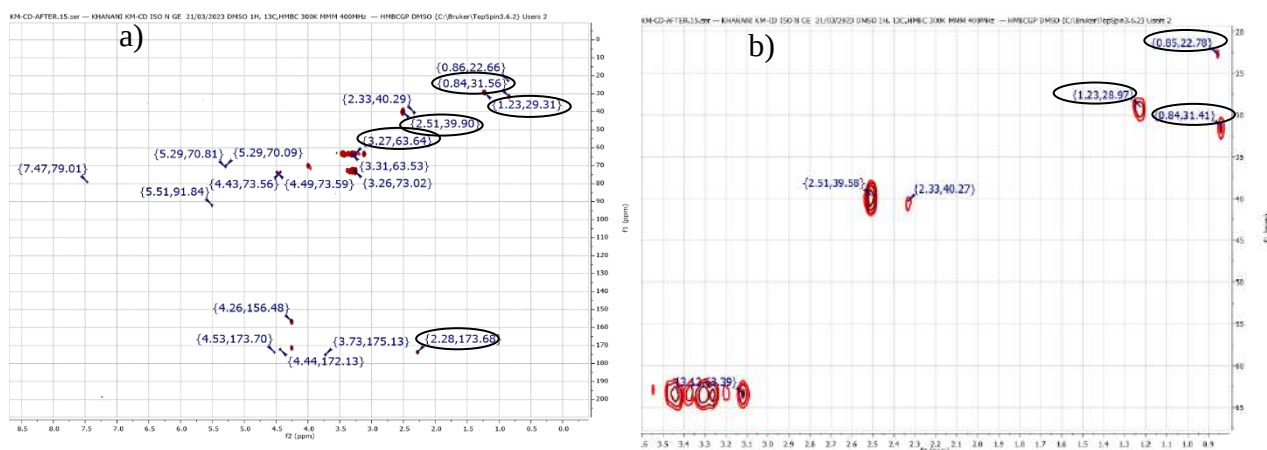


Figure 6.13 The HMBC NMR of the a) CDs after dialysis and b) the allylic region between 2.0 and 0.4 ppm

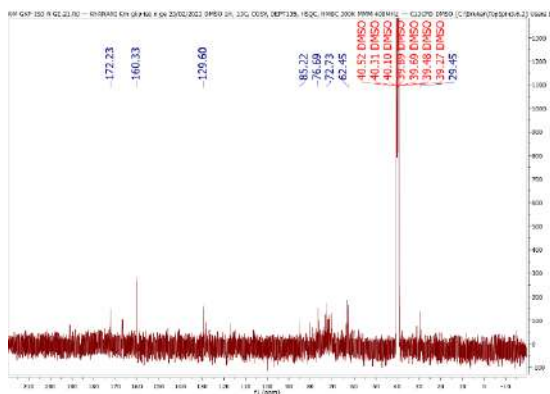
Table 6.6 The summarized NMR data of the short chain confirming the pentanal structure.

¹ H NMR	¹³ C NMR	COSY	HMBC
0,83	14,30	0,83-1,25	0,84-31,56
1,25	29,34	1,24-1,48	1,23-29,31
1,48	24,97	1,50-2,27	
2,28	33,99	-	2,28-173,68
2.49	43.05		
8,37	-		

Figure 6.14a of the *GKP-Iso-N-Ge*-CDs shows multiple carbon peaks at 63.49, 72.91, 78.49, 129.60, 160.19, and 172.23 ppm that did not correspond to the protons found on the ¹H NMR. The ¹³C NMR of the *Iso-N-Ge*-CDs seen in **Figure 6.14b** show the prominent carbons before conjugation to clearly distinguish the carbons present after conjugation. Any correlation may have been helpful in proving more information about the CH₃CH₂CH₂CH₂ chain. Fortunately, the one peak that was seen to correlate with 173.68 on HMBC was the

2.28 ppm proton peak. The corresponding protons of these carbons determined by HSQC NMR are shown in **Table 6.7**. These peaks were identified because they could possibly interact with the peptide after conjugation.

a)



b)

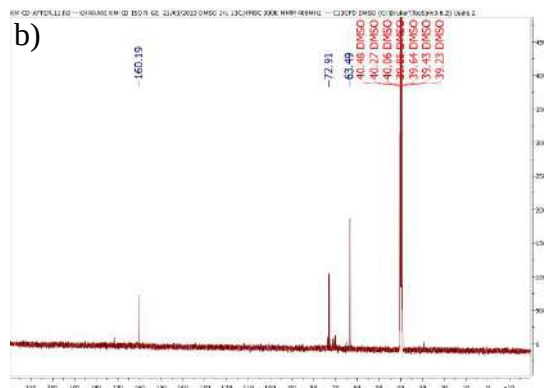


Figure 6.14 The ^{13}C NMR of the a) GKP-Iso-N-Ge-CDs conjugate and the b) Iso-N-Ge-CDs

Table 6.7 The summarized NMR data of the carbon dots

^1H NMR (HSQC)	^{13}C NMR	COSY	HMBC
3,35	63,49	1.62	63.49
3,43	72,91		
4,17	78,49		
	129,60		
-	160,19		
	172.23		

6.4.4.1 ^1H NMR comparison between the GKP-Iso-N-Ge-CDs conjugate and the GKP peptide

The freshly prepared GKP-Iso-N-Ge-CDs conjugate sample was also analyzed using ^1H NMR. The data revealed that the conjugate displayed similar peaks as the bare peptide sample. This indicated that the peptide was still intact after conjugation. In **Figure 6.15**, drawn in red, are the spectra of the GKP-Iso-N-Ge-CDs conjugate, and the blue/green spectra represent the GKP peptide.

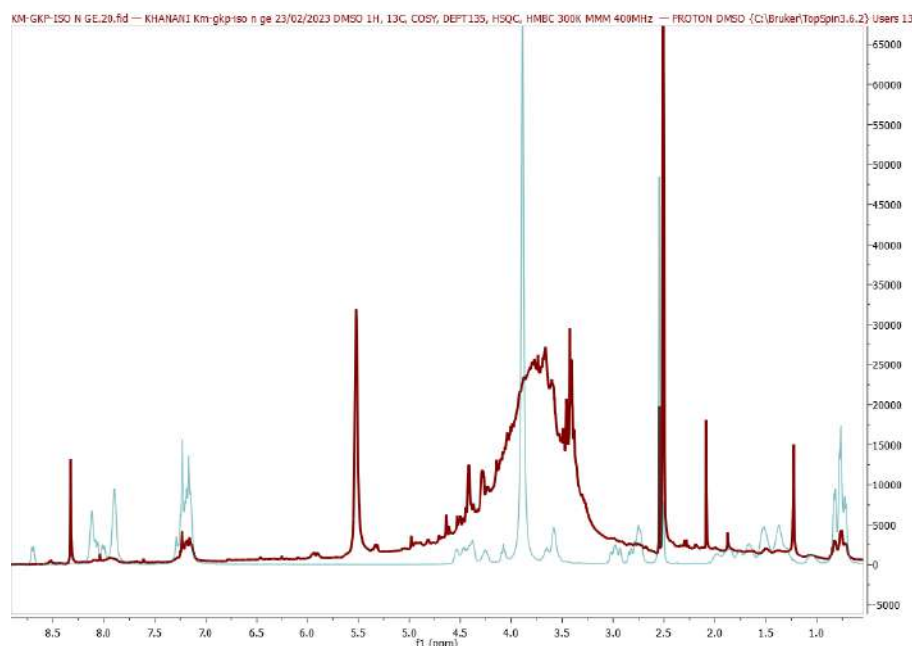


Figure 6.15 The overlapping ^1H NMR spectra. Indicated in red and blue/green, are the spectra of the GKP-Iso-NGe-CDs conjugate and GKP peptide

Interestingly, when comparing the two spectra, a shift to the right was detected in the aromatic region between 7.0 and 7.25 ppm and between 0.5 and 0.75 ppm in the methyl region. Another possible interaction is the π - π stacking between the aromatic rings on the carbon dots and the aromatic rings in the phenylalanine groups. However, the phenylalanine peaks are still distinct after conjugation, which shows a low affinity for the CDs.³⁹ Moreover, the amide protons in the backbone of the peptide appeared to be significantly affected after conjugation due to the poor resolution of the amide protons and reduced peak intensity between the region 8.6 ppm and 7.7 ppm. This indicated that the peptide could possibly be wrapped around the CDs.^{40, 41} Additionally, the amide proton peak from lysine disappeared in the conjugate spectra. The computational studies mentioned in Chapter 5 showed that the dihedral angle of the lysine was 130.8 degrees, ascribed to the antiparallel feature, while the other residues were between -95 and -35, which is characteristic of the helical structure. This implies that the lysine NH is exposed while the other NH protons are in line with each other in a helical coil. Moreover, lysine is the only hydrophilic amino acid in the peptide and since the functional groups of the CDs are also hydrophilic, they have high affinity towards each other. This was attributed to the possibility that the NH of lysine was embedded in the CDs which resulted in the disappearance of the lysine NH.

The ROESY NMR of the conjugate was used to investigate whether there are any long-range correlations between the peptide and the CDs. The spectra in **Figure 6.16b)** showed a

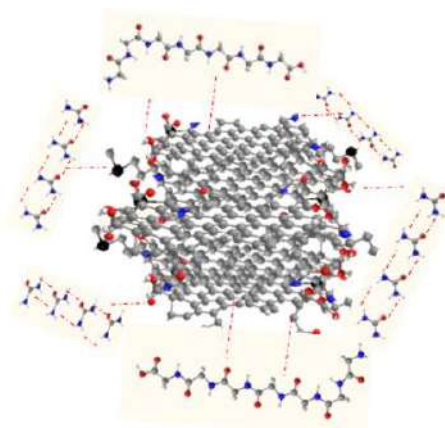


Figure 6.17 Peptide wrapping around the CDs

The data gathered from the NMR experiments provided insight into the mechanisms of the CDs formed as well as the possible structure of the final conjugates. **Figure 6.18** illustrates the monomer which was used after heating using a microwave, which led to crosslinking and polymerization. This further reacted to form graphene layers which interact through pi stacking or amorphous layers, which was confirmed in the HRTEM images and as a graphitic peak in XRD patterns $2\theta = 22.4^\circ$ (002) plane reflection mentioned in section 3.5.1 and 3.5.4, respectively. The resulting carbon dots seem to interact with the unreacted urea through electrostatic force and the alpha protons of the glycine and proline amino acids.

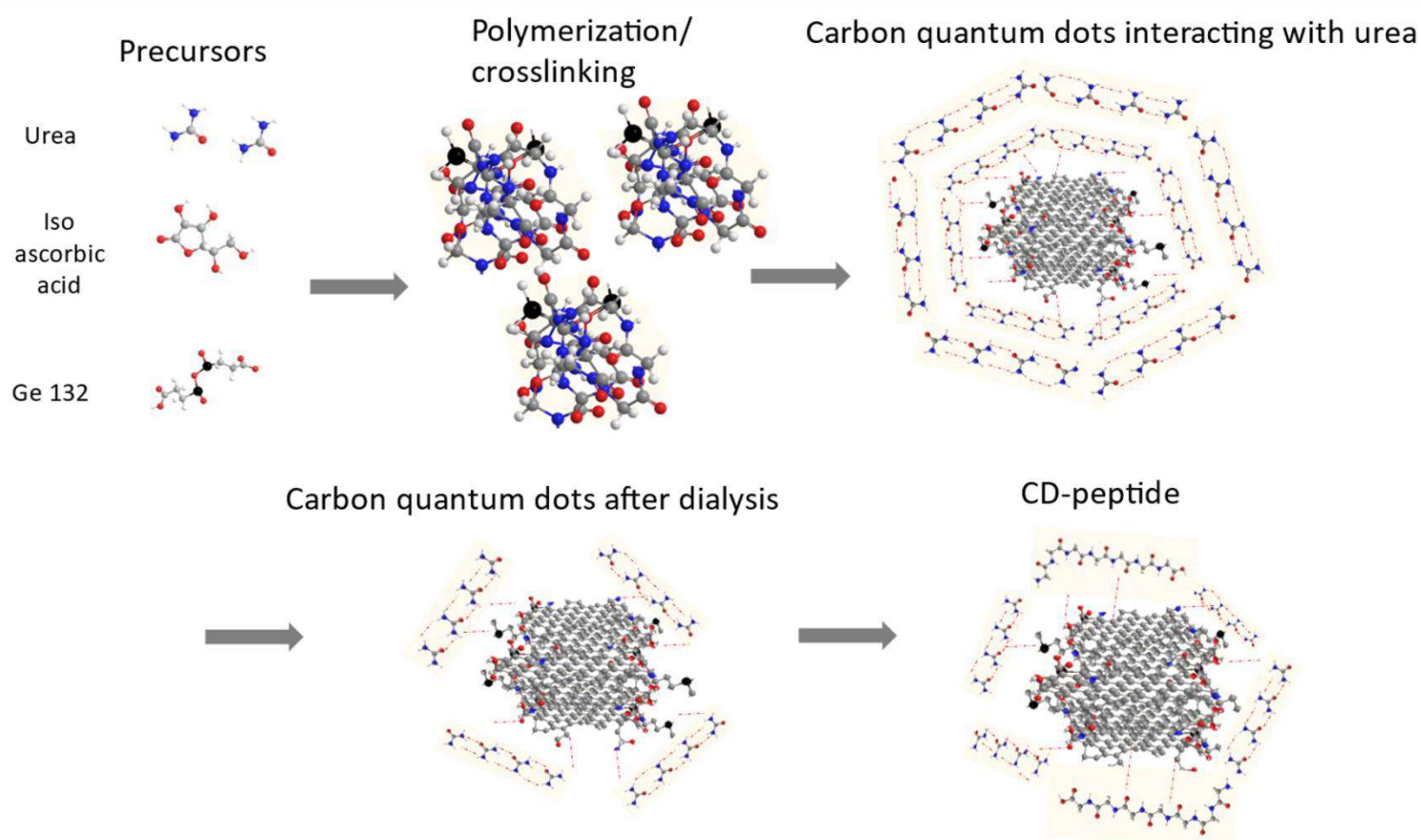


Figure 6.18 The mechanisms of the CDs formed as well as the possible structure of the final conjugates.

6.4.4.2 2-D COSY NMR comparison between the GKP-Iso-N-Ge-CDs conjugate and the GKP peptide

In **Figure 6.19**, the COSY NMR spectra show the ^1H - ^1H correlations within the a) GKP and b) GKP-Iso-N-Ge-CDs conjugate. Due to the various chemical environments in which the proton can exist, peaks that were previously overlapped in the peptides' ^1H NMR spectra are now seen as distinct peaks.

The area between 5.5 and 3.0 ppm commonly exhibits CH groups and hetero-atom-containing moieties, and this region seems to be the most populated area. This may be a combination of the CH groups found on the surface of the carbon dot and the CH groups found on the peptide. Interestingly, some of these protons appear to be correlating to the aromatic protons around 7.5 and 8.5 ppm for the conjugate. These are more likely to be

attributed to the aromatic protons from the peptide rather than the aromatic protons from the carbon dots.

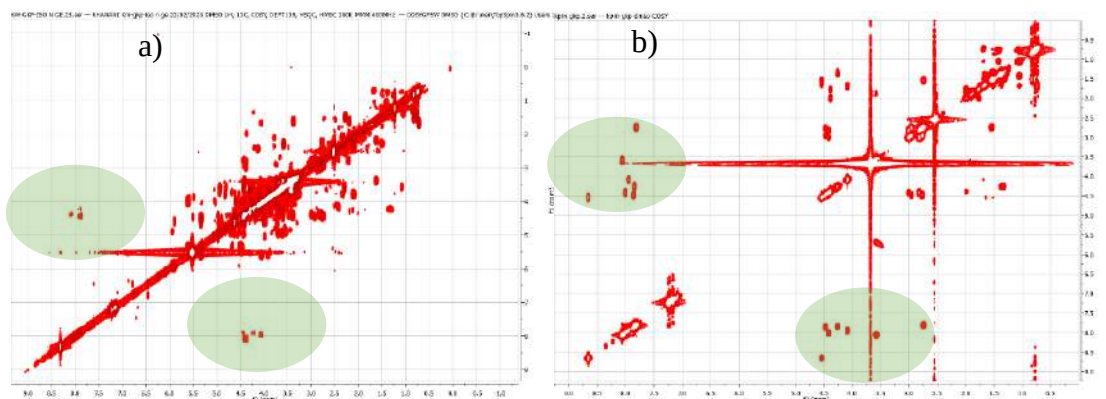


Figure 6.19 The COSY NMR spectra show the ^1H - ^1H correlations within the a) GKP-Iso-N-Ge-CDs conjugate and b) GKP.

Illustrated in **Figure 6.20** are the overlapped COSY spectra of the peptide and the conjugate, with the peptide in red and the conjugate in green. The comparison between the two shows a huge difference; however, the regions which are overlapping should provide insight into the conjugate. Due to the different intensities of the samples, the similarities are not clearly exhibited.



Figure 6.20 The overlapping of the peptide and the conjugate COSY spectra, with the peptide in red and the conjugate in green.

When a carbon dot is conjugated to a peptide, the peptide can have a complex effect on the carbon atoms' NMR chemical shift, resulting in portions of the carbon dot's NMR spectrum that are both shielded and deshielded.⁴² For instance, if the peptide has groups that can give electrons to the carbon dot, like amino or hydroxyl groups, this can shelter the carbon atoms that are close to the carbon dot. On the other hand, if the peptide contains electron-withdrawing groups like carbonyl or nitro groups, they may pull electrons away from the carbon dot, deshielding the carbon atoms nearby.

Additionally, the peptide may engage in noncovalent interactions with the carbon dot, such as hydrogen bonds, which may potentially have an impact on the carbon atoms in the carbon dot's NMR chemical shift. These interactions occasionally cause localized regions of shielded or unshielded carbon atoms to form in the carbon dot. Thus, the NMR chemical shift of a carbon dot attached to a peptide can be complicated and reliant on a variety of variables. Thus, it's critical to carefully examine the NMR spectrum before conjugation to comprehend the carbon dot's chemical environment.

Four regions that were overlapping were analyzed to see if a shift could be observed relative to the peaks of the peptide. In **Figure 6.21a**) the region between 4.60 to 4.04 ppm F2 (X) and 3.1 to 1.3 ppm F1(Y) illustrates the alpha-beta correlation of leucine, isoleucine and the two phenylalanine at (1.51,4.6) ppm, (4.08, 1.70) ppm and (4.48- 4.39, 3.00, 2.76) respectively. The conjugate in green appears to shift upfield in this particular region in the F2 (X). This shielding phenomenon often occurs when the atoms are being shielded from the magnetic field, which suggests that the alpha protons of the phenylalanine are being shielded as a result of conjugation to the carbon dots.

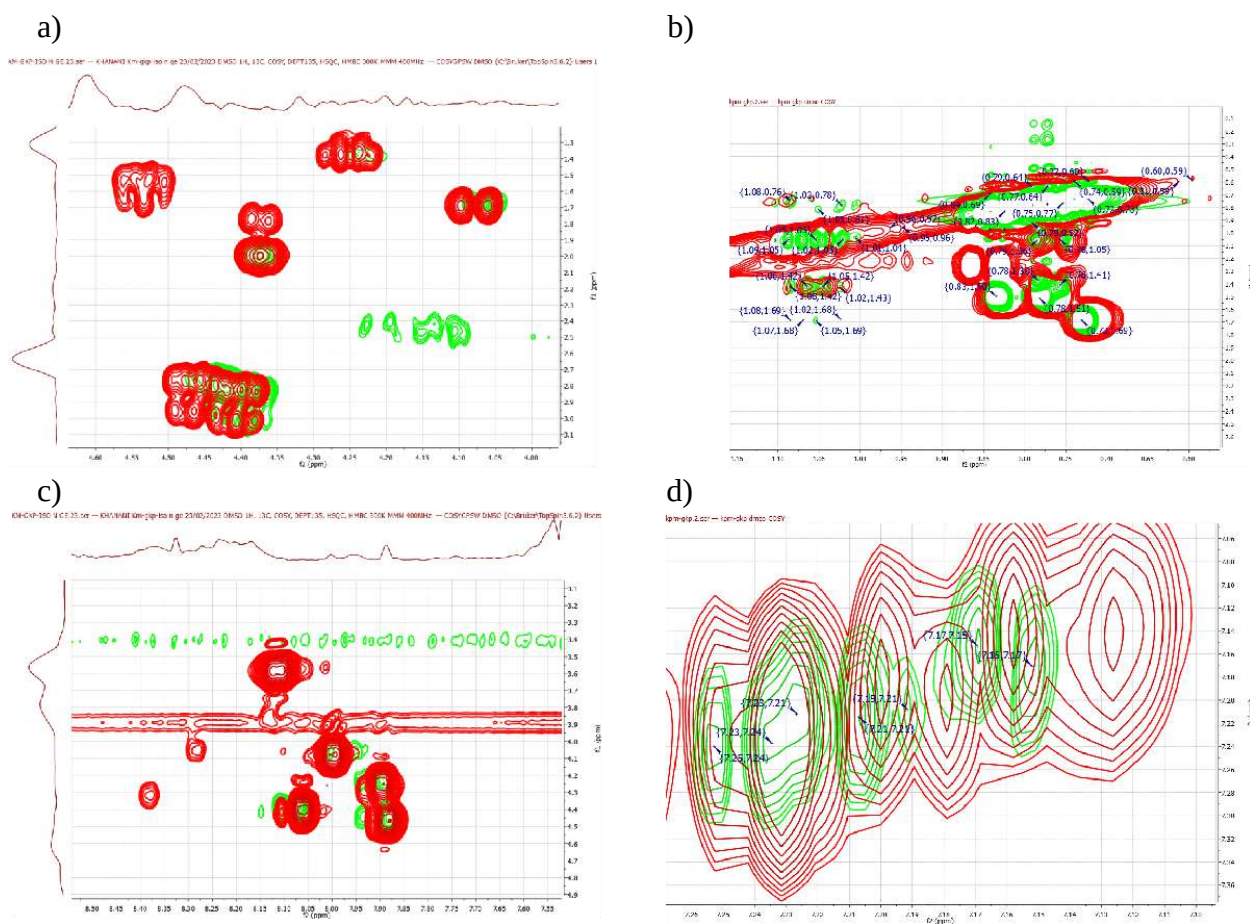


Figure 6.21 COSY NMR of four overlapping sections with the peptide in red and the conjugate in green.

In **Figure 6.21 b)**, the chemical shifts for the CH₃ groups of isoleucine and leucine are observed at (1.69,0.73) ppm and (1.51,0.83) ppm, respectively. The elucidation of the short chain, which was assigned above, also had a peak at 0.83 ppm, however, the COSY revealed that a (0.83,1.25) ppm correlation was observed, which eliminated the possibility of it being from the short chain-CDs. Other correlations around these protons could be seen; however, more importantly, a downfield shift is observed in this area. This indicates that the functional groups which are further away from the peptide backbone are exposed to the magnetic field. Moreover, **Figure 6.21c)** also displays a downfield shift for the amides of isoleucine, leucine, and phenylalanine at (7.85,4.08), (7.94, 4.26) and (7.86,4.48) ppm.

Finally, in **Figure 6.21 d)** six peaks in the aromatic region were investigated to analyze the effect of conjugation in this region. Interestingly, the four peaks between 7.18 and 7.13 were

shifted downfield; however, the peaks at 7.25 and 7.23 ppm appear to shift downfield slightly. This data indicates that a carbon dot attached to a peptide may exhibit deshielded and shielded NMR chemical shifts in different regions. The magnetic field surrounding the target nucleus in NMR spectroscopy determines the chemical shift.

6.4.4.3 2-D HSQC comparison between the GKP-Iso-N-Ge-CDs conjugate and the GKP peptide

The HSQC experiment is used to determine proton-carbon single bond correlations of the conjugate, where the protons lie along the observed F2 (X) axis, and the carbons are along the F1 (Y) axis. In this section, conjugation was analyzed to compare the effects of conjugation concerning carbon atoms. In **Figure 6.22**, the HSQC NMR of the peptide in red and the conjugate in green was assessed.

The first thing which was noticed was the significant difference in the region between 4.50 and 0.50 ppm. The carbons on the peptide appear to be more evenly distributed, while the conjugate is more concentrated between 5.5 ppm and 3.0 ppm. This may be because peptides have a wide variety of alkanes while carbon dots predominantly have hetero-atom containing protons and anilines (due to nitrogen doping) as well as other non-cyclic alkanes which may be the functional groups present on the surface of the carbon dots.

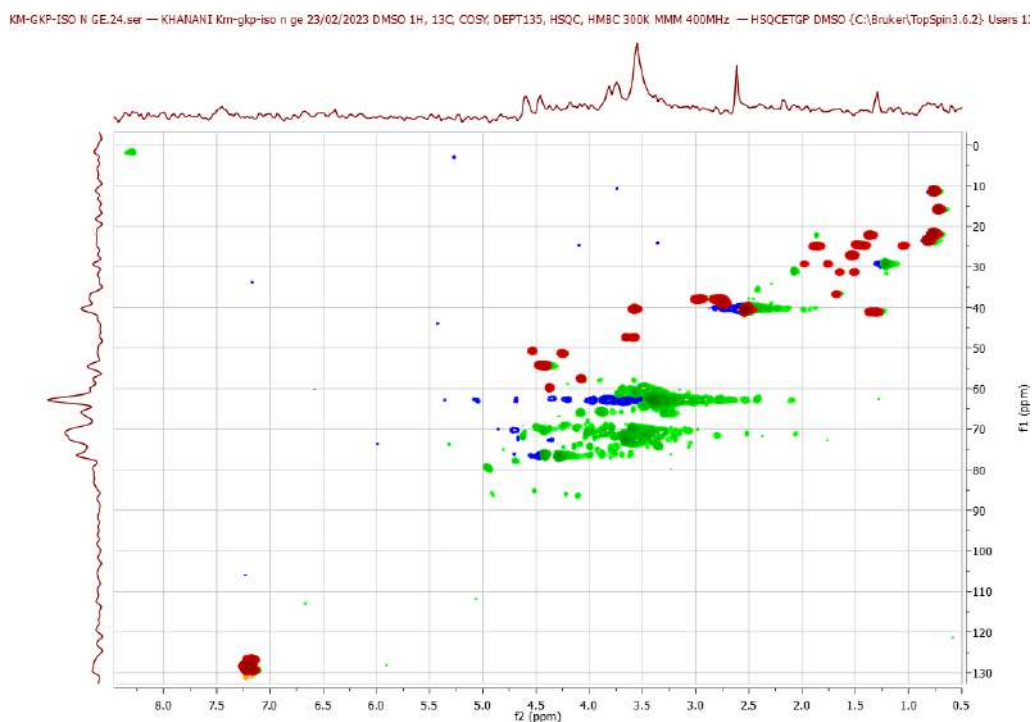


Figure 6.22 This shows the HSQC NMR of the peptide in red and the conjugate in green.

In **Figure 6.23a)** the aromatic region is observed, which appears to be shifted upfield in the direction F2 (X) axis and slightly downward in the downfield direction in the F1(Y) axis. This demonstrated that the carbon atoms are also affected by the conjugation of the carbon dots. The combination of shielding and deshielding seen in this section is consistent with what was observed in the COSY NMR. The CH₃ groups from leucine and isoleucine are observed in Figure 6.23b) and appear to be shifting upfield.

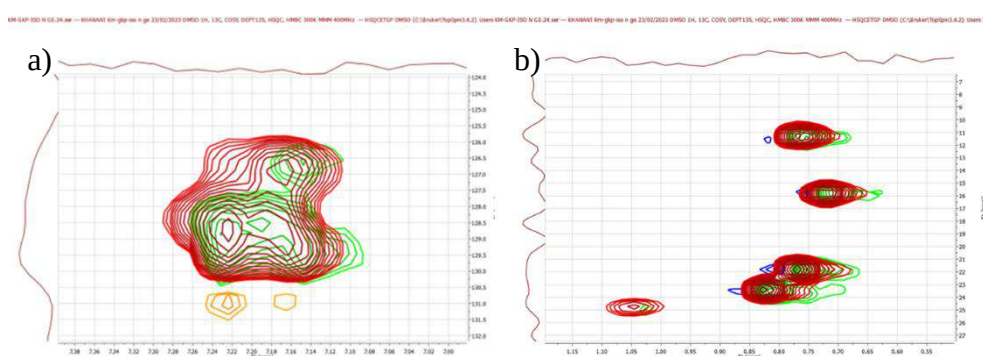


Figure 6.23 This illustrates the HSQC NMR of the a) aromatic region and b) methyl region of the peptide in red and the conjugate in green.

6.4.4.4 Comparison of other conjugates

Other GKP peptide derivatives were conjugated to the *Iso-N-Ge*-CDs, as seen in **Figure 6.24**. One common feature that seems to be observed in all the samples is the presence of a broad peak from 5.5 ppm to 3.0 ppm, although, in a) GKP-*Iso-N-Ge*, d) D-GKP-*Iso-N-Ge*-CDs and e) D-GKP-*ada-Iso-N-Ge*-CDs the broad peak appears more distinctly. Although the broad peak is small, it appears to have shifted downfield and wider for b) GKP-*ada-Iso-N-Ge*-CDs, c) GKP-*pal-Iso-N-Ge*-CDs and f) D-GKP-*pal-Iso-N-Ge*-CDs. This suggests that different peptides were affected differently due to the carbon dots after conjugation, and less intensity and broadening of peaks usually indicate poor structure integrity or homogeneity. Sample b) GKP-*ada-Iso-N-Ge*-CDs and c) GKP-*pal-Iso-N-Ge*-CDs also had larger FWHM compared to the other conjugates. This further suggests that the conjugates have inhomogeneity, and hence these conjugates might not be suitable for biological studies.

Therefore, based on the obtained data the following conjugates will be sent for biological testing: a) GKP-*Iso-N-Ge*-CDs, d) D-GKP-*Iso-N-Ge*-CDs and e) D-GKP-*ada-Iso-N-Ge*-CDs.

The selection criteria involve the narrow FWHM of PL spectra indicative of CDs of a smaller size distribution for higher selectivity of the spectral range. The UV-Vis data clearly demonstrates conjugation for all the samples, however there is no additional information which can indicate if they have better properties for biological testing. The NMR of the conjugates suggests that the helical structure may be affected due to the significant shift in the peaks. Since the helical structure may be contributing to its activity, the NMR spectra with less structural difference in the peptide may have better biological properties.

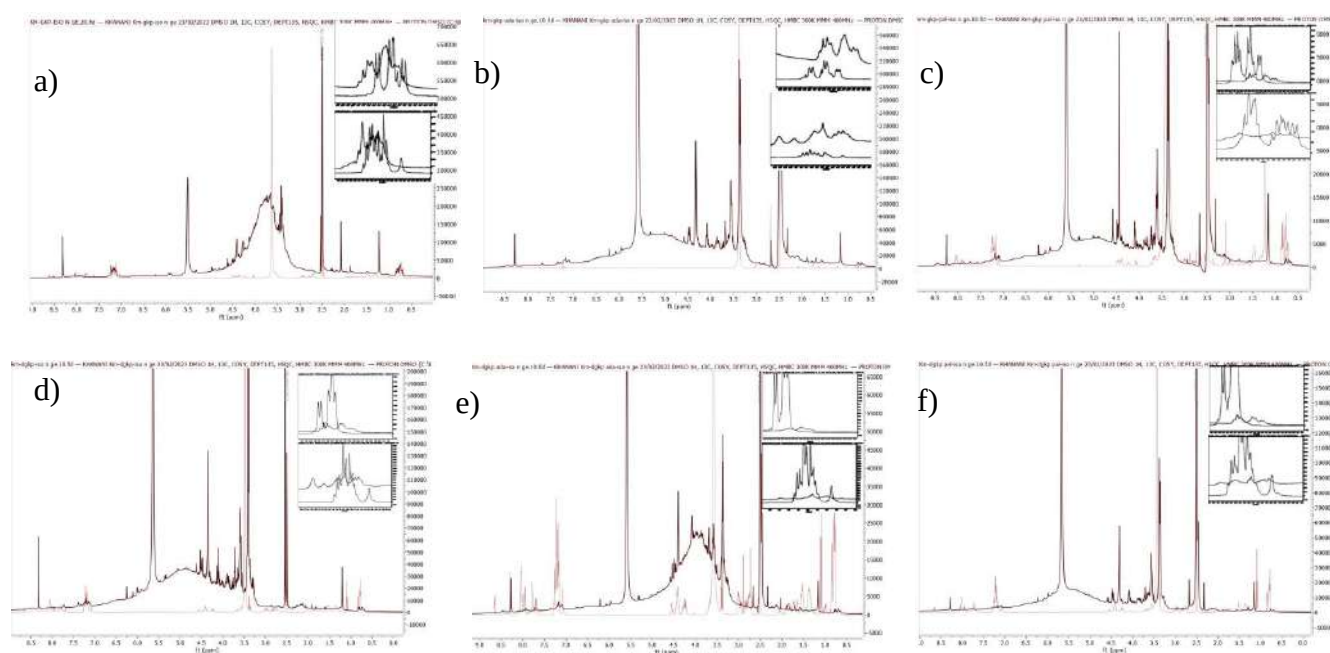


Figure 6.24 This shows the ^1H NMR of the a) GKP-Iso-N-Ge-CDs for b) GKP-ada-Iso-N-Ge-CDs c) GKP-pal-Iso-N-Ge-CDs d) D-GKP-Iso-N-Ge-CDs and e) D-GKP-ada-Iso-N-Ge-CDs f) D-GKP pal-Iso-N-Ge-CDs of the conjugate in red and the peptides in blue.

A similar trend was seen for the conjugates synthesized from the BP peptide derivatives (**Figure 6.25**). However, a significant shift appears in the region between 3.0 and 4.5 ppm which has been assigned to the functional groups from the CDs. Therefore, based on the data the following conjugates will be sent for biological testing: c) BP pal-Iso-N-Ge-CDs, and d) DBP ada-Iso-N-Ge-CDs.

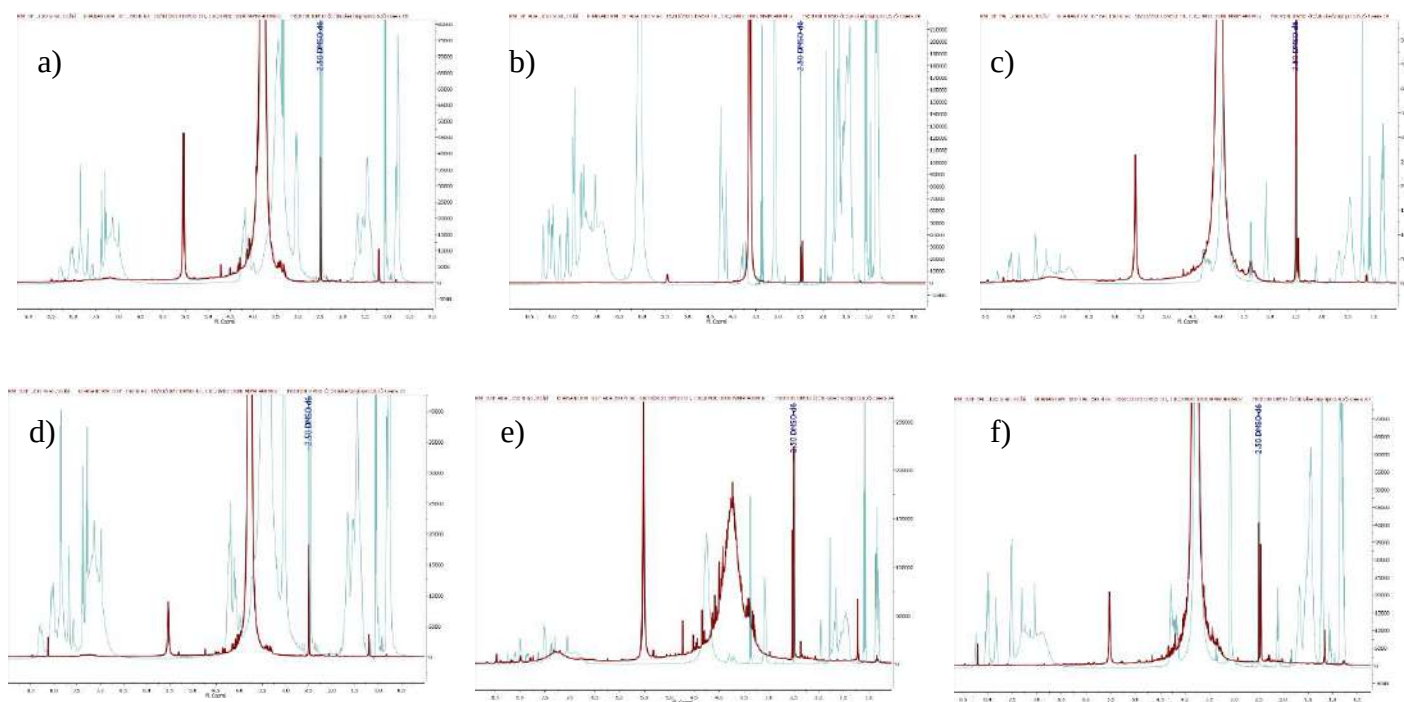


Figure 6.25 The ^1H NMR of the a) BP-Iso-N-Ge-CDs for b) BP-ada-Iso-N-Ge-CDs c) BP-pal-Iso-N-Ge-CDs, d) D-BP-Iso-N-Ge-CDs, and e) D-BP-ada-Iso-N-Ge-CDs, f) D-BP-pal-Iso-N-Ge-CDs of the conjugate in red and the peptides in blue.

6.5 Conclusion

The key to identifying molecular species is the use of nuclear magnetic resonance, which proves to be an effective tool. Our work highlights the need to include nuclear magnetic resonance as a standard characterization technique for carbon-based nanomaterials, to reduce the possibility of observing properties that arise from molecular species, rather than the target carbon nanodots.

The characteristics of the conjugates were determined using the photoluminescent spectrometer, and the UV-Vis spectrometer. This data demonstrated that the conjugates have excellent photoluminescent properties and the shift in the peaks when comparing the CDs alone with the conjugated counterparts indicated successful conjugation. Furthermore, the shift in the UV peaks of the conjugates corroborates the data revealed by the photoluminescence. Additionally, the significant effects on the ^1H NMR of the conjugates also indicated the interactions in the backbone of the peptide. Therefore, the characterization techniques indicate successful conjugation of the CDs to the peptide.

In summary, the critical role of NMR spectroscopy demonstrated that inconsistencies and errors when classifying CDs may occur, and only NMR can clearly determine the unreacted starting material and impurities. Additionally, unreacted starting material and impurities that can interfere with the properties of the CDs or conjugates can be determined using NMR techniques.

We investigated the CDs before and after dialysis using NMR experiments, and the data revealed that dialysis reduced the amount of unreacted urea and other impurities which are not part of the CDs or conjugate. More importantly, the NMR data revealed the presence of urea and a small molecule which could not be explicitly revealed using other characterization techniques. 2-D NMR was used to analyze the effects of conjugation and long-range interactions on the ROESY NMR of the peptide to carbon dots through various atoms clearly correlating in the NMR of the resulting conjugate. The confirmation of conjugation was not only achieved through UV-Vis and PL techniques but also through the detection of long-range interactions between the peptide and CDs in the ROESY NMR. Additionally, the NMR analysis of the conjugates suggests that the helical structure may be influenced after conjugation, as indicated by significant peak shifts. Since the helical structure is likely crucial for the peptide's bioactivity and enhanced stability, NMR spectra showing fewer structural changes in the peptide could potentially lead to improved biological properties.

For future investigations, the samples that exhibit promising results and can be sent for biological testing include a) GKP-*Iso-N-Ge-CDs*, d) D-GKP-*Iso-N-Ge-CDs* and e) D-GKP-*ada-Iso-N-Ge-CDs*, c) BP-*pal-Iso-N-Ge-CDs*, and d) DBP-*ada-Iso-N-Ge-CDs*. These candidates still display distinguishable peaks from the original peptide, making them worthy of further exploration.

Moreover, this chapter demonstrates that NMR is a powerful technique which should be incorporated when analyzing CDs or conjugates. NMR has proven to be an essential technique to minimize the risk of residual molecular species in the final material since it can reveal the presence of molecular species in a fast and easy way.

6.6 References

1. Jiang, K., Wang, Y., Gao, X., Cai, C. & Lin, H. Facile, Quick, and Gram-Scale Synthesis of Ultralong-Lifetime Room-Temperature-Phosphorescent Carbon Dots by Microwave Irradiation. *Angew. Chem. Int. Ed.* **57**, 6216–6220 (2018).
2. Wu, P., Li, W., Wu, Q., Liu, Y. & Liu, S. Hydrothermal synthesis of nitrogen-doped carbon quantum dots from microcrystalline cellulose for the detection of Fe³⁺ ions in an acidic environment. *RSC Adv.* **7**, 44144–44153 (2017).
3. Chhabra, V. A., Kaur, R., Kumar, N., Deep, A., Rajesh, C. & Kim, K.-H. Synthesis and spectroscopic studies of functionalized graphene quantum dots with diverse fluorescence characteristics. *RSC Adv.* **8**, 11446–11454 (2018).
4. Dai, H., Chen, S., Li, Y., Zeng, B., Zhang, S., Hong, Z. & Lin, Y. Photoelectrochemical biosensor constructed using TiO₂ mesocrystals based multipurpose matrix for trypsin detection. *Biosens. Bioelectron.* **92**, 687–694 (2017).
5. Balbo Block, M. A. & Hecht, S. Wrapping Peptide Tubes: Merging Biological Self-Assembly and Polymer Synthesis. *Angew. Chem. Int. Ed.* **44**, 6986–6989 (2005).
6. Chirvony, V. S., Suárez, I., Rodríguez-Romero, J., Vázquez-Cárdenas, R., Sanchez-Diaz, J., Molina-Sánchez, A., Barea, E. M., Mora-Seró, I. & Martínez-Pastor, J. P. Inhomogeneous Broadening of Photoluminescence Spectra and Kinetics of Nanometer-Thick (Phenethylammonium)₂PbI₄ Perovskite Thin Films: Implications for Optoelectronics. *ACS Appl. Nano Mater.* **4**, 6170–6177 (2021).
7. Oh, G.-H., Kim, B.-S., Song, Y. & Kim, S. Acid treatment to tune the optical properties of carbon quantum dots. *Appl. Surf. Sci.* **605**, 154690 (2022).
8. Zhang, Q., Wang, R., Feng, B., Zhong, X. & Ostrikov, K. (Ken). Photoluminescence mechanism of carbon dots: triggering high-color-purity red fluorescence emission through edge amino protonation. *Nat. Commun.* **12**, 6856 (2021).

9. Toyozawa, Y. Theory of Line-Shapes of the Exciton Absorption Bands. *Prog. Theor. Phys.* **20**, 53–81 (1958).
10. Traven, V. F., Cheptsov, D. A., Svetlova, J. I., Ivanov, I. V., Cuerva, C., Lodeiro, C., Duarte, F., Dunaev, S. F. & Chernyshev, V. V. The role of the intermolecular $\pi \cdots \pi$ interactions in the luminescence behavior of novel coumarin-based pyrazoline materials. *Dyes Pigments* **186**, 108942 (2021).
11. Yamaguchi, M., Tanaka, K. & Chujo, Y. Design of Conjugated Molecules Presenting Short-Wavelength Luminescence by Utilizing Heavier Atoms of the Same Element Group. *Chem. – Asian J.* **13**, 1342–1347 (2018).
12. Garcia, Y. S., Barros, M. R., Ventura, G. T., de Queiroz, R. M., Todeschini, A. R. & Neves, J. L. Probing the interaction of carbonaceous dots with transferrin and albumin: Impact on the protein structure and non-synergetic metal release. *J. Mol. Liq.* **292**, 111460 (2019).
13. Lin, Y., Zheng, Y., Guo, Y., Yang, Y., Li, H., Fang, Y. & Wang, C. Peptide-functionalized carbon dots for sensitive and selective Ca^{2+} detection. *Sens. Actuators B Chem.* **273**, 1654–1659 (2018).
14. Distefano, A., Cali, F., Gaeta, M., Tuccitto, N., Auditore, A., Licciardello, A., D’Urso, A., Lee, K.-J., Monasson, O., Peroni, E. & Grasso, G. Carbon dots surface chemistry drives fluorescent properties: New tools to distinguish isobaric peptides. *J. Colloid Interface Sci.* **625**, 405–414 (2022).
15. Ding, H., Wei, J.-S., Zhang, P., Zhou, Z.-Y., Gao, Q.-Y. & Xiong, H.-M. Solvent-Controlled Synthesis of Highly Luminescent Carbon Dots with a Wide Color Gamut and Narrowed Emission Peak Widths. *Small* **14**, 1800612 (2018).
16. Yuan, F., Wang, Y.-K., Sharma, G., Dong, Y., Zheng, X., Li, P., Johnston, A., Bappi, G., Fan, J. Z., Kung, H., Chen, B., Saidaminov, M. I., Singh, K., Voznyy, O., Bakr, O. M.,

- Lu, Z.-H. & Sargent, E. H. Bright high-colour-purity deep-blue carbon dot light-emitting diodes via efficient edge amination. *Nat. Photonics* **14**, 171–176 (2020).
17. Zheng, X. T., Ananthanarayanan, A., Luo, K. Q. & Chen, P. Glowing Graphene Quantum Dots and Carbon Dots: Properties, Syntheses, and Biological Applications. *Small* **11**, 1620–1636 (2015).
18. Stepanidenko, E. A., Skurlov, I. D., Khavlyuk, P. D., Onishchuk, D. A., Koroleva, A. V., Zhizhin, E. V., Arefina, I. A., Kurdyukov, D. A., Eurov, D. A., Golubev, V. G., Baranov, A. V., Fedorov, A. V., Ushakova, E. V. & Rogach, A. L. Carbon Dots with an Emission in the Near Infrared Produced from Organic Dyes in Porous Silica Microsphere Templates. *Nanomater. Basel Switz.* **12**, 543 (2022).
19. Yoshinaga, T., Shinoda, M., Iso, Y., Isobe, T., Ogura, A. & Takao, K. Glycothermally Synthesized Carbon Dots with Narrow-Bandwidth and Color-Tunable Solvatochromic Fluorescence for Wide-Color-Gamut Displays. *ACS Omega* **6**, 1741–1750 (2021).
20. Mirzov, O. & Scheblykin, I. G. Photoluminescence spectra of a conjugated polymer: from films and solutions to single molecules. *Phys. Chem. Chem. Phys.* **8**, 5569–5576 (2006).
21. Handelman, A., Kuritz, N., Natan, A. & Rosenman, G. Reconstructive Phase Transition in Ultrashort Peptide Nanostructures and Induced Visible Photoluminescence. *Langmuir* **32**, 2847–2862 (2016).
22. Chun Li, Liu, W., Sun, X., Pan, W., Yu, G. & Wang, J. Excitation dependent emission combined with different quenching manners supports carbon dots to achieve multi-mode sensing. *Sens. Actuators B Chem.* **263**, 1–9 (2018).
23. García-Plazaola, J. I., Fernández-Marín, B., Duke, S. O., Hernández, A., López-Arbeloa, F. & Becerril, J. M. Autofluorescence: Biological functions and technical applications. *Plant Sci.* **236**, 136–145 (2015).

24. Xu, A., Wang, G., Li, Y., Dong, H., Yang, S., He, P. & Ding, G. Carbon-Based Quantum Dots with Solid-State Photoluminescent: Mechanism, Implementation, and Application. *Small* **16**, 2004621 (2020).
25. Zheng, J., Wang, Y., Zhang, F., Yang, Y., Liu, X., Guo, K., Wang, H. & Xu, B. Microwave-assisted hydrothermal synthesis of solid-state carbon dots with intensive emission for white light-emitting devices. *J. Mater. Chem. C* **5**, 8105–8111 (2017).
26. Yu, J., Yong, X., Tang, Z., Yang, B. & Lu, S. Theoretical Understanding of Structure–Property Relationships in Luminescence of Carbon Dots. *J. Phys. Chem. Lett.* **12**, 7671–7687 (2021).
27. Shen, C.-L., Liu, H.-R., Lou, Q., Wang, F., Liu, K.-K., Dong, L. & Shan, C.-X. Recent progress of carbon dots in targeted bioimaging and cancer therapy. *Theranostics* **12**, 2860–2893 (2022).
28. Qin, J., Gao, X., Chen, Q., Liu, H., Liu, S., Hou, J. & Sun, T. pH sensing and bioimaging using green synthesized carbon dots from black fungus. *RSC Adv.* **11**, 31791–31794 (2021).
29. Yang, Y. Z., Xiao, N., Liu, S. G., Han, L., Li, N. B. & Luo, H. Q. pH-induced aggregation of hydrophilic carbon dots for fluorescence detection of acidic amino acid and intracellular pH imaging. *Mater. Sci. Eng. C* **108**, 110401 (2020).
30. Zhang, B., Duan, Q., Li, Y., Wang, J., Zhang, W. & Sang, S. pH and redox dual-sensitive drug delivery system constructed based on fluorescent carbon dots. *RSC Adv.* **11**, 2656–2663 (2021).
31. Yang, T., Du, G., Cui, Y., Yu, R., Hua, C., Tian, W. & Zhang, Y. pH-sensitive doxorubicin-loaded polymeric nanocomplex based on β -cyclodextrin for liver cancer-targeted therapy. *Int. J. Nanomedicine* **14**, 1997–2010 (2019).

32. Gao, L., Xie, Z. & Zheng, M. Highly efficient carbon dots for quantitatively visualizing pH fluctuations in cells, zebrafish, mice and tumors. *J. Photochem. Photobiol. B* **238**, 112620 (2023).
33. Tao, S., Zhu, S., Feng, T., Xia, C., Song, Y. & Yang, B. The polymeric characteristics and photoluminescence mechanism in polymer carbon dots: A review. *Mater. Today Chem.* **6**, 13–25 (2017).
34. Walther, T. Measuring Non-Destructively the Total Indium Content and Its Lateral Distribution in Very Thin Single Layers or Quantum Dots Deposited onto Gallium Arsenide Substrates Using Energy-Dispersive X-Ray Spectroscopy in a Scanning Electron Microscope. *Nanomaterials* **12**, 02220 (2022).
35. Ruiz, V., Yate, L., García, I., Cabanero, G. & Grande, H.-J. Tuning the antioxidant activity of graphene quantum dots: Protective nanomaterials against dye decoloration. *Carbon* **116**, 366–374 (2017).
36. Arroyave, J. M., Ambrusi, R. E., Robein, Y., Pronsato, M. E., Brizuela, G., Di Nezio, M. S. & Centurión, M. E. Carbon dots structural characterization by solution-state NMR and UV–visible spectroscopy and DFT modeling. *Appl. Surf. Sci.* **564**, 150195 (2021).
37. Bichenkova, E. V., Raju, A. P. A., Burusco, K. K., Kinloch, I. A., Novoselov, K. S. & Clarke, D. J. NMR detects molecular interactions of graphene with aromatic and aliphatic hydrocarbons in water. *2D Mater.* **5**, 015003 (2017).
38. Cailotto, S., Amadio, E., Facchin, M., Selva, M., Pontoglio, E., Rizzolio, F., Riello, P., Toffoli, G., Benedetti, A. and Perosa, A., 2018. Carbon dots from sugars and ascorbic acid: role of the precursors on morphology, properties, toxicity, and drug uptake. *ACS medicinal chemistry letters*, 9(8), pp.832-837.

39. Xie, H., Becraft, E. J., Baughman, R. H., Dalton, A. B. & Dieckmann, G. R. Ranking the affinity of aromatic residues for carbon nanotubes by using designed surfactant peptides. *J. Pept. Sci. Off. Publ. Eur. Pept. Soc.* **14**, 139–151 (2008).
40. Antonucci, A., Kupis-Rozmysłowicz, J. & Boghossian, A. A. Noncovalent Protein and Peptide Functionalization of Single-Walled Carbon Nanotubes for Biodelivery and Optical Sensing Applications. *ACS Appl. Mater. Interfaces* **9**, 11321–11331 (2017).
41. Ramezani-Dakhel, H., Bedford, N. M., Woehl, T. J., Knecht, M. R., Naik, R. R. & Heinz, H. Nature of peptide wrapping onto metal nanoparticle catalysts and driving forces for size control. *Nanoscale* **9**, 8401–8409 (2017).
42. Parenti, F., Tassinari, F., Libertini, E., Lanzi, M. & Mucci, A. π -Stacking Signature in NMR Solution Spectra of Thiophene-Based Conjugated Polymers. *ACS Omega* **2**, 5775–5784 (2017).

7. Final conclusions and recommendations

The simple microwave synthesis of CDs was confirmed using TEM and the synthesized CDs were all found to be ≤ 10 nm in size with a sphere-like shape. The FTIR spectra showed that the CDs were successfully doped with nitrogen for the novel *Iso-N-Ge*-CDs as shown by the characteristic band at approximately 3247 cm^{-1} (N-H) and 1392 cm^{-1} (C-N). Moreover, the N1s spectrum in the XPS revealed the presence of graphitic-N (399.6 eV) and NO_x (401.3 eV),³³ with the relative abundance of N1s estimated to be 15.9 w %. The incorporation of GeO₂ on the carbon dots was also confirmed by the XPS data with the Ge2p₃ peak at 1219.6 eV, which is evidence of the presence of Ge=O. The photoluminescent qualities of the CDs were examined using PL and the results show that the CDs are fluorescent and when exposed to UV light the CDs exhibited a wide range of colors. PL data also showed that the CDs have substantial photoluminescent qualities. According to data obtained from the BX63 Upright Microscope, *Iso-N-Ge*-CDs display fluorescence that can be used for bio-imaging applications.

On the other hand, the 11 novel peptides derived from the peptide accelerating sequence GKPIILFF and the cancer-selective peptide RLRLRIGRR have been successfully synthesized as well as the 2 original sequences. The chromatograms of the GKP peptides before and after tuning also show how the synthesis process was improved, which decreased coupling time and increased purity. The retention time of the peptides seemed to be lengthened by the addition of adamantane and palmitic acid. This is due to the hydrophobic nature of the moieties and the overall change in the secondary structure. The RGDGKP peptide appeared at a lower retention time of 8.75 min compared to the GKP peptide at 8.95, owing to the addition of hydrophilic residues such as arginine and aspartic acid. The yields varied from 20 to 62%, which suggests that the cleavage and purification step significantly affected the final yield. Moreover, the impurities found in the samples varied, which affected how each peptide was purified. This issue may be investigated in the future by varying the cleavage conditions for optimum results.

To provide a better understanding of the secondary structure of the peptides, the GKPIILFF peptide was fully elucidated using a combination of NMR techniques. The D-GKPIILFF peptide was also evaluated to study the effect of the use of D amino acids on the structure. The ¹H NMR peaks of the peptide showed similar chemical shifts which posed a challenge for assignments. Therefore, several two-dimensional NMR spectroscopic techniques were

used such as COSY, HSQC, DEPT, TOCSY and ROESY NMR. The data revealed that the L- GKPIIFF penetration acceleration peptide sequence (Pas) conformation adopts a helical secondary structure, which has been verified by the ROESY NMR method via the through-space interaction between the Pro 3 and Phe 7 amino acid residues. Peptides with helical structure often have enhanced stability which improves the bioactivity of the peptide. Furthermore, we propose that the α -helix structure of the peptide may be the contributing factor that facilitates the pore formation and binding to the phospholipids, which results in the acceleration of the penetration of its cargo. For future work, permeability studies will be conducted on the synthesized peptides and computational studies will be used to check if the DPPE1 enzyme found in the cell membrane binds to the peptides.

The conjugates were then investigated using a photoluminescent spectrometer, and the UV-Vis spectrometer. This data demonstrated that the conjugates have photoluminescent properties and the shift in the peaks when comparing the CDs alone with the conjugated counterparts which indicated successful conjugation. Furthermore, the shift in the UV peaks of the conjugates corroborates the data revealed by the photoluminescence. Additionally, the ^1H NMR of the conjugates also indicated the interactions in the aromatic region which is influenced by the π - π stacking between the phenylalanine groups of the peptide and the aromatic core of the CDs. Moreover, the amide protons from the peptide backbone appeared to be more significantly affected by the conjugation compared to the peptide residues from phenylalanine, isoleucine, and leucine. Additionally, the amide proton from lysine was shown through molecular dynamics that were not aligned with the alpha helix of the other residues. This caused it to be embedded in the CDs which resulted in the amide of lysine disappearance in the ^1H NMR. Therefore, the data indicates that the peptide wrapped around the CDs through π stacking between the aromatic core of the CDs and the backbone of the peptide.

Additionally, the NMR analysis of the carbon dots before conjugation demonstrated the need to include nuclear magnetic resonance as a standard characterization technique for carbon-based nanomaterials, to reduce the possibility of observing properties that arise from molecular species, rather than the target carbon dots. Not only was the conjugation confirmed using UV-Vis and PL, long-range interactions between the peptide and CDs were detected in the ROESY NMR. Moreover, the NMR analysis of the conjugates suggests that the helical structure could be affected, as evidenced by the notable shift in peaks. Since the helical structure likely influences its bioactivity through enhanced stability, the NMR spectra

showing fewer structural changes in the peptide structure might have improved biological properties. This means for future work, the samples which can be sent for biological testing as promising candidates are a) GKP-*Iso-N-Ge*, b) GKP-*ada-Iso-N-Ge*, c) BP *pal-Iso-N-Ge*, and d) DBP-*ada-Iso-N-Ge*, which still retain distinguishable peaks from the original peptide.

The critical role of NMR spectroscopy demonstrated that inconsistencies and errors when classifying CDs may occur and only NMR can clearly determine the unreacted starting material and impurities. Moreover, unreacted starting material and impurities that can interfere with the properties of the CDs or conjugates can be determined using NMR techniques.

Furthermore, the other impurity formed may require other purification techniques such as liquid-liquid extraction using dichloromethane and water or chromatographic techniques such as prep-HPLC. More importantly, the NMR data revealed the presence of urea and a small molecule which couldn't be explicitly revealed using other characterization techniques. 2-D NMR was used to analyze the effects of conjugation of the peptide to carbon dots and various atoms could be identified.

Finally, the structural differences in the peptide after conjugates may not be a desirable effect since the alpha-helical conformation plays a key role in the penetration of cells. This reveals the importance of understanding the structure of the conjugates and using techniques such as NMR to monitor the effects of conjugation. For future work, different types of linkers can be investigated to help maintain the crucial secondary structure of the peptide to prevent potential loss of activity.

8. Experimental procedures

8.1 Reagents and chemicals

8.1.1 Reagents for peptides

Piperidine, *N, N, N', N'*-tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU), Fmoc amino acids, paraformaldehyde, *p*-toluenesulfonic acid, toluene, triethylsilane, chlorotriptyl resin, triisopropylsilane (TIPS), formic acid, trifluoroacetic acid (TFA), 1,2-ethanedithiol (EDT), diisopropylethylamine (DIPEA), *N*-Methylmorpholine (NMM) were purchased from Dld Scientific. Iodine, ascorbic acid, and dichloromethane (DCM) were purchased from Sigma Aldrich. DCM was dried upon distillation over calcium hydride. Dimethylformamide (DMF), methanol, acetonitrile, hexane, dimethyl sulfoxide, and ether were purchased from Radchem.

8.1.2 Reagents for carbon dots

Iso-ascorbic acid ($C_6H_8O_6$), urea (CH_4N_2O), and bis (2-carboxyethyl germanium (IV) sesquioxide) (Ge-132) were all purchased from Sigma Aldrich, South Africa, and used as received. The experiments utilised distilled water and hydrochloric acid (HCl 32 %, Sigma Aldrich, South Africa) as solvents.

8.2 Instrumentation

8.2.1 Instrumentation used for peptides and amino acids

8.2.1.1 *Ultra High-Pressure Liquid Chromatography (UHPLC)*

Analysis and purity assessment were carried out using a Thermo Scientific Dionex Ultimate 3000 UHPLC coupled to a Diode Array Detector (215 nm and 254 nm) and a Bruker Compact Q-TOF mass spectrometer. The compounds were separated using a binary solvent system where solvent A consisted of H_2O and 0.1% FA (v/v) and solvent B consisted of ACN and 0.1% FA (v/v) at a flow rate of 0.30 ml/min and on a Phenomenex C18 column (5 m, 100 m, 4.60 mm x 150 mm). An electrospray ionization (ESI) source operating in the positive mode was used for the mass spectrometry study.

8.2.1.2 Mass spectroscopic analysis

The Advion expression-MS (ESI) mass spectrometer was used in the positive mode to analyze amino acids and short peptide sequences.

Analytical LC-MS analysis was performed using a High-Resolution Mass Spectrometer (Bruker Compact quadrupole time of flight) connected to a UHPLC Thermo Scientific Ultimate 3000, RS coupled to a diode array detector (215 and 254 nm). The autosampler was set at 20.0°C with an injection volume of 10 μ L. The electrospray ionization (ESI) source was utilized and the ion source was kept at 220 °C in the positive ion mode. The MS scan range was set at m/z 100-3000. Nitrogen gas (N_2) was used as the dry and nebulizer gas. The nebuliser gas was operated at a pressure of 1.8 bars whereas the drying gas was operated at a flow rate of 9 L/min.

8.2.1.3 Preparative High-Pressure Liquid Chromatography (PREP-HPLC)

The Agilent 1260 Infinity preparative equipment HPLC with a UV/VIS detector (215 nm and 254 nm) and an automated fraction collector were used to purify the peptides on a C-18 (5 μ m 100 Å 250 mm x 21.2 mm) column at a flow rate of 15 mL/min. Formic acid served as the ion-pairing agent in a two-buffer system. Buffers A and B contained 0.1% formic acid/ H_2O (v/v) and 0.1% formic acid/acetonitrile (v/v), respectively.

8.2.1.4 Ultraviolet-visible spectroscopy

A Cary Series UV-Vis spectrophotometer from Agilent Technologies was used to collect the absorption spectra. The theoretical yield of the peptides was calculated using the absorbance at 301 nm; see section 4.1.3 for details.

8.2.1.5 Nuclear magnetic resonance spectroscopy (NMR)

^1H -NMR spectra were acquired on a Bruker Avance 300 MHz, Bruker Avance 400 MHz, and a Bruker III 500 MHz spectrometer. The chemical shifts (δ) readings are referenced to the internal standard, tetramethylsilane, and provided in parts per million (ppm) relative to deuterated dimethyl sulfoxide ($DMSO-d_6$, 2.49 ppm) (TMS, 0.00 ppm). The first-order analysis of the spectra resulted in Hertz values for the coupling constant (J) (Hz). Signal multiplicity is denoted by the following symbols: singlet (s), broad singlet (br), doublet doublets (dd), triplet (t), quartet (q), and multiplet (m).

Bruker Avance 300 MHz, Bruker Avance 400 MHz, and Bruker III 500 MHz spectrometers were used to record ^{13}C -NMR spectra. The chemical shift values are expressed in (ppm) relative to internal standards such as DMSO-d₆ 39.52 ppm and TMS as an internal standard.

With a 500 MHz NMR Bruker III spectrometer, 2D NMR spectra from the COSY, ^{13}C -HSQC, TOCSY, NOESY, and ROESY experiments were captured at 293, 300, and 308 K. All 2D spectra were captured using time-proportional phase increase in the phase-sensitive mode (TPPI). A pre-saturation pulse with a 2 s length decreased the DMSO-d₆ residual water peak.

The TOCSY signal was captured with mixing times of 48, 64, 70, 80, and 100 ms; each increment was the sum of 32 scans with a relaxation delay of 2.0 s; 2048 data points were gathered for each experiment.

8.2.1.6 Circular Dichroism (CD)

CD spectra were recorded on a JASCO J-18 spectropolarimeter between the range of 190 to 250 nm in the specified solvents (water and phosphate buffer), with 10 scans at 20°C. The quartz cuvette used had a pathlength of 0.2 cm with a resolution of 0.2 nm, a bandwidth of 1.0 nm and a sensitivity of 10-20 mdeg. The peptides (0, 00061 mol L⁻¹) were prepared in water and a 20 mM phosphate buffer (pH 8). The value of absorption was measured as molar ellipticity per residue deg.cm². dmol⁻¹). The signals obtained were converted to mean residue ellipticity, $[\theta]$ using the equation below:

$$[\theta] = \frac{(MRW \times \theta)}{10 \times d \times c} \text{ deg.cm}^2.\text{dmol}^{-1}$$

where MWR is the mean residue weight, θ the observed ellipticity in degrees, c is peptide concentration in g/ml and d is the path length in cm.

8.2.2 Instrumentation used for carbon dots and their conjugates.

8.2.2.1 Transmission electron microscopy (TEM)

The FEI Tecnai-12 microscope was used to obtain transmission electron microscopy (TEM) pictures with a 200 KV electron acceleration voltage and a 10-100 nm beam size. The particle size and shape of the CDs were determined using TEM. After being dissolved in ethanol or water, the CDs underwent a 20-minute ultrasonic treatment. The 10 mg CDs were

dissolved in 5 ml of water and two drops of the solution were added to the copper grid that had been coated. The grid was then left to dry for 24 hours at room temperature. The FEI Tecnai T12 electron microscope was used to capture the TEM pictures, which operate at an acceleration voltage of 120 kV.

8.2.2.2 Ultraviolet-visible spectroscopy

Samples in solution were subjected to UV-Vis absorption measurements using a Varian Cary Eclipse (Cary 50) UV-Vis absorption spectrophotometer (200-800 nm wavelengths). The measurements were conducted using a quartz cuvette with a 1 cm optical length.

8.2.2.3 Bruker Tensor 27 FTIR analyzer

An FTIR analyzer called the Bruker Tensor 27 was used to determine the functional groups of the CDs (Figure 3.7). The measurements were made using a transmission mode with an accumulation of 64 scans and a resolution of 4.0 cm^{-1} on solid samples.

8.2.2.4 X-ray photoelectron spectroscopy (XPS) data

The surface composition of the CDs was determined using XPS. In this method, the material is exposed to X-ray radiation to extract the photoelectrons. The spectra were acquired using a quantum 2000 scanning ESCA lab 250Xi microprobe instrument operated at 300 W with monochromatic Al $\text{K}\alpha$ (1486.7 eV) (1486.7 eV). The CSIR was responsible for sample preparation and analysis. The application known as Origin85 software was used to analyze the obtained CDs' XPS spectra.

8.2.2.5 Photoluminescence (PL) spectroscopy

PL spectroscopy was utilized to examine the CD's fluorescence characteristics, conjugates were dissolved in PBS solutions while the carbon dots were dissolved in water. The emissions were measured in the wavelength range of 300-700 nm and the width of the slit was 5 nm. The Varian Cary Eclipse EL04103870 fluorescence spectrophotometer was used to collect the PL data.

8.2.2.6 X-ray diffraction (XRD)

The PXRD is an effective method that was used to identify the phases of the materials while they were being synthesized and to determine their crystallinity based on their diffraction patterns. Moreover, the purity of the produced goods can be checked using the PXRD. The

samples were reduced to powder and placed into a sample container with zero background. A glass slide was employed to achieve a level and homogeneous surface. The Bruker D2 PHASER powder X-ray diffractometer, equipped with an LYNXEYE detector, utilized the co- α irradiation with a 30 KV x-ray and 10 mA current in 0.0260° increments for 10 minutes to produce the PXRD patterns. PXRD patterns were gathered between 2Theta = 10 and 90°.

8.2.3 General procedure for synthesizing carbon dots

The carbon dots were synthesized in a closed vessel CEM Discovery SP microwave with a maximum power and pressure output of 150 W and 250 psi, respectively. The amounts used to synthesize each of the CDs were presented in Table 1. The starting material was dispersed, by shaking, in distilled water (8.0 ml and 1 mM Formic acid (2 ml) until a homogeneous mixture was formed. The mixture was then transferred to a 30 ml microwave reaction vessel, placed in the microwave oven, and irradiated at 150 W for 5-10 minutes. Following the reaction, the reaction mixture changed color from clear to black brown, indicating carbonization. The samples were purified by centrifugation at 14000 rpm for 20 minutes to separate the large particles. The collected supernatant was then filtered using a 0.22 μ m filter membrane, and a rotary evaporator was then used to evaporate the water from the CD solution. The obtained product was allowed to dry in an oven at 100 °C overnight.

Table 8.1 List of the precursors for the synthesis of the carbon dots

Product name	Precursor amount (g)		
	Iso-ascorbic acid	Ge132	Urea
1. Iso- CDs	2.00	-	-
2. Iso-Ge- CDs	2.00	0.50	-
3. Iso-N- CDs	2.00	-	0.50
4. Iso-N-Ge-CDs	2.00	0.50	0.50

8.2.4 General procedure for conjugating carbon dots to peptides

Peptide-CDs were fabricated by adding 20 mg of CDs and 2 mg of peptide in 1 mM of PBS pH 7.4 and 5% DMSO. The mixture was gently stirred at room temperature overnight. Subsequently, the conjugate (composite) was dialyzed against Milli Q water for 24 hours to remove free peptide and unreacted material and then finally dried using a freeze drier.

8.2.5 General procedure for the swelling, activation, and coupling of the first amino acid to rink amide resin

In a 15 ml centrifuge tube, the rink amide resin (600 mg, 0.160 mmol/g) was swelled in DMF (15.0 ml) for 3 hours. The resin was subsequently dried using suction in a reaction vessel equipped with a fritted filter. The resin was deprotected twice by adding 20% piperidine in DMF for 5 minutes each time. The resin was then rinsed with DMF (3 x 15 ml) and DCM (3 x 15 ml). To couple the first amino acid to the free NH_2 on the resin, HBTU (0.863 g, 0.19 M), NMM (1.0 M, 12.0 ml), and phenylalanine (0.2 M, 1.86 g) for GPKILFF peptide or arginine (0.2 M, 3.12 g) for RLRLRIGRR peptide, were added to the reaction vessel and the reaction was gently mixed using a steady stream of nitrogen gas. After 10-30 minutes, the mixture was filtered under suction. Lastly, DMF (3 x 15.0 ml) and DCM (3 x 15.0 ml) were used to wash the resin.

8.2.6 General procedure for analysing the substitution of the first amino acid

The first amino acid attached to the resin was weighed out in duplicate 5.0–10.0 mg samples into Eppendorf tubes. Subsequently, 20% piperidine in DMF (1.0 ml) was then added to the resin to remove the Fmoc protective group, and the mixture was agitated for 20 minutes before centrifugation. 100 μl of the supernatant from the centrifuged material was placed into a tube containing DMF (10.0 ml). Afterwards, 2.0 ml of the solution and 2.0 ml of DMF were pipetted into separate cuvette cells. The *spectrophotometer* was zeroed using the cuvette with the DMF solution. The sample's absorbance at 301 nm was measured three times, and the substitution was calculated using the equation:

$$\text{Subs} = 101(A)/7.8(w)$$

A = absorbance

w = mg of resin

The theoretical yield was then determined using the calculated loading capacity (sub) as shown in the equation below.

$$\text{Theoretical yield} = \text{sub (mmol/g)} \times \text{mass of resin (g)} \times \text{Mr of peptide (g/mol)}$$

8.2.7 General procedure for the synthesis of peptides

After coupling the first amino acid using the procedure mentioned in section 1.1.6, the first amino acid (phenylalanine) was deprotected by treating the resin with 20% piperidine in DMF (20.0 ml) for five minutes. DMF (3 x 15.0 ml) was then used to wash the resin. A solution of the second amino acid (0.2 M) in DMF was added to the resin along with NMM (1.0 M, 12.0 ml) and HBTU (0.863 g, 0.19M). This combination was reacted for 30 minutes with gentle mixing by bubbling nitrogen gas, after which suction was used to remove the solvent. The resin was washed with DMF (3 x 15.0 ml), treated twice for 5 minutes with 20% piperidine in DMF (12.0 ml), and then washed again with DMF (3 x 15.0 ml). The following amino acid was weighed according to the calculated masses presented in Table 2., dissolved in DMF and added to the resin together with NMM (1 M, 12.0 ml) and HBTU (0.86 g 0.19M). after the mixture had been reacted for 10-30 minutes, the resin was then rinsed with DMF (3 x 15.0 ml). This process was continued until the entire peptide was produced.

Table 8.2 List of the amino acid (0.2 M) added to the resin together with NMM (1.0 M) and HBTU (0.19 M) during synthesis.

Mass of Fmoc Protected Derivatives amino acids for a 0.2M in 6 ml vial	for 150mg resin	
Amino acid	Moles	Mass (g)
1. Phenylalanine (Phe, F)	0,0012	0,465
2. Aspartic acid (Asp,D)	0,0012	0,494
3. proline (Pro, P)	0,0012	0,405
4. Glycine (Gly, G)	0,0012	0,358
5. lysine (Lys, K)	0,0012	0,563
6. Aspartic acid (Asp,D)	0,0012	0,494
7. Glycine (Gly, G)	0,0012	0,714
8. Arginine (Arg, R)	0,0012	0,779
9. Cysteine (Cys, C)	0,0012	0,703
10. Leucine (Leu, L)	0,0012	0,424
11. Isoleucine (Ile, I)	0,0012	0,424
Other Reagents		
HATU	0,0011 4	0,433
HBTU	0,0011 4	0,433
Palmitic	0,0012	0,307
Adamantane	0,0012	0,216

8.2.8 General procedure for the synthesis of peptides containing adamantane and palmitic acid on the N-terminal

The N-terminal palmitic acid analogues were synthesized by mixing the resin-bound peptide with palmitic acid (0.2 M, 615 mg), NMM (1.0 M, 12.0 ml), and HBTU (0.863 g 0.19 M) in DMF. After 30 minutes of reaction time and gentle mixing, the solvent was removed using vacuum suction and the resin was washed with DMF (3 x 15.0 ml) followed by DCM (3 x 15.0 and then dried under vacuum.

1-Adamantanecarboxylic acid (0.2 M, 0,432 g) with the same quantities of NMM (1.0 M) and HBTU (0.19 M) were added to the resin-bound peptide to produce the N-terminal adamantane analogues. The resin was then washed with DMF (3 x 15.0 ml) followed by

DCM (3 x 15.0 ml) and dried before the peptide was cleaved using the procedure described in section 1.1.10.

8.2.9 General procedure for the cleavage of the peptides from the resin

The process of detaching the peptide from the solid support also includes removing the protective groups from the side chains. In a 50 ml tube, 96:2.5:1.5 (v/v) TFA: H₂O: TIPS were combined to create a cleavage mixture/cocktail. After that, the dried resin was placed into a 50 ml centrifuge tube with 20 ml of the cleavage mixture, and the tube was shaken for three hours. Upon filtration, the supernatant and the washings (resin was washed with 5 ml of the cleavage mixture) were transferred into a centrifuge tube and 60.0 ml of cold diethyl ether was added to the supernatant, which caused the peptide to precipitate. The tubes were centrifuged at 5000 rpm for 5 minutes and the ether/TFA solution was decanted. The resulting peptide precipitate was washed with cold ether three times.

9. Appendix

9.1 Chapter 3

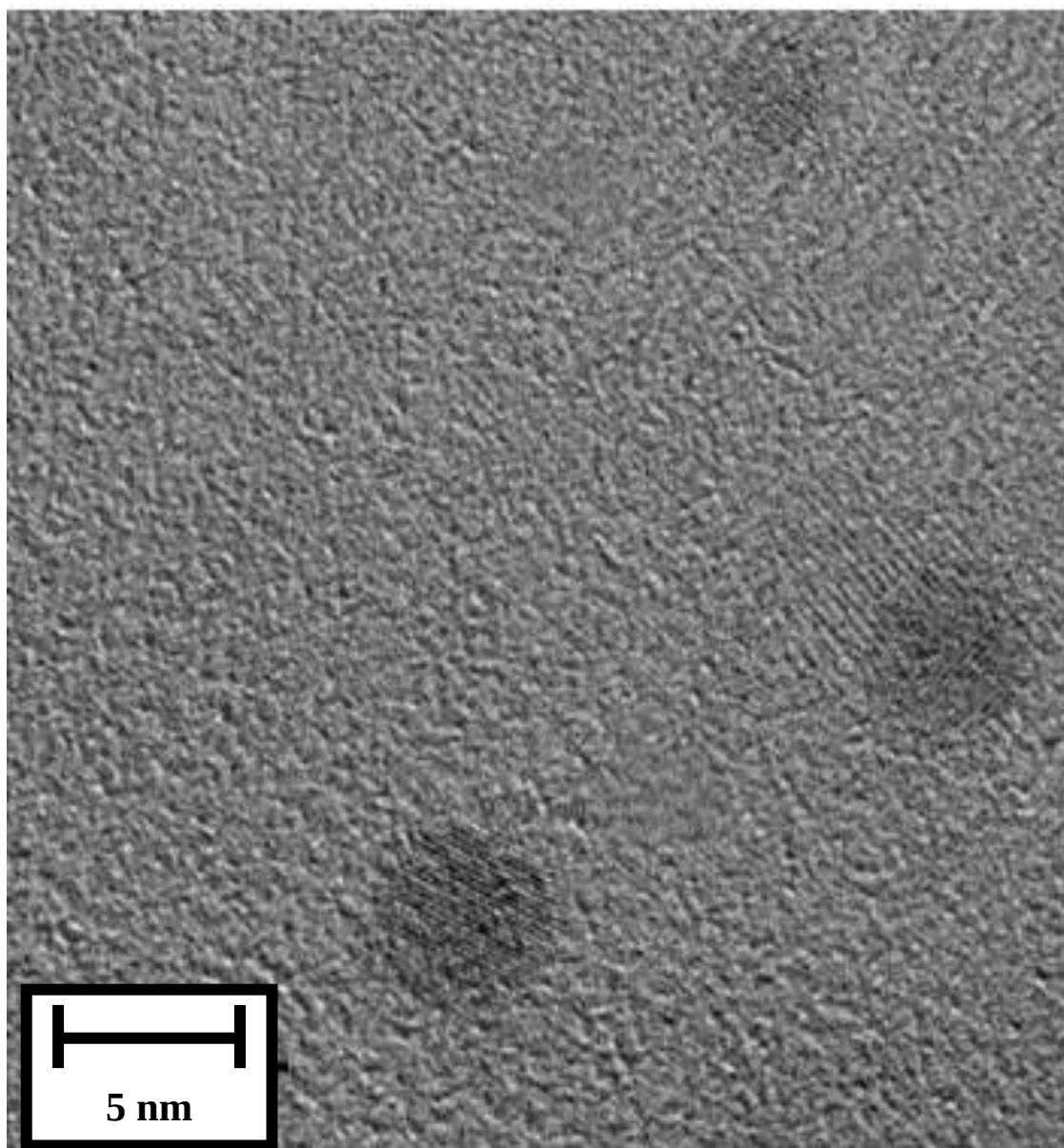


Figure S3.1. TEM micrograph displaying the lattice fringes of *Iso*-CDs.

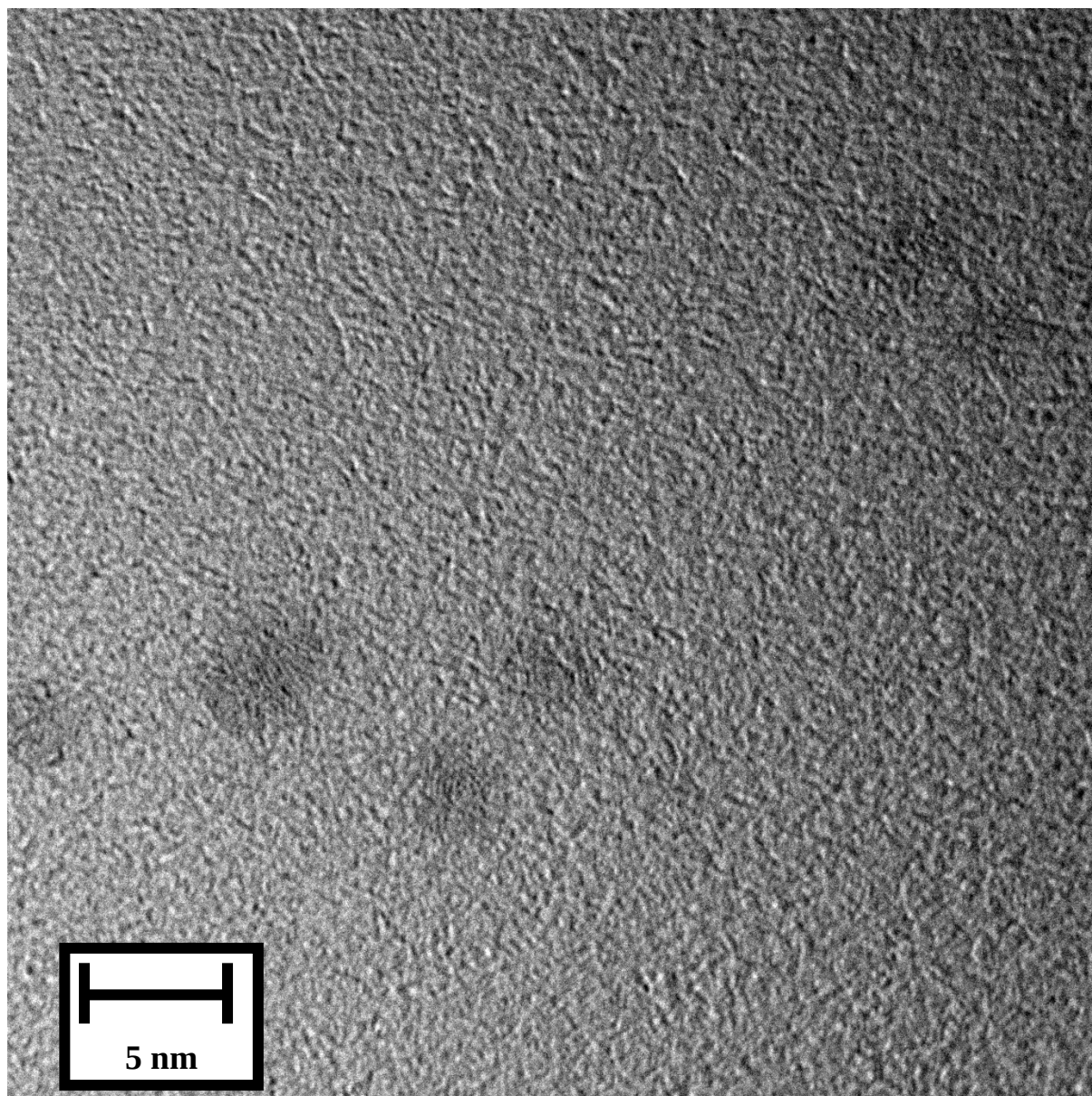


Figure S3.2. TEM micrograph displaying the lattice fringes of *Iso-Ge-CDs*

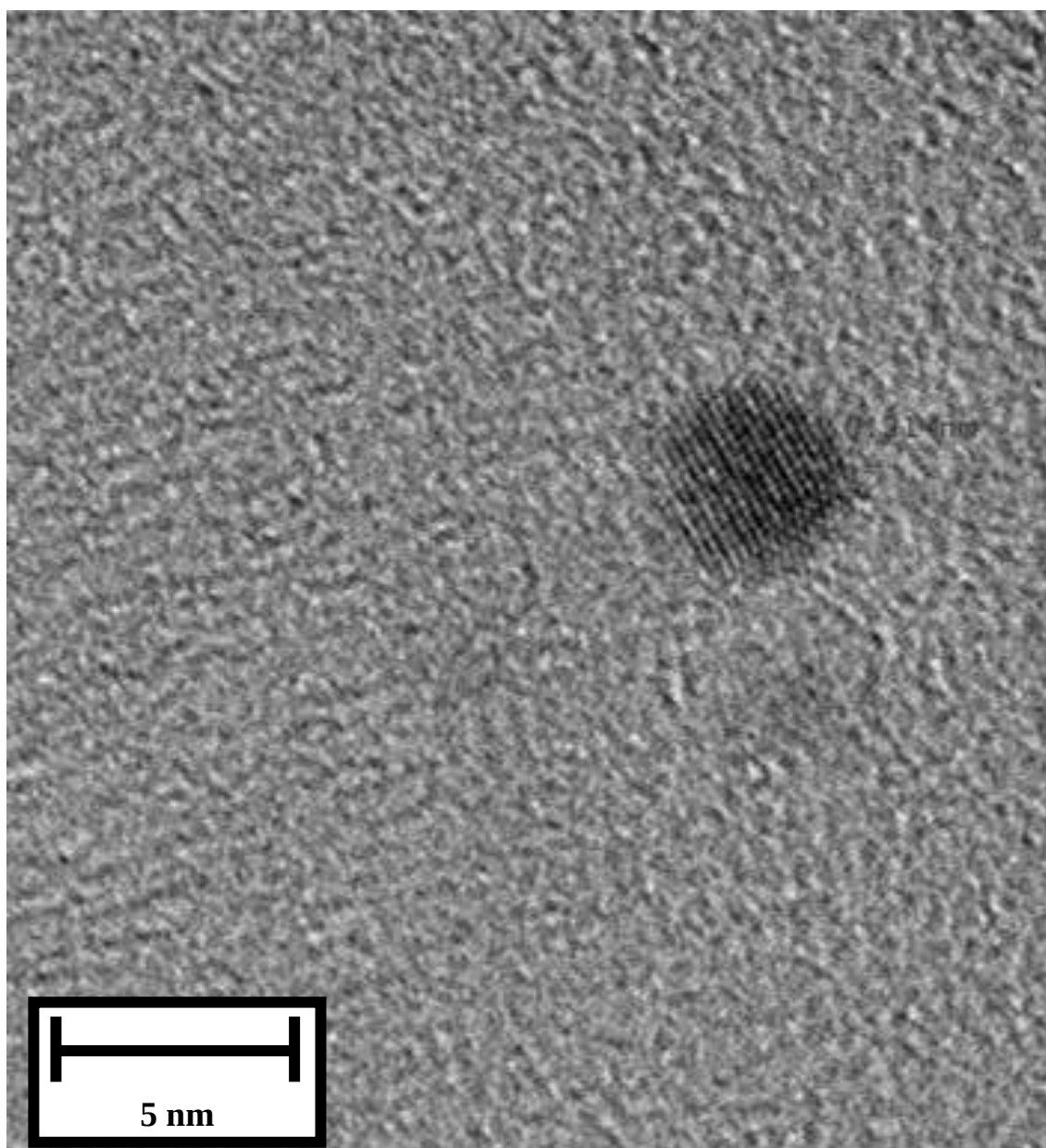


Figure S3.3. TEM micrograph displaying the lattice fringes of *Iso-N*-CDs

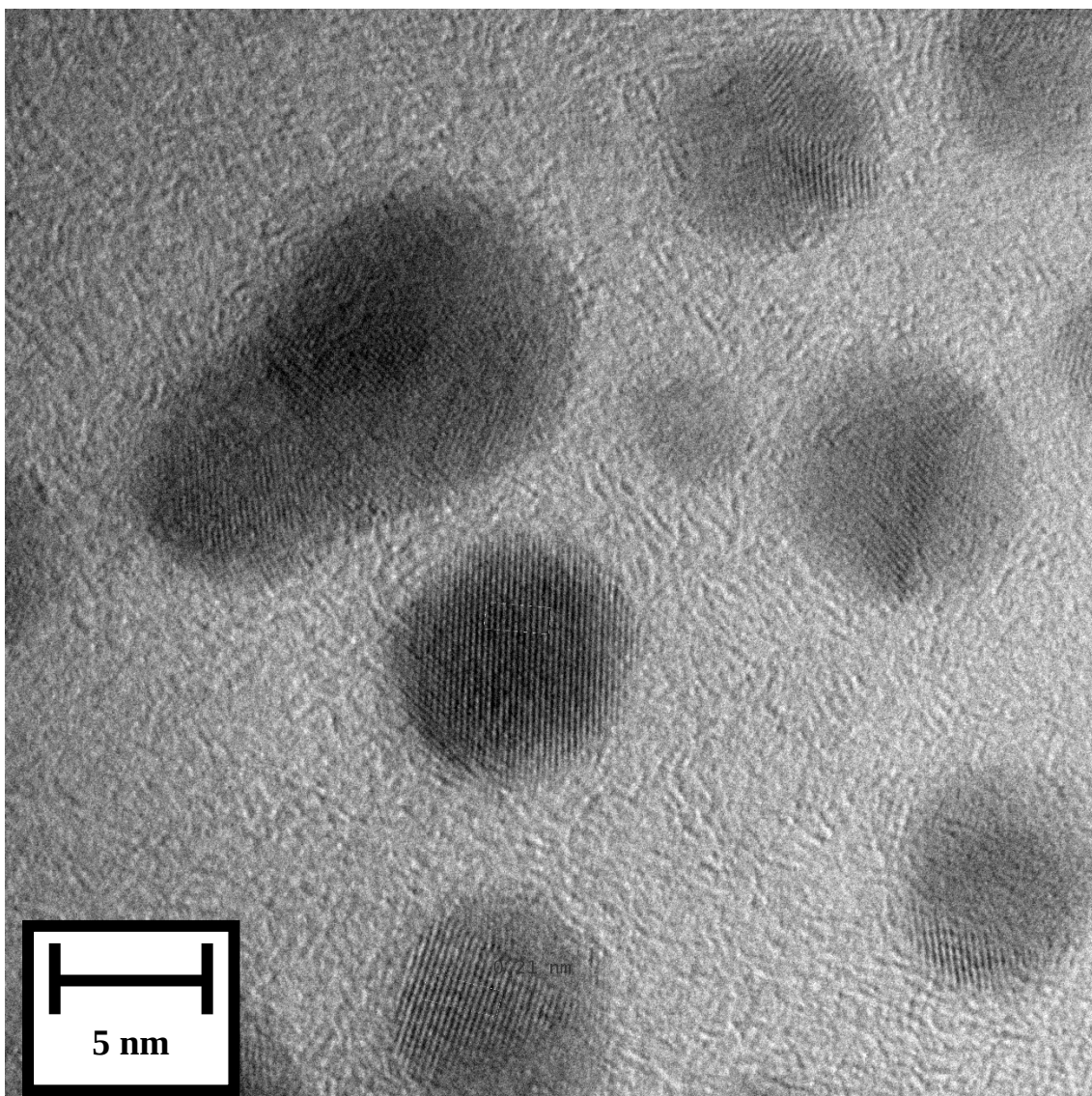
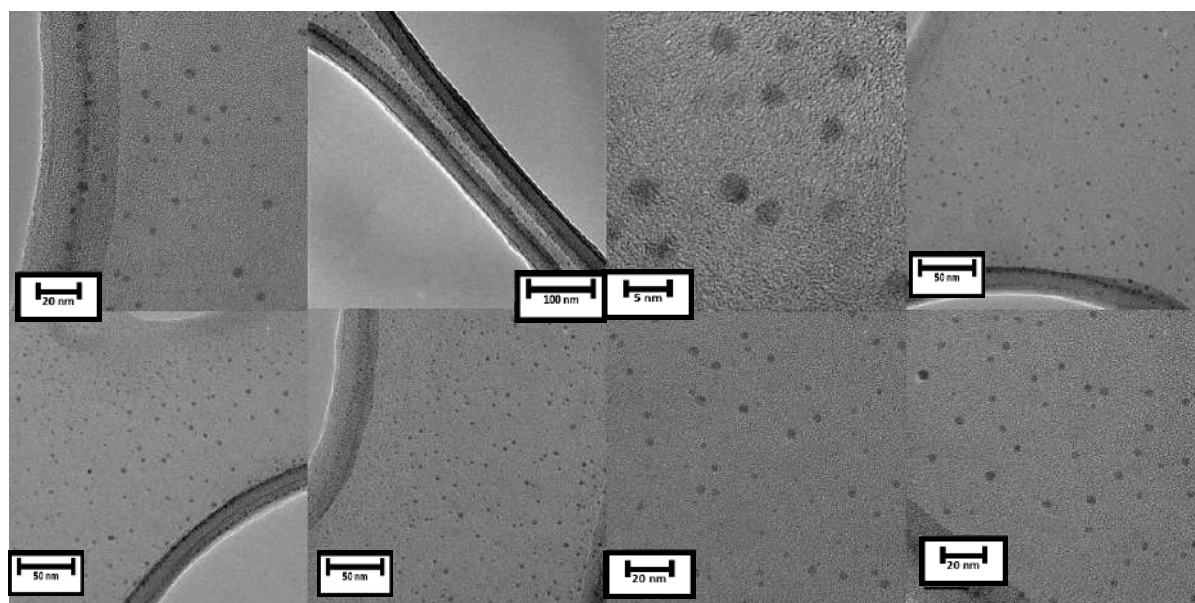
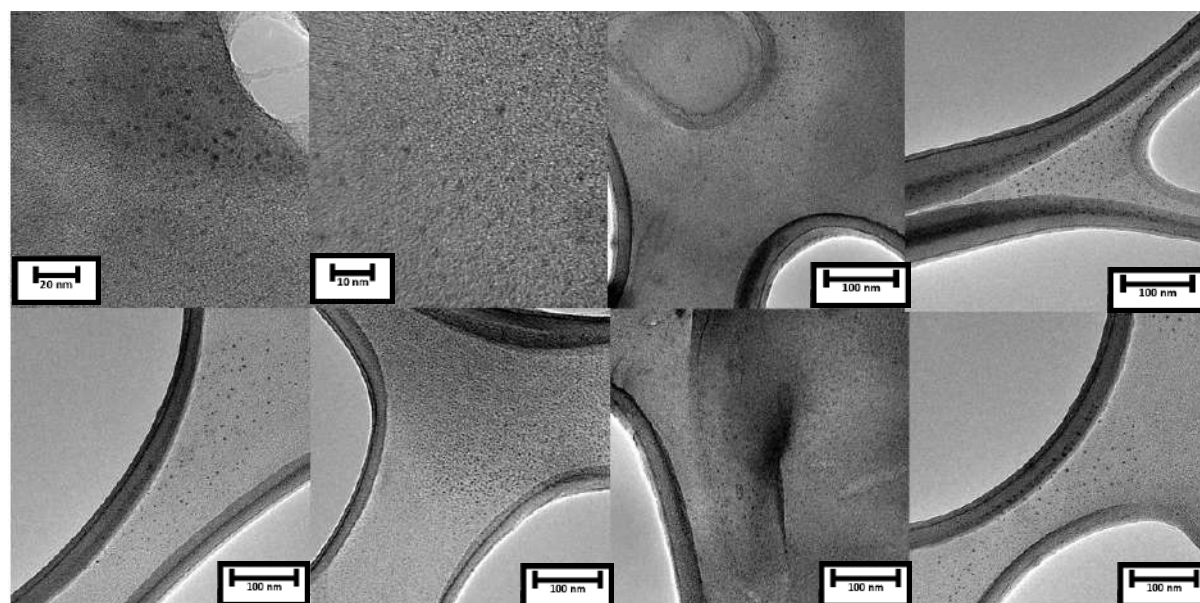


Figure S3.4. TEM micrograph displaying the lattice fringes of *Iso-N-Ge-CDs*



Iso - CDs

Figure S3.5. TEM micrograph displaying the size distribution and morphology of *Iso*-CDs.



Iso Ge -CDs

Figure S3.6. TEM micrograph displaying the size distribution and morphology of *Iso-Ge*-CDs.

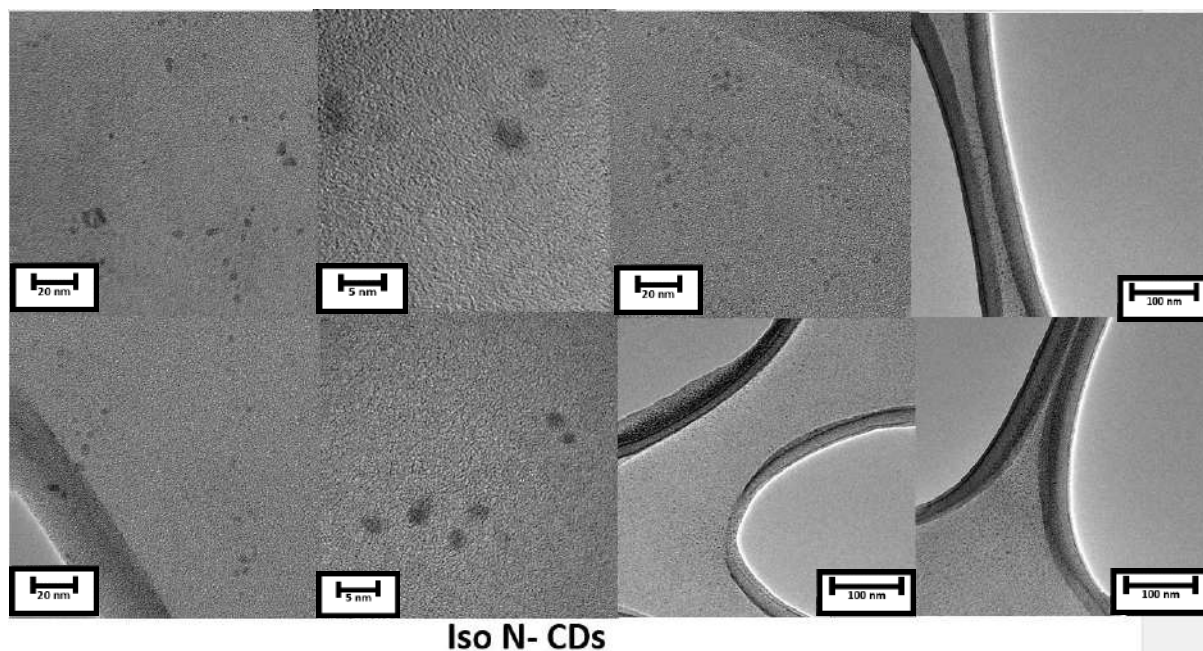


Figure S3.7. TEM micrograph displaying the size distribution and morphology of *Iso-N*-CDs.

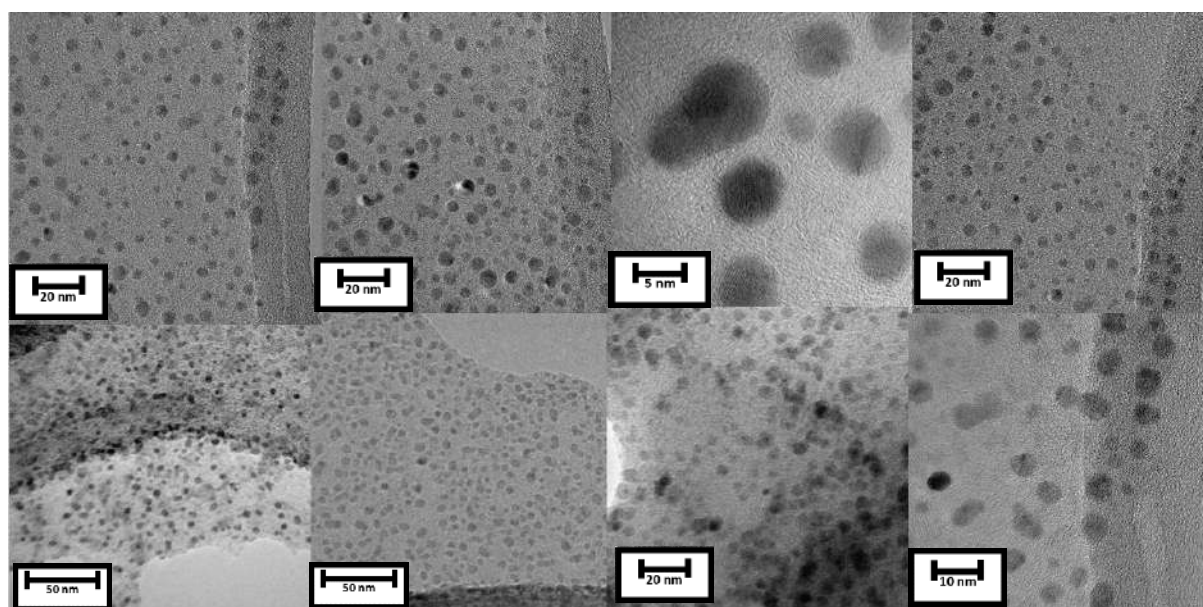


Figure S3.8. TEM micrograph displaying the size distribution and morphology of *Iso-N-Ge*-CDs.

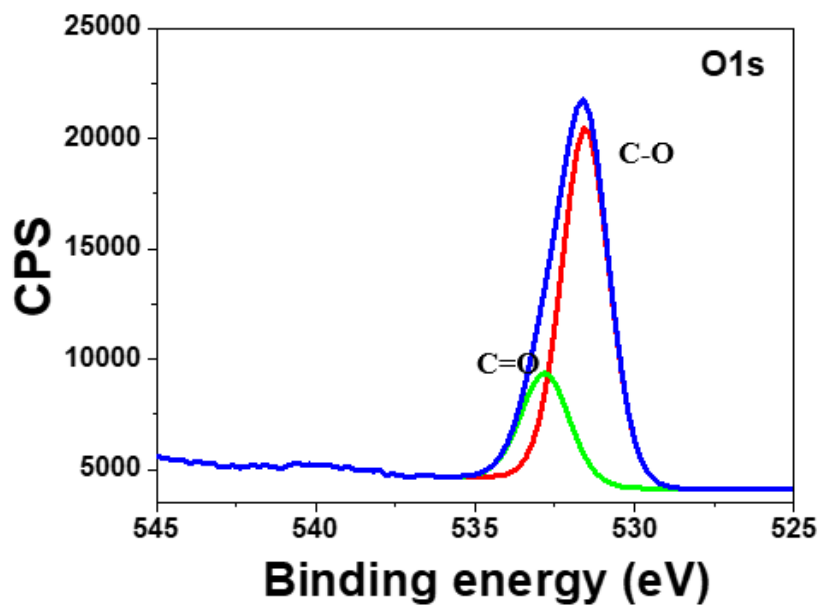


Figure S3.9. Expanded survey scan of O1s XPS spectrum of *Iso-N-Ge-CDs*.

Table S3.1: Elemental ID and Quantification of *Iso-N-Ge-CDs*.

Name	Peak BE	FWH M eV	Atomic %
C1s	285.4	4.1	55.4
O1s	532	3.3	25.9
N1s	399.9	3	15.9
Ge2p 3	1219. 8	3.2	2.8

Table S3.2: Breakdown of C, N, O, and Ge species found in *Iso-N-Ge-CDs*.

Name	Peak BE	FWHM eV	Atomic %
[C-C]	284.6	1.4	25.1
[C-O/C-N]	286	1.4	18.7
C1s [C=O]	288	1.4	5.7
C1s [O-C=O/ N-C=O]	288.7	1.4	6.1
N1s [Graphitic N]	399.6	1.6	15.6
N1s [NO _x]	401.3	1.6	1.2
O1s [C-O]	531.5	1.8	19.6
O1s [C=O]	532.8	1.8	5.8
Ge2p3 [GeO ₂ /GeO]	1219. 6	1.7	2.1

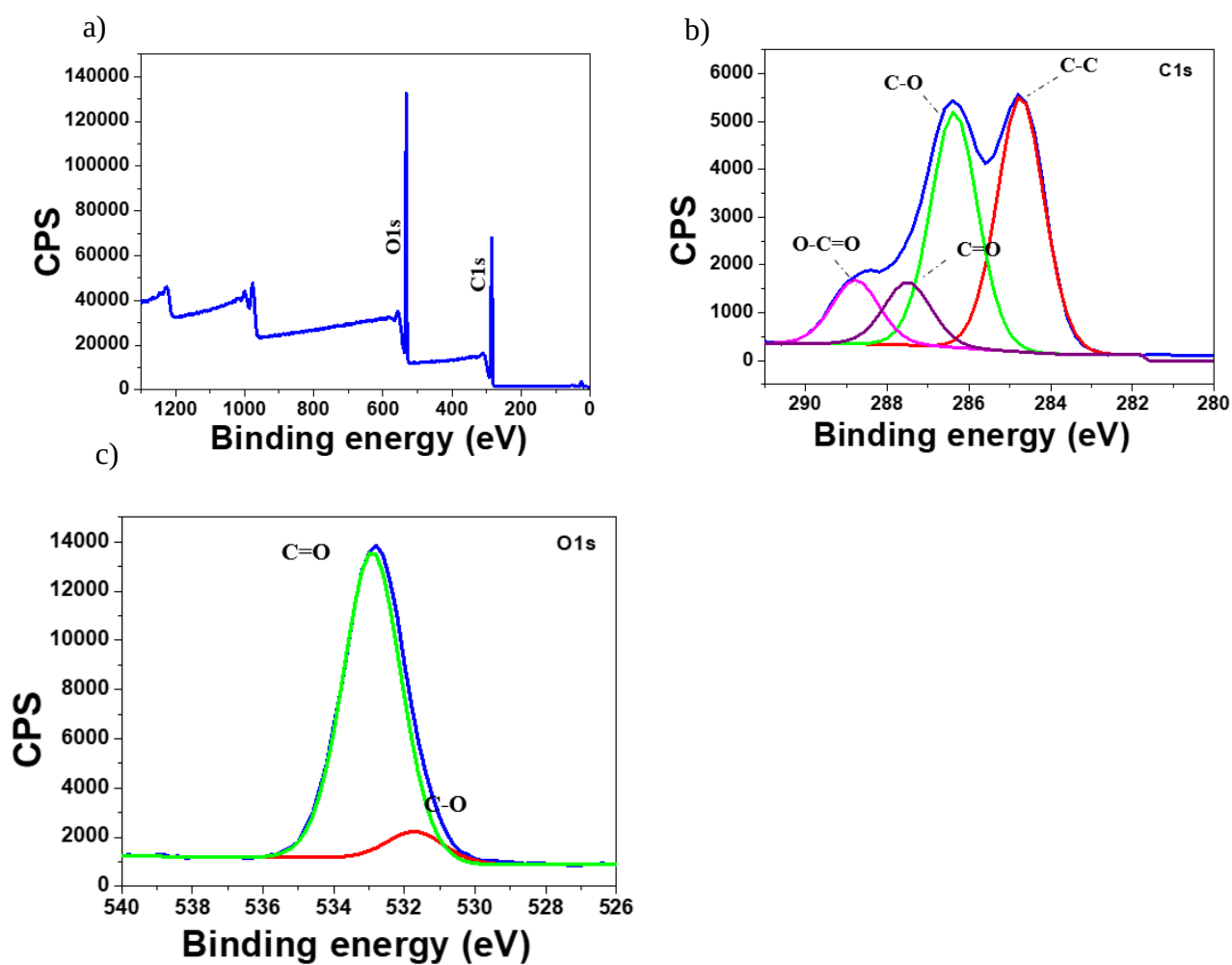


Figure S3.10. XPS data of *Iso*-CDs showing a) the full survey scan, (b) the C1s expanded scan and (c) the O1s expanded scan.

Table S3.3: Elemental ID and Quantification of *Iso*-CDs

Name	Peak BE	FWHM eV	Atomic %
C1s	285.4	4.2	64.6
O1s	532.6	3.3	35.4

Table S3.4: Breakdown of C, N, O and Ge species found in *Iso*-CDs

Name	Peak BE	FWHM eV	Atomic %
[C-C]	284.7	1.4	26.4
[C-O]	286.4	1.4	24.3
C1s [C=O]	287.5	1.4	6.5
C1s [O-C=O]	288.8	1.4	6.6
O1s [C-O]	531.7	1.9	3.4
O1s [C=O]	532.9	1.9	32.7

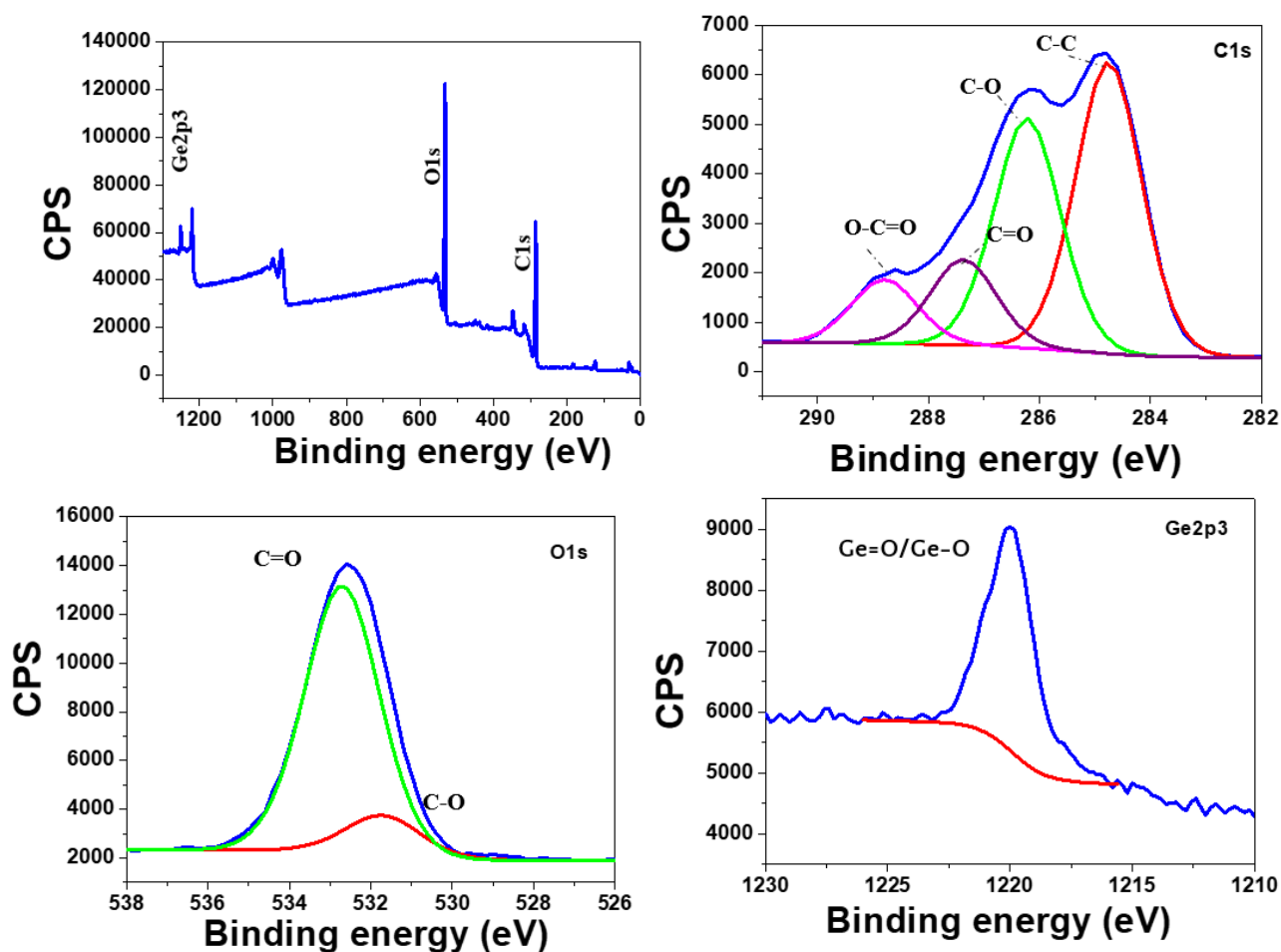


Figure S3.11. XPS data of *Iso-Ge-CDs* showing (a) full survey spectrum showing peaks from the survey scan, (b) C1s expanded scan, (c) O1s expanded scan and (d) Ge2p₃ expanded scan.

Table S3.5: Elemental ID and Quantification of *Iso-Ge-CDs*

Name	Peak BE	FWHM eV	Atomic %
C1s	285.4	4.1	63.4
O1s	532.3	3.5	34.5
Ge2p ₃	1219.9	3.7	2.1

Table S3.6: Breakdown of C, N, O and Ge species found in *Iso-Ge-CDs*

Name	Peak BE	FWHM eV	Atomic %
[C-C]	284.8	1.4	27.6
[C-O]	286.2	1.4	21.8
C1s [C=O]	287.4	1.4	8.1
C1s [O-C=O]	288.8	1.4	6.1
O1s [C-O, GeO ₂]	531.7	2.2	4.7
O1s [C=O]	532.7	2.2	29.8
Ge2p3 [GeO ₂]	1220	2.3	2

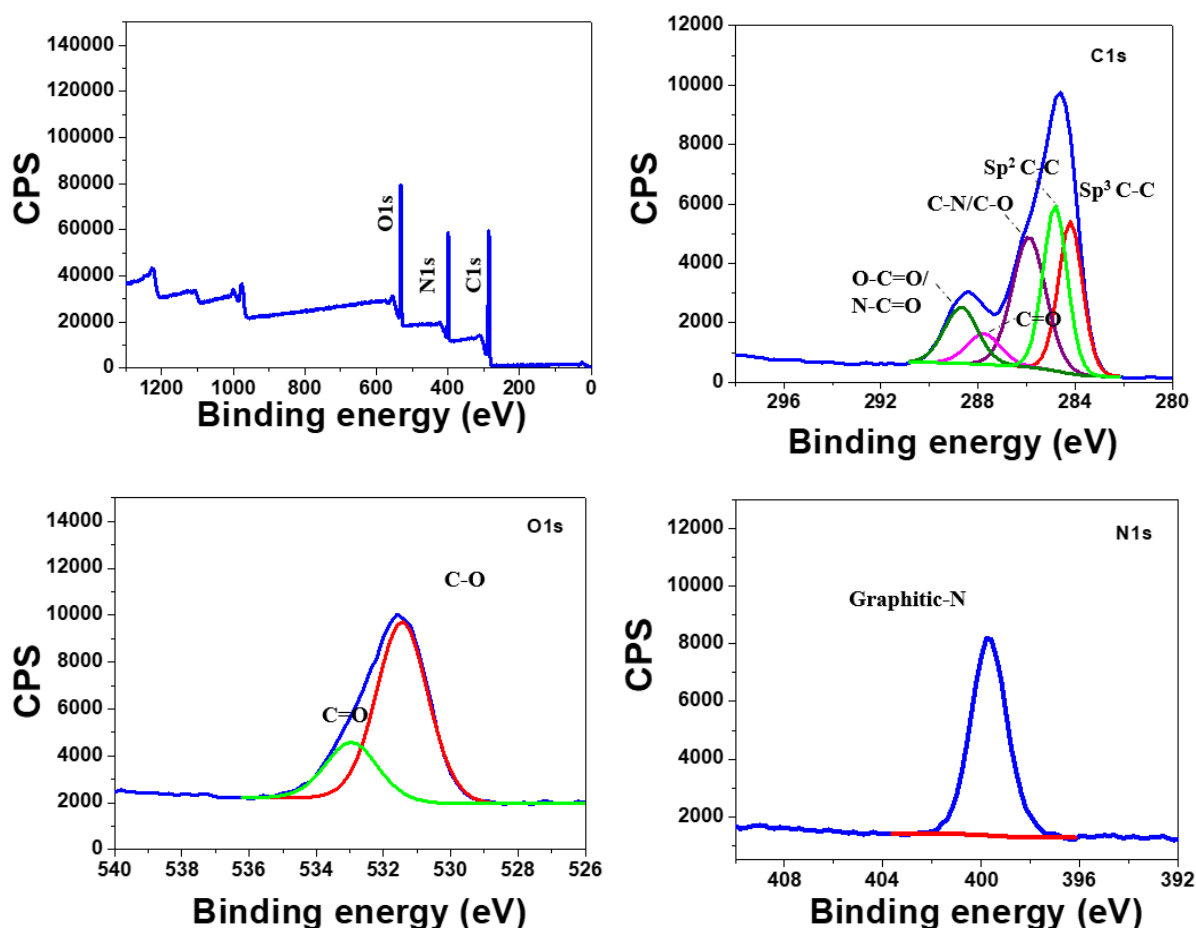


Figure S3.12. XPS data of *Iso-N-CDs* showing (a) full survey spectrum, (b) C1s expanded scan, (c) O1s expanded scan and (d) N1s expanded scan.

Table S3.7: Elemental ID and Quantification of *Iso-N*-CDs

Nam e	Peak BE	FWHM eV	Atomic %
C1s	285.1	4	58.9
N1s	399.7	2.9	21.4
O1s	531.7	3.4	19.7

Table S3.8: Breakdown of C, N, O, and Ge species found in *Iso-N*-CDs

Name	Peak BE	FWHM eV	Atomic %
Sp ³ [C-C]	284.2	1.2	15.6
Sp ² [C-C]	284.8	1.2	16.9
[C-O/C-N]	285.9	1.6	18.6
C1s [C=O]	287.8	1.6	4.4
C1s [O-C=O/ N-C=O]	288.7	1.6	8
Graphitic N [C-N]	399.7	1.7	18.9
O1s [C-O]	531.4	1.8	13.4
O1s [C=O]	533	1.8	4.2

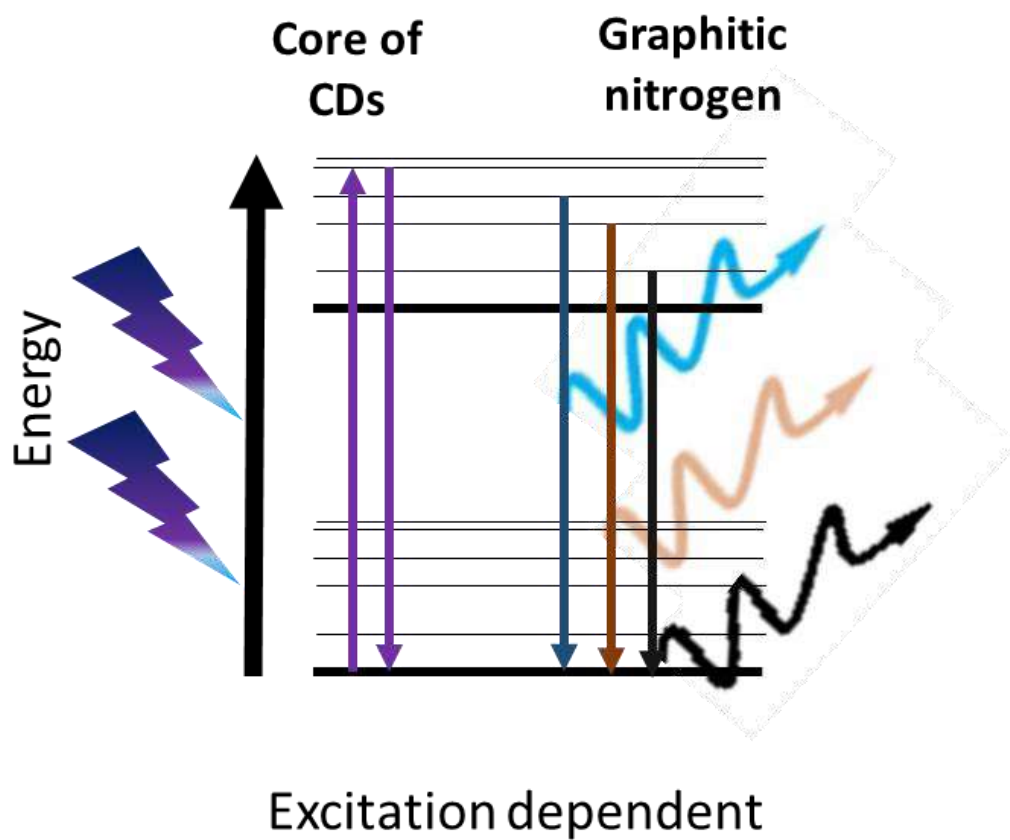


Figure S3.13. Illustration of how the surface functional groups affect the photoluminescent properties.

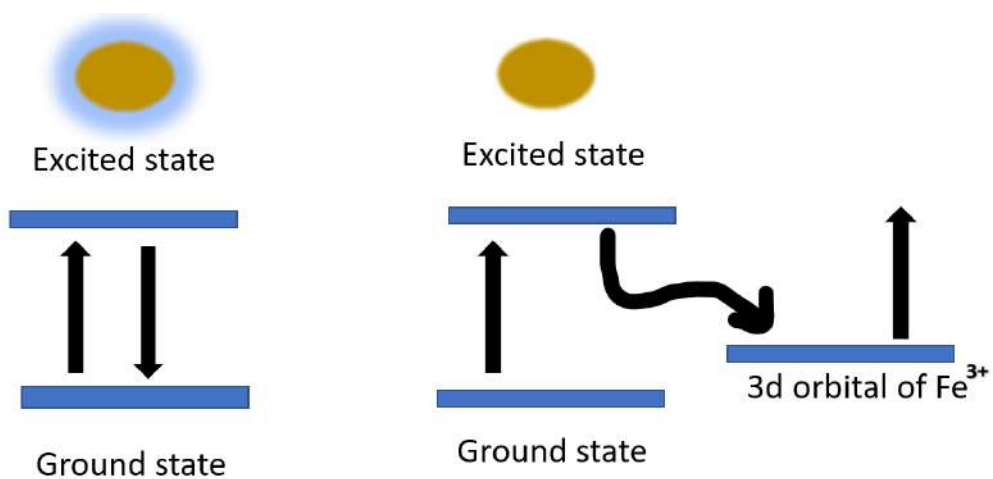


Figure S3.14. CDs' fluorescence quenching mechanism when exposed to Fe^{3+} ions.

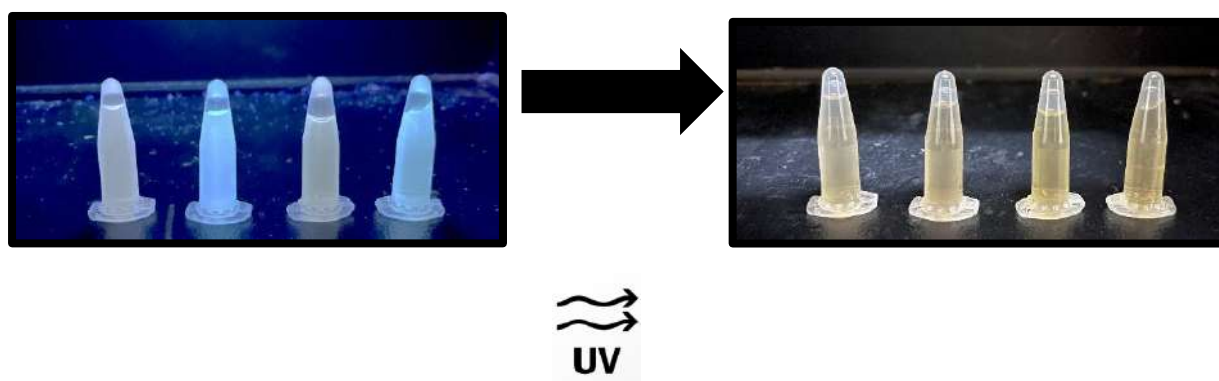


Figure S3.15. Luminescence of (from left to right) *Iso*-CDs, *Iso-N*-CDs, *Iso-Ge*-CDs, and *Iso-N-Ge*-CDs under UV lamp.

9.2 Chapter 4

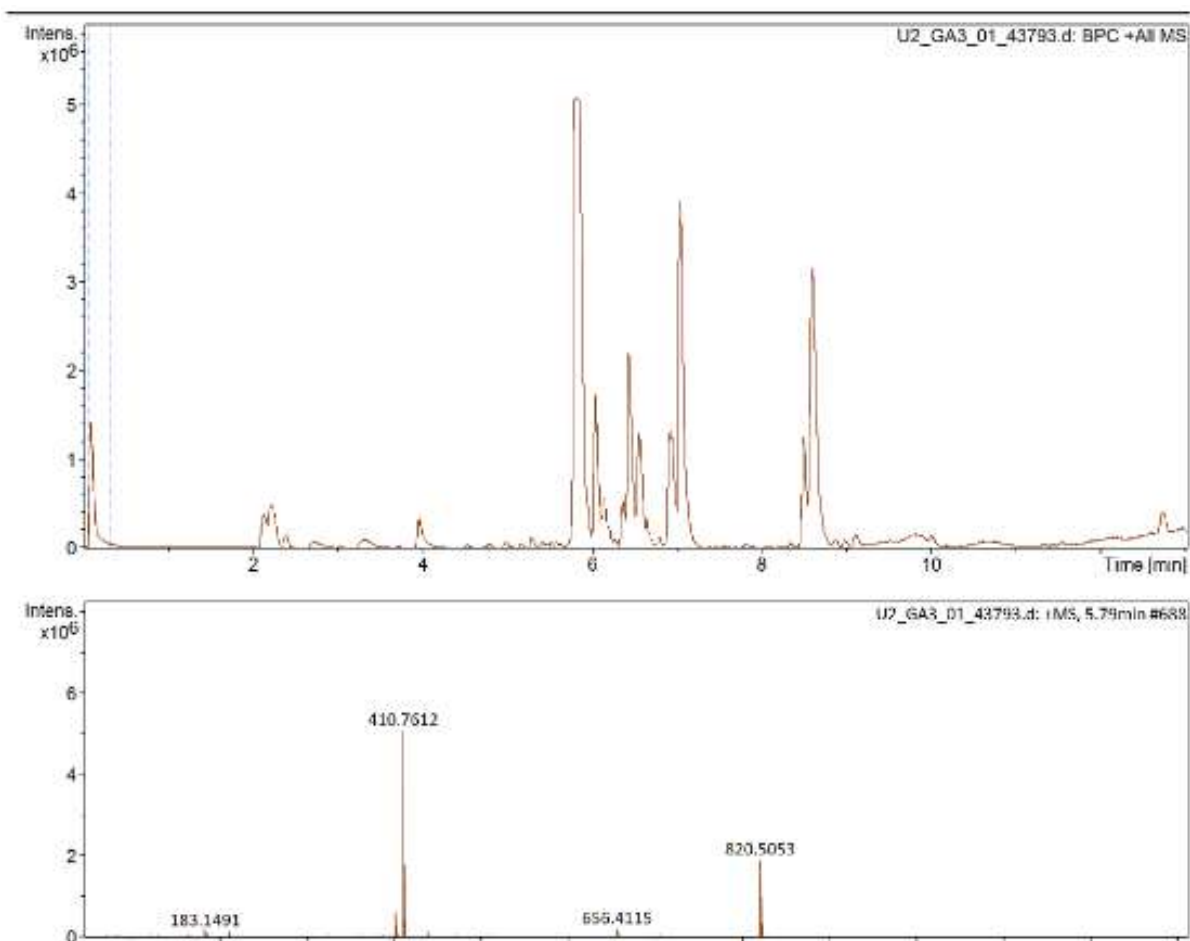


Figure S4.1 LCMS chromatogram of the crude GKP peptide (Full sequence and mass) obtained using method 1 outlined in **section 4.4.3**. (Fmoc-amino acids coupled and HBTU for 35 minutes and the Fmoc protecting group was removed using 20% piperidine for 5 minutes twice)

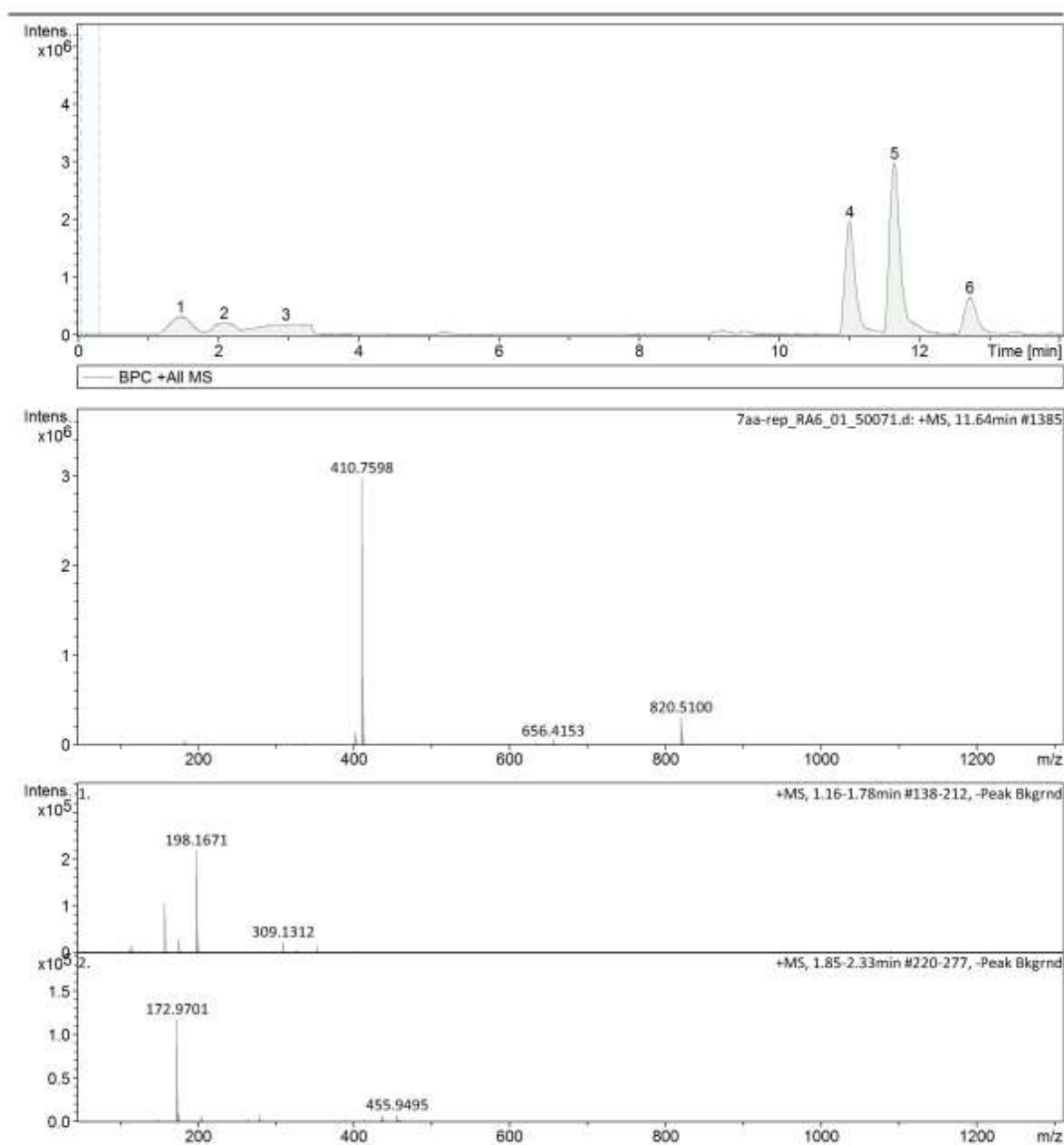


Figure S4.2 LCMS chromatogram of the crude GKP peptide (Full sequence and mass) obtained using method 2 outlined in **section 4.4.3**. (Fmoc-amino acids coupled and HATU for 20 minutes and the Fmoc protecting group was removed using 20% piperidine for 5 minutes twice)

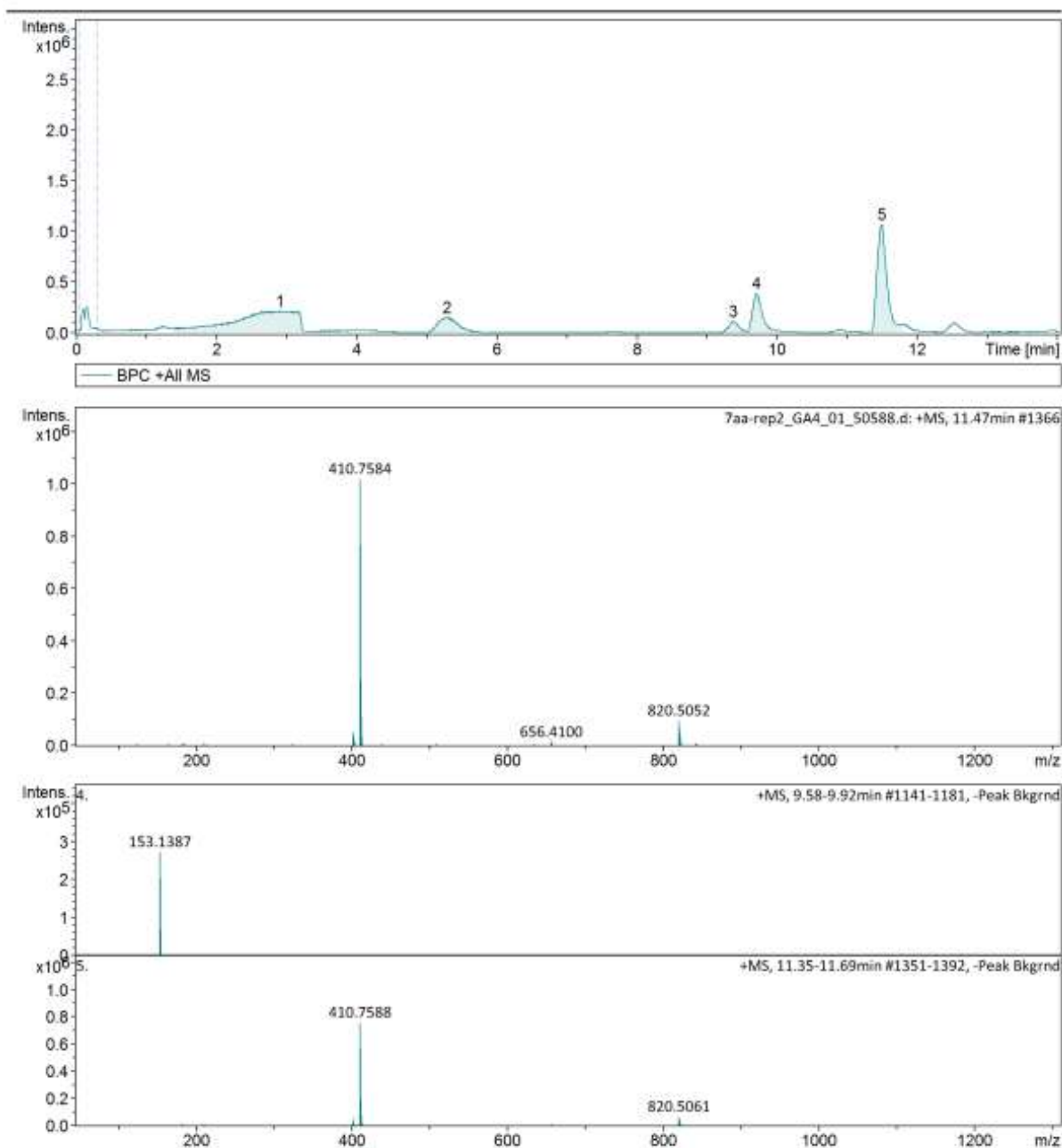


Figure S4.3 LCMS chromatogram of the crude GKP peptide (Full sequence and mass) obtained using method 3 outlined in **section 4.4.3**. (Fmoc-amino acids coupled and HBTU for 35 minutes, and the Fmoc protecting group was removed using 20% piperidine 1% DBU for 5 minutes twice)

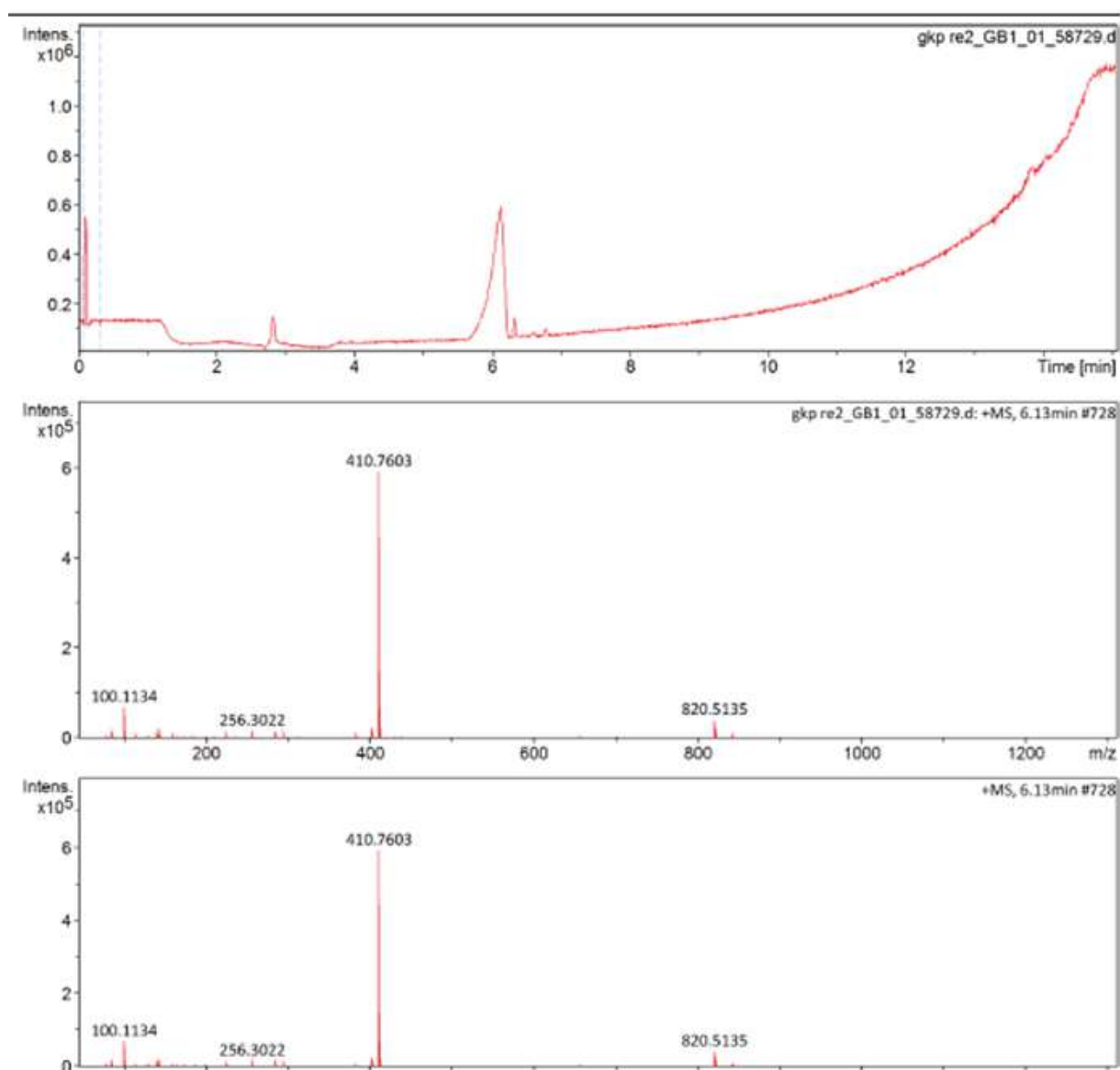


Figure S4.4 LCMS chromatogram of the crude GKP peptide (Full sequence and mass) obtained using method 4 outlined in **section 4.4.3**. (Fmoc-amino acids coupled and HBTU for 15 minutes and the Fmoc protecting group was removed using 20% piperidine 1% DBU for 5 minutes twice)

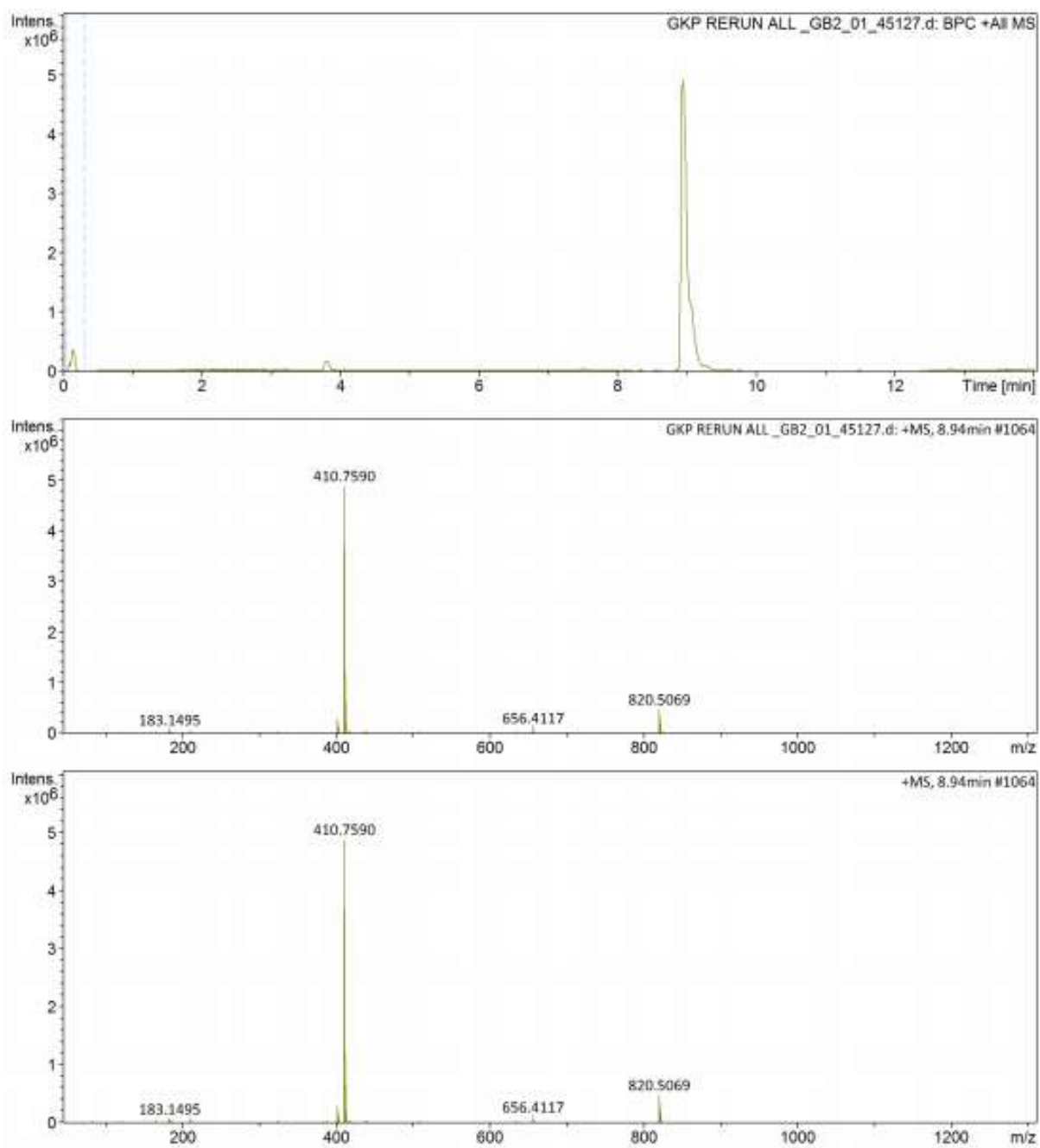


Figure S4.5 LCMS chromatogram of the GKP peptide after purification.

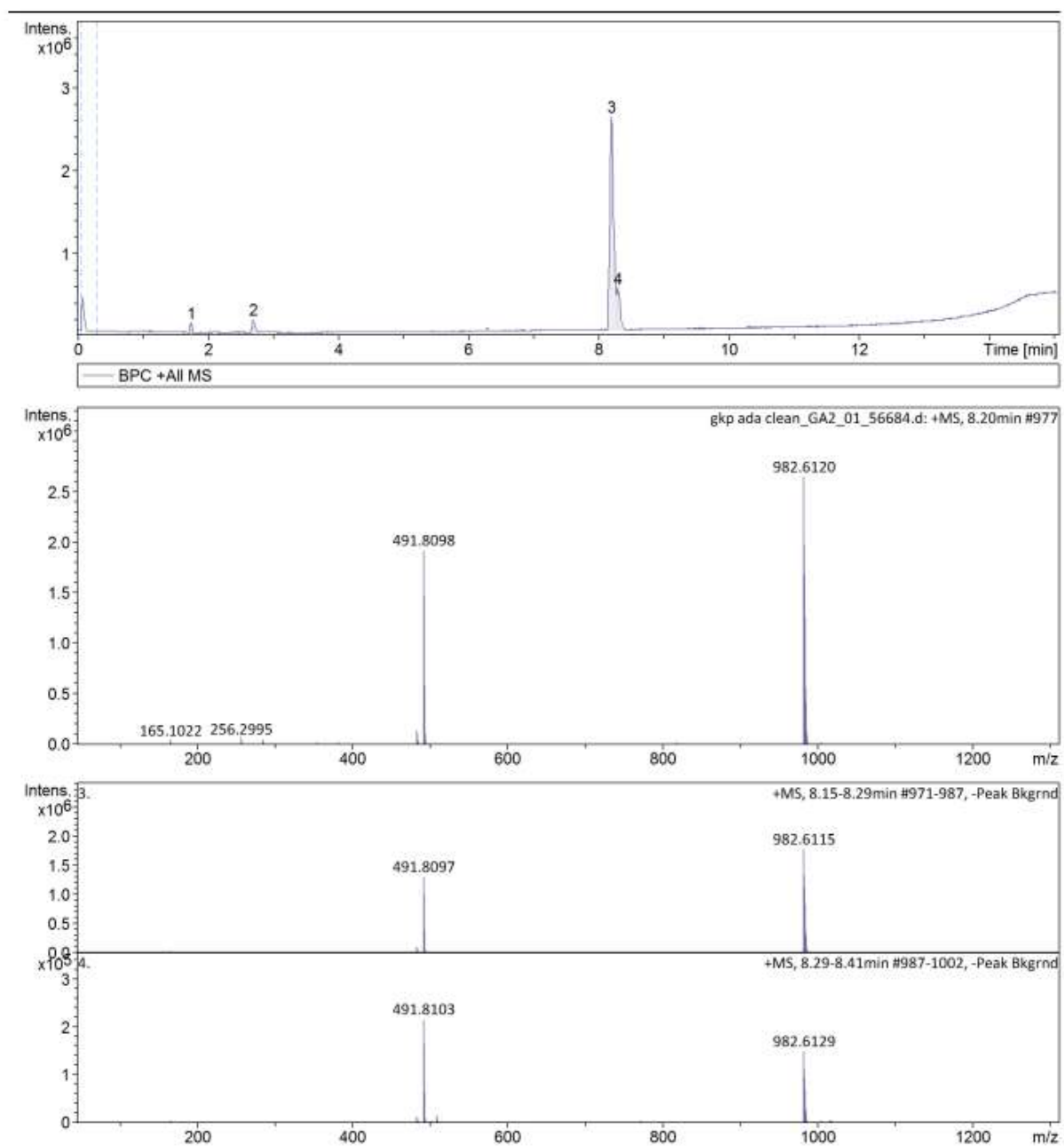


Figure S4.6 LCMS chromatogram of the GKP-ada peptide after purification.

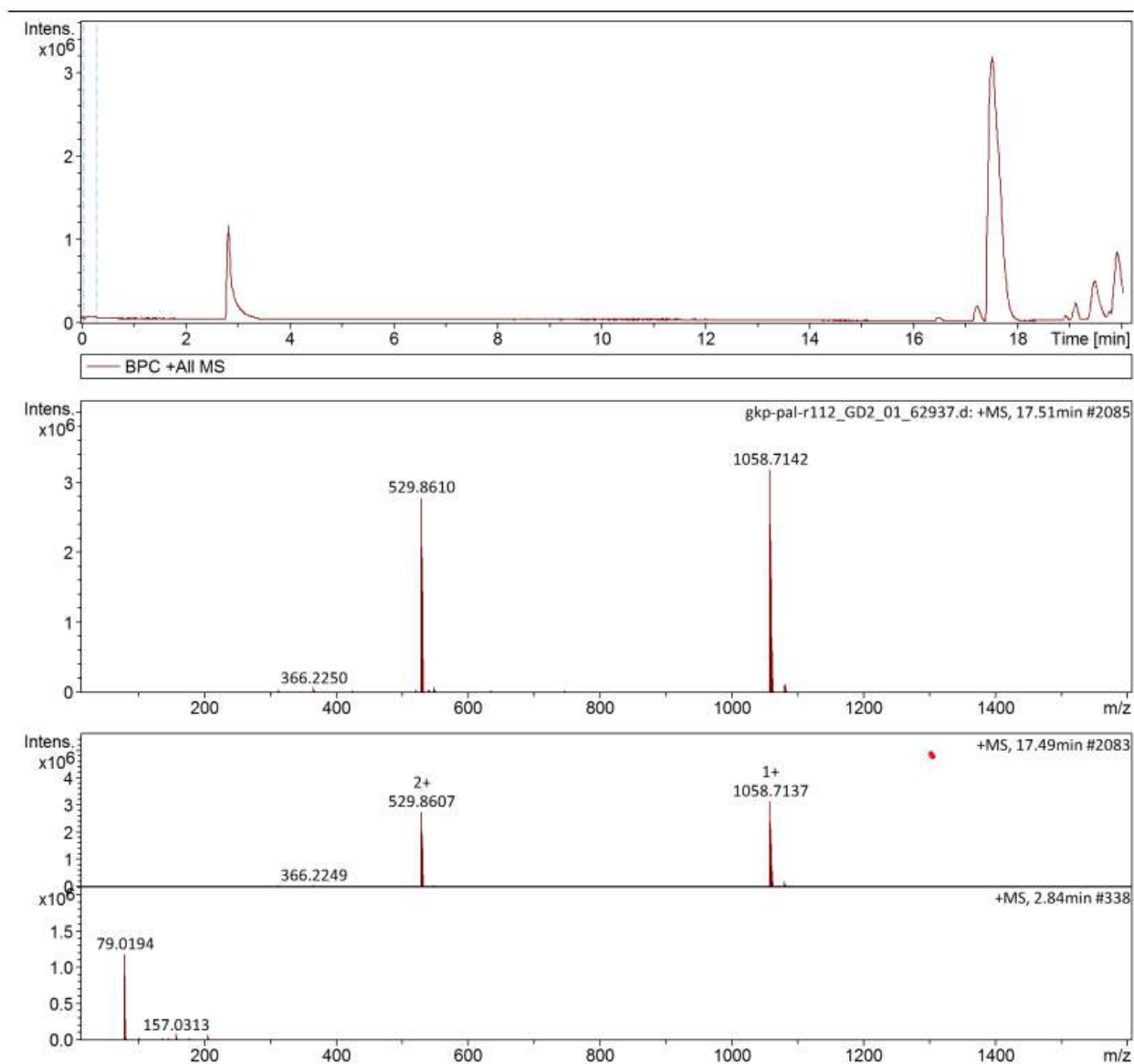


Figure S4.7 LCMS chromatogram of the GKP-pal peptide after purification.

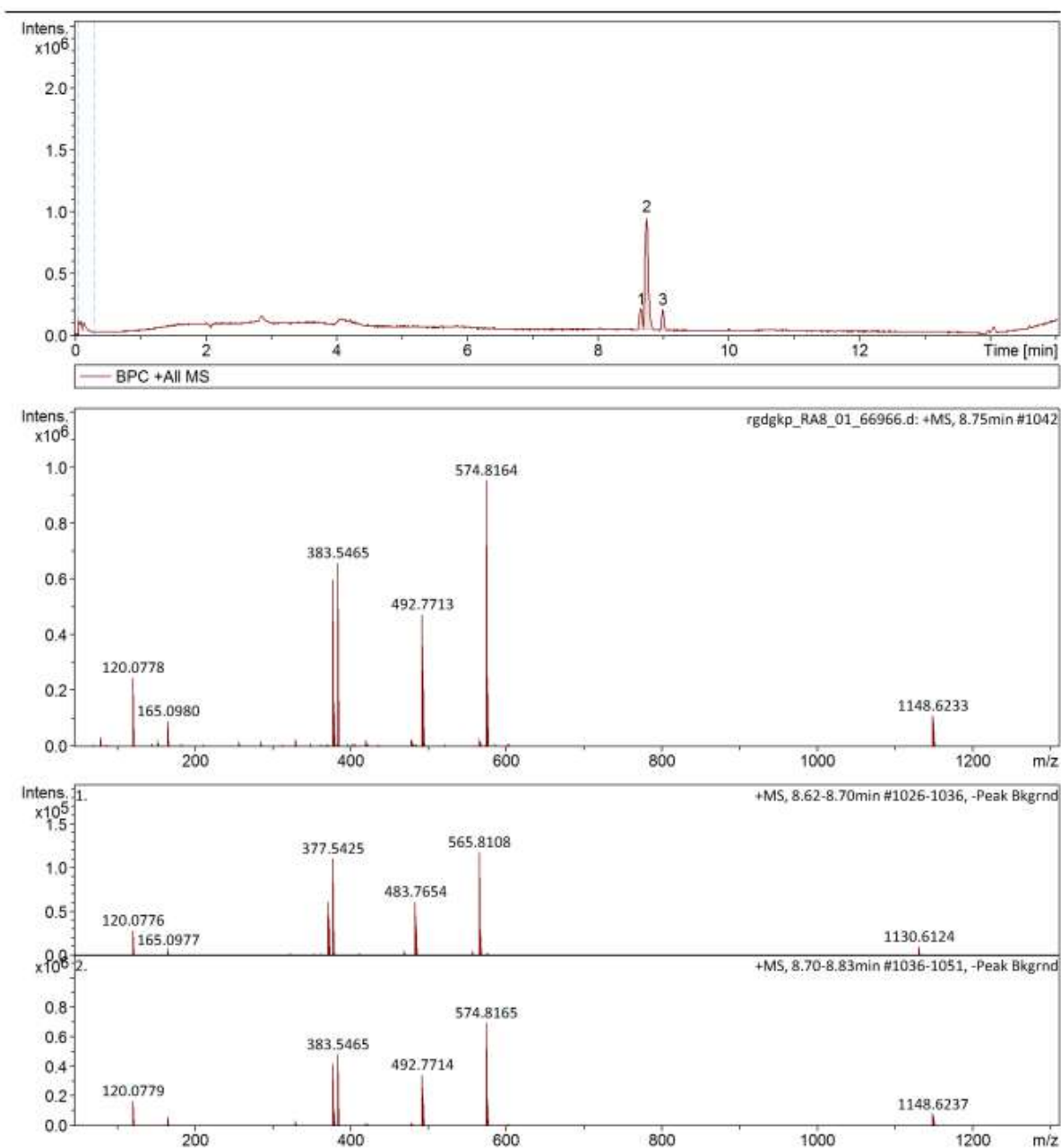


Figure S4.8 LCMS chromatogram of the RGD-GKP-ada peptide after purification.

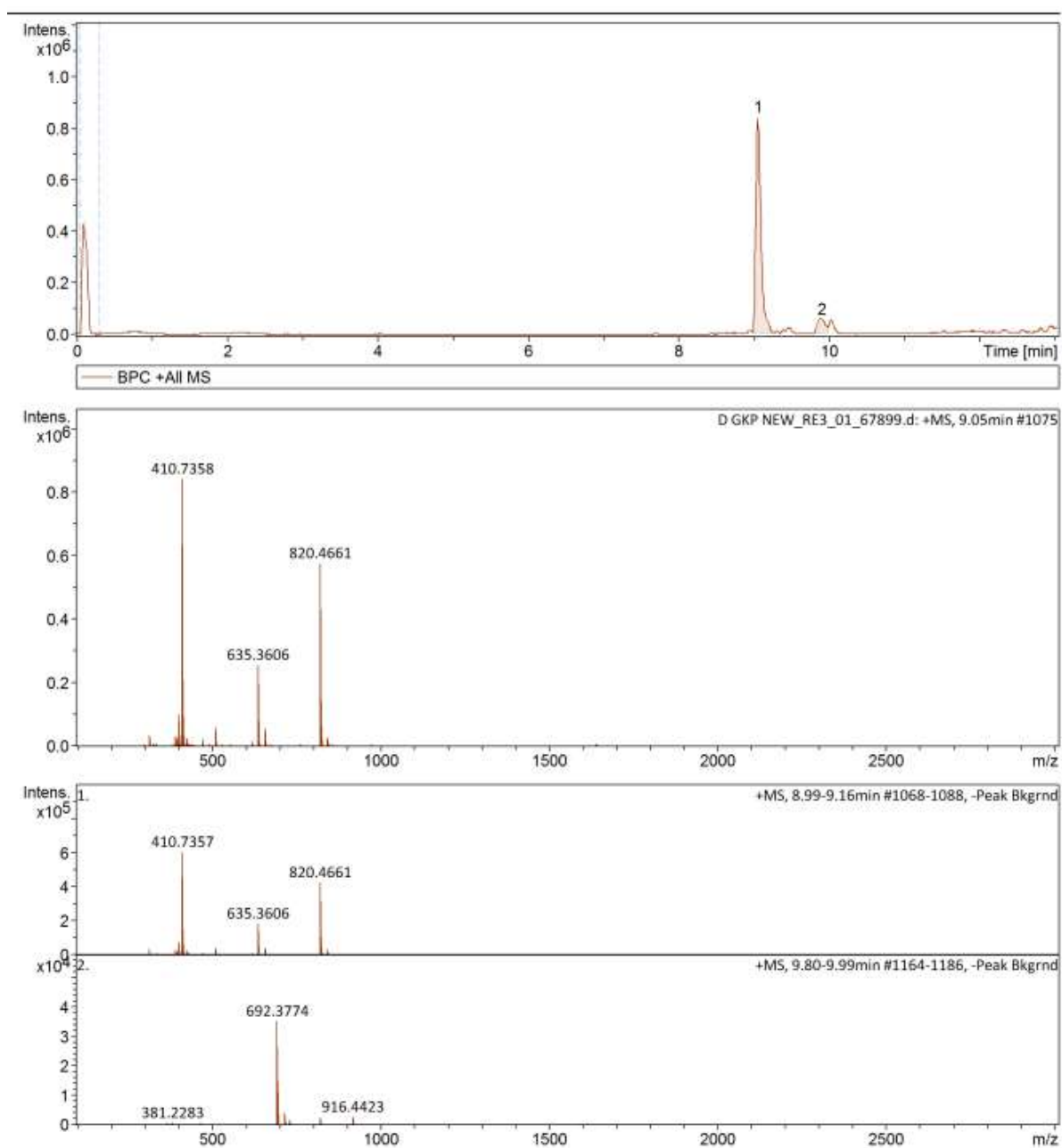


Figure S4.9 LCMS chromatogram of the D-GKP peptide after purification.

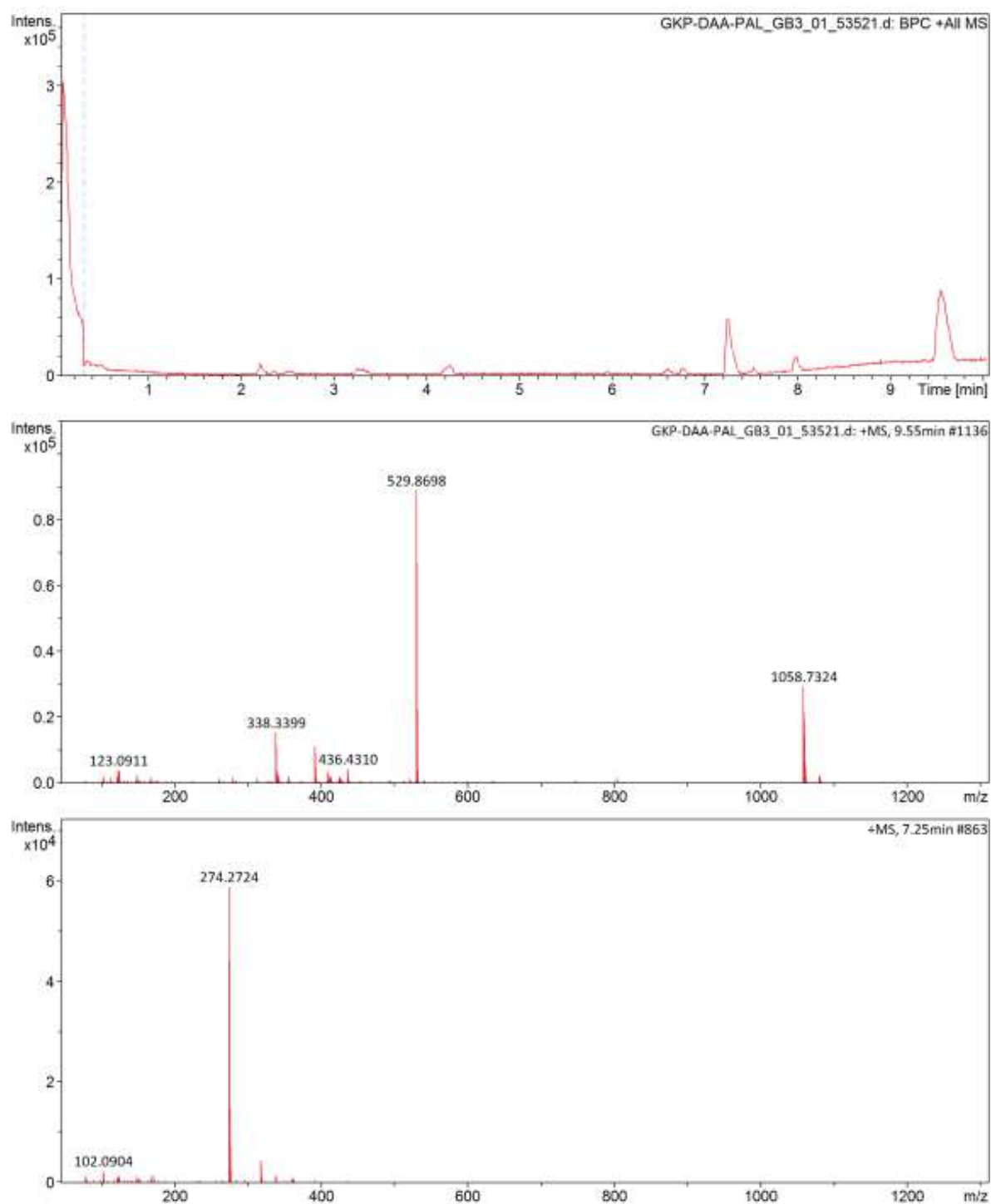


Figure S4.10 LCMS chromatogram of the D-GKP-pal peptide after purification.

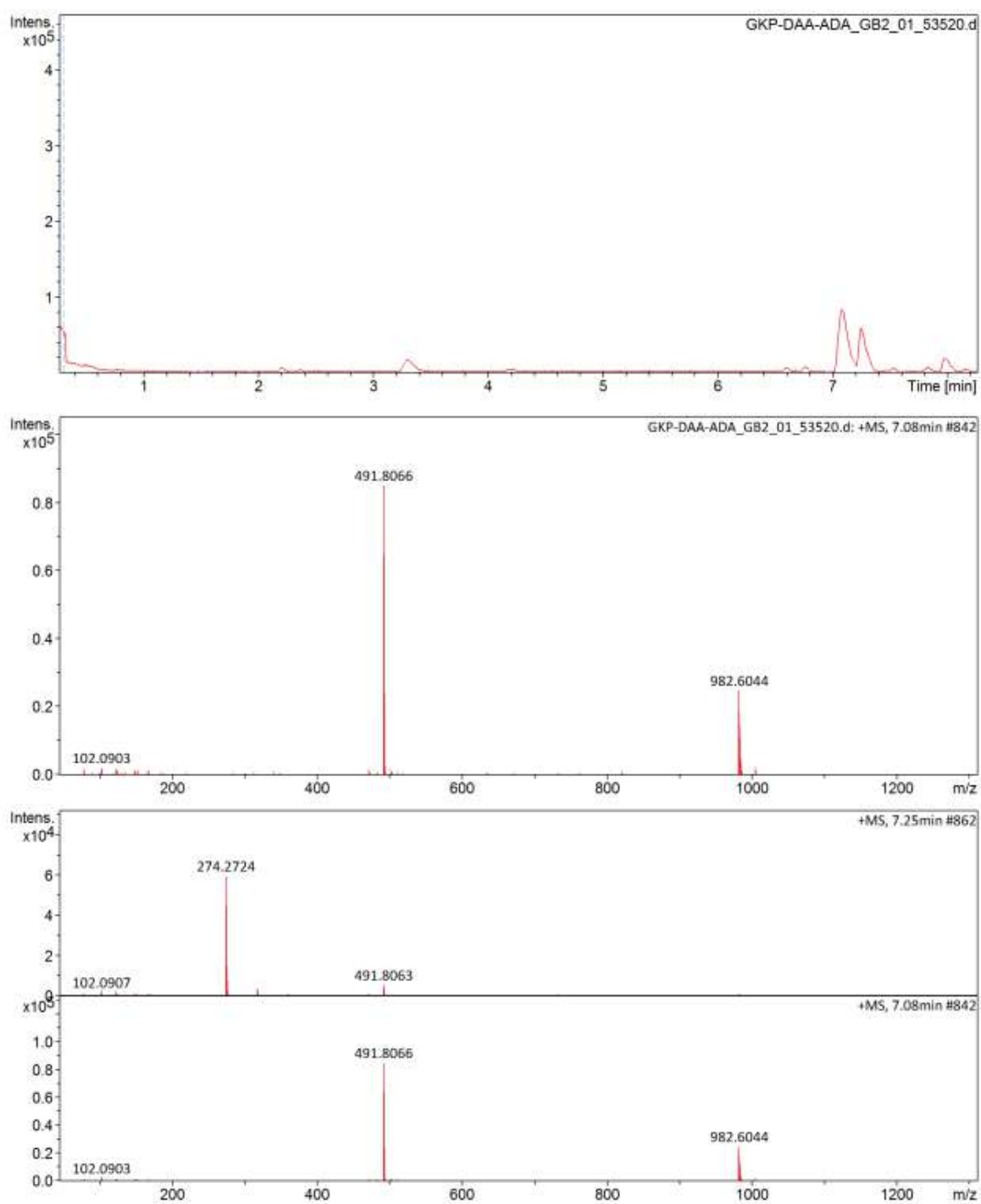


Figure S4.11 LCMS chromatogram of the D-GKP-ada peptide after purification.

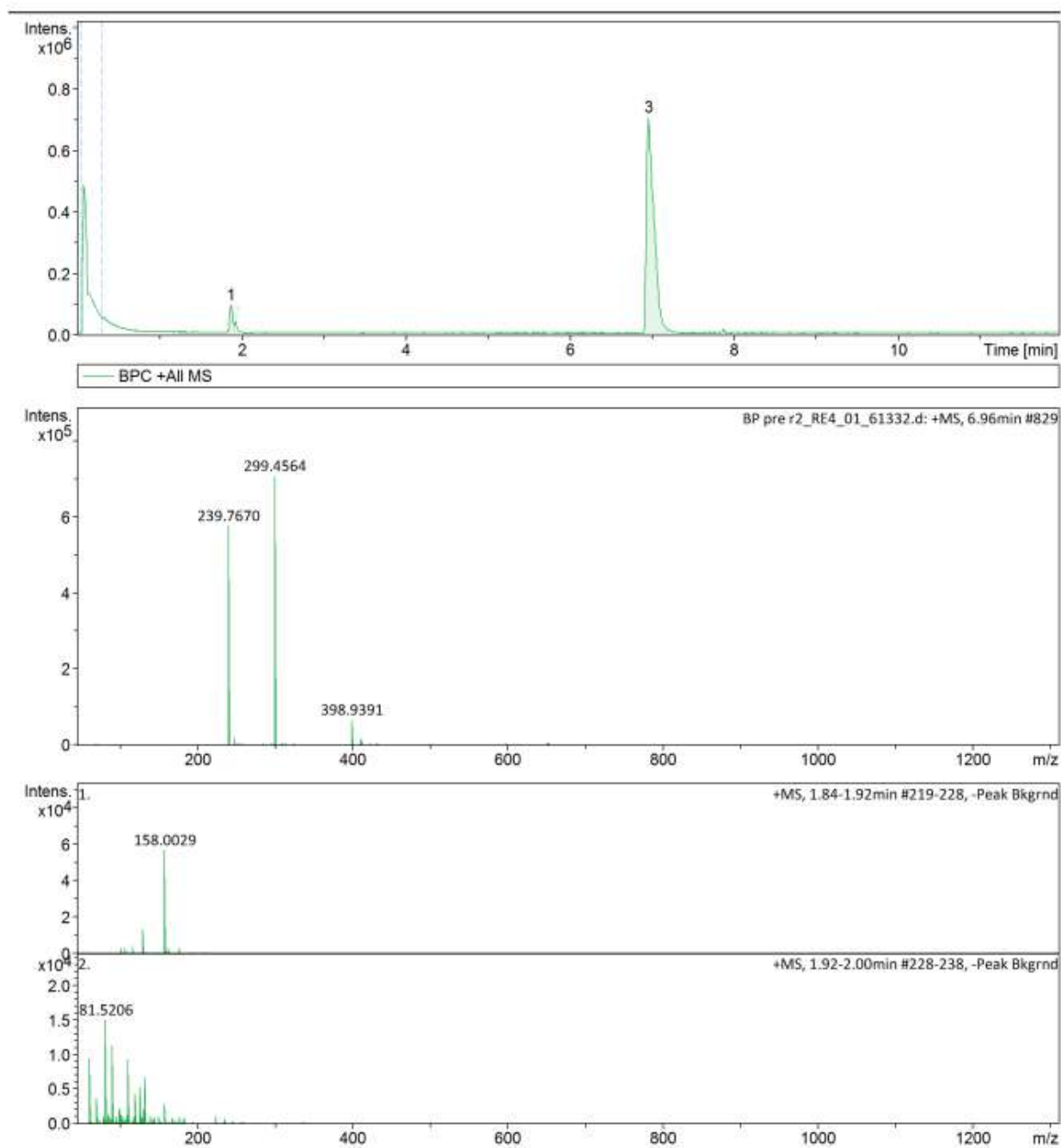


Figure S4.12 LCMS chromatogram of the BP peptide after purification.

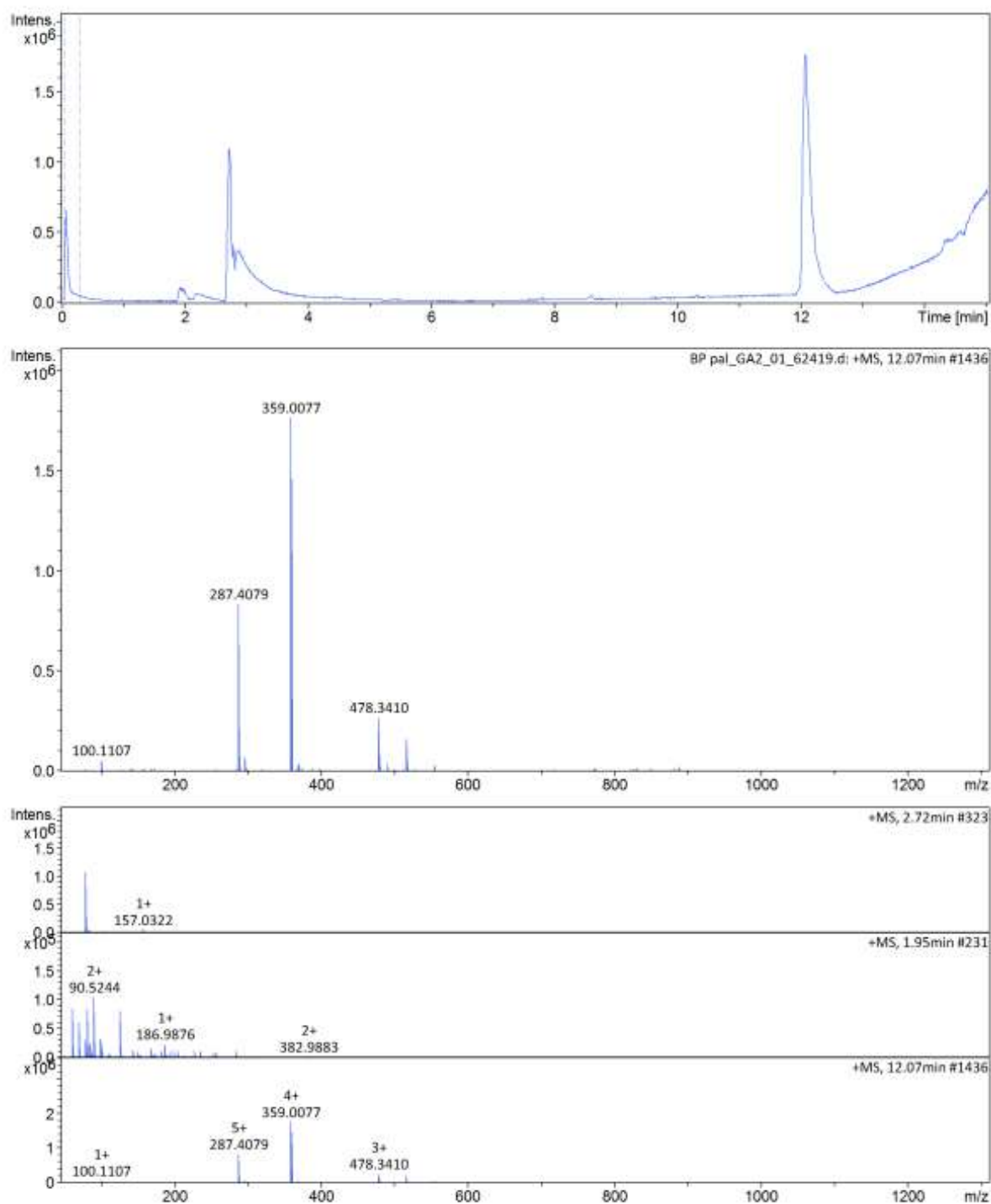


Figure S4.13 LCMS chromatogram of the BP-pal peptide after purification.

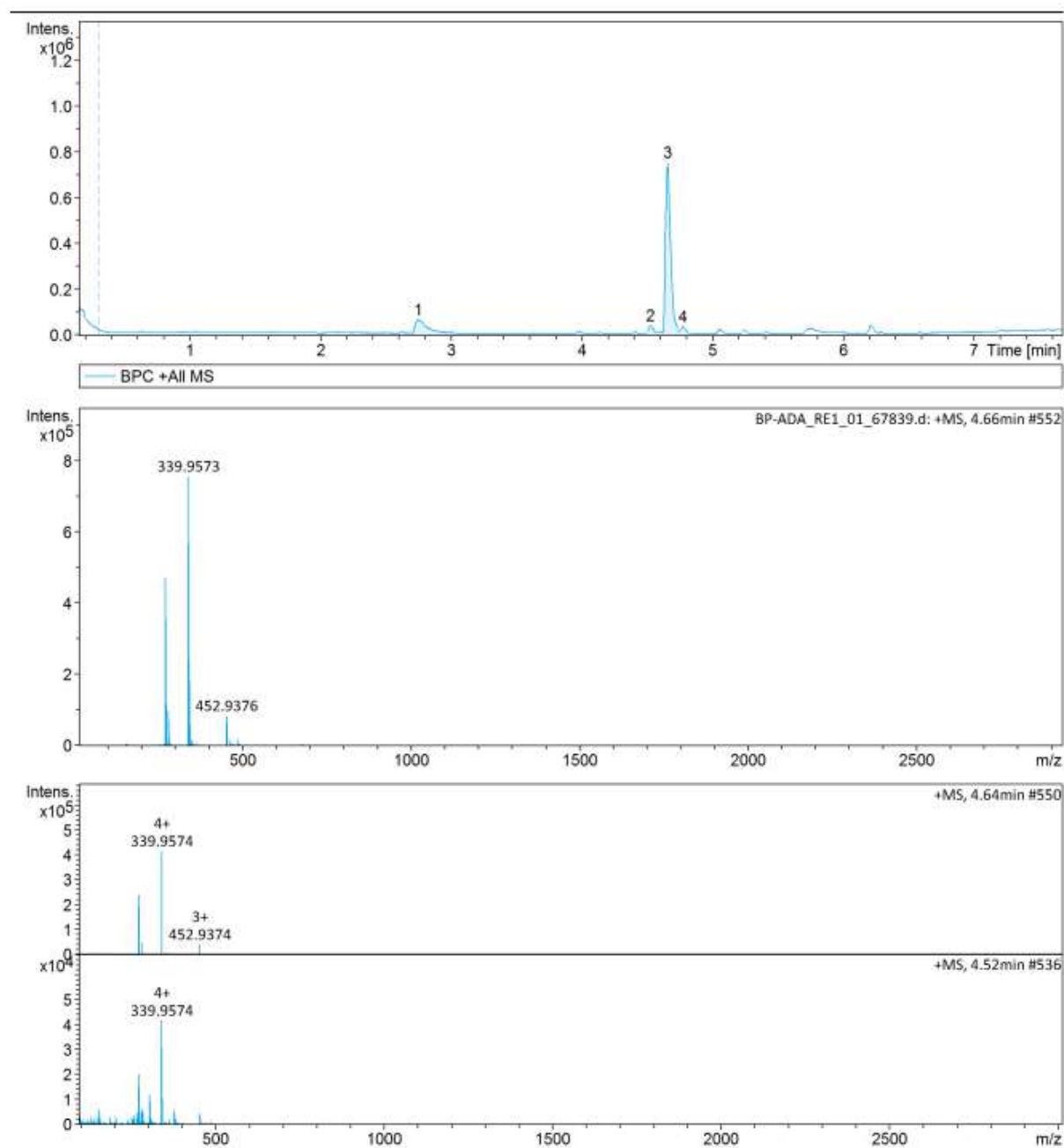


Figure S4.14 LCMS chromatogram of the BP-ada peptide after purification.

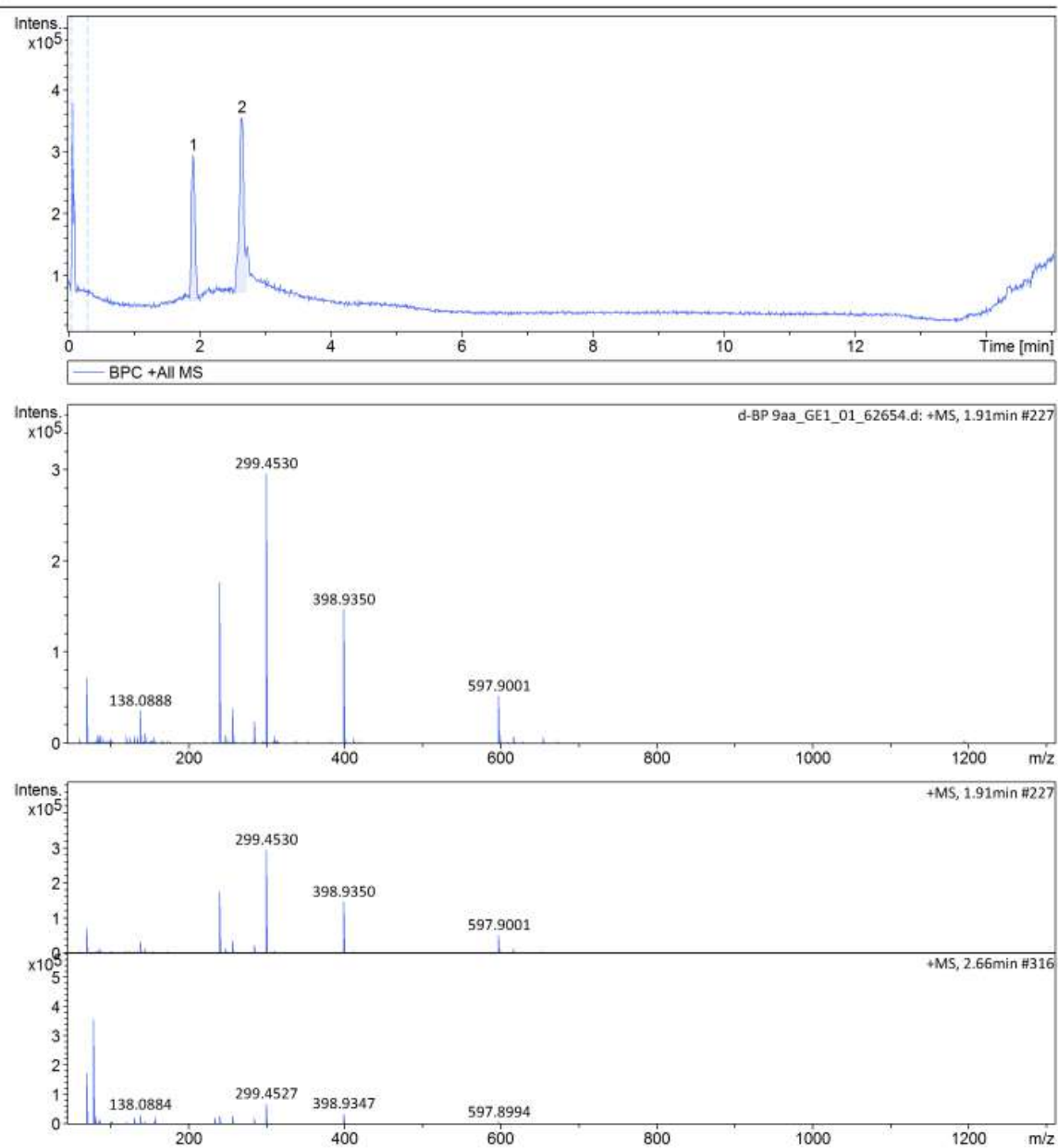


Figure S4.15 LCMS chromatogram of the D-BP peptide after purification.

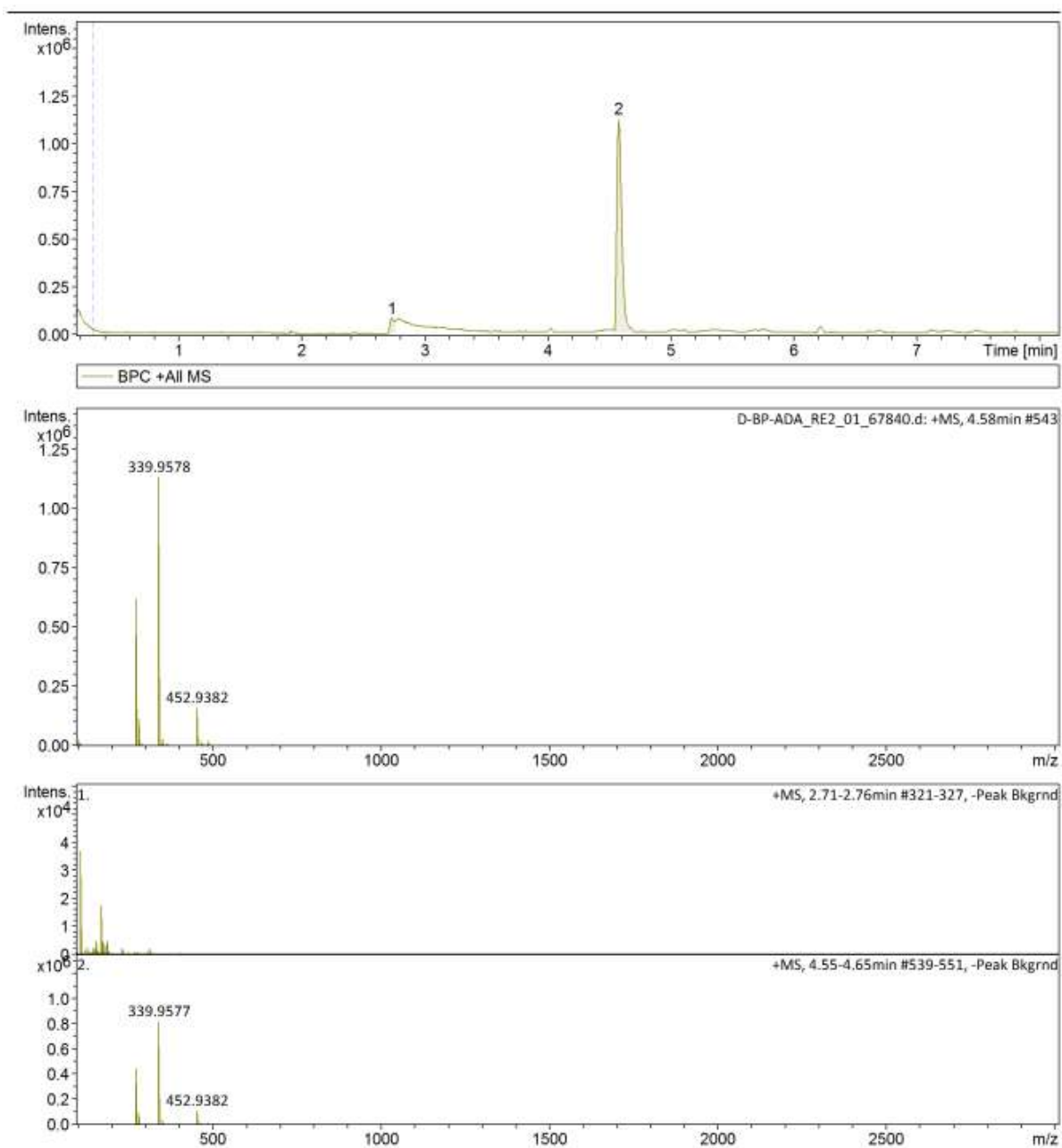


Figure S4.16 LCMS chromatogram of the D-BP-ada peptide after purification.

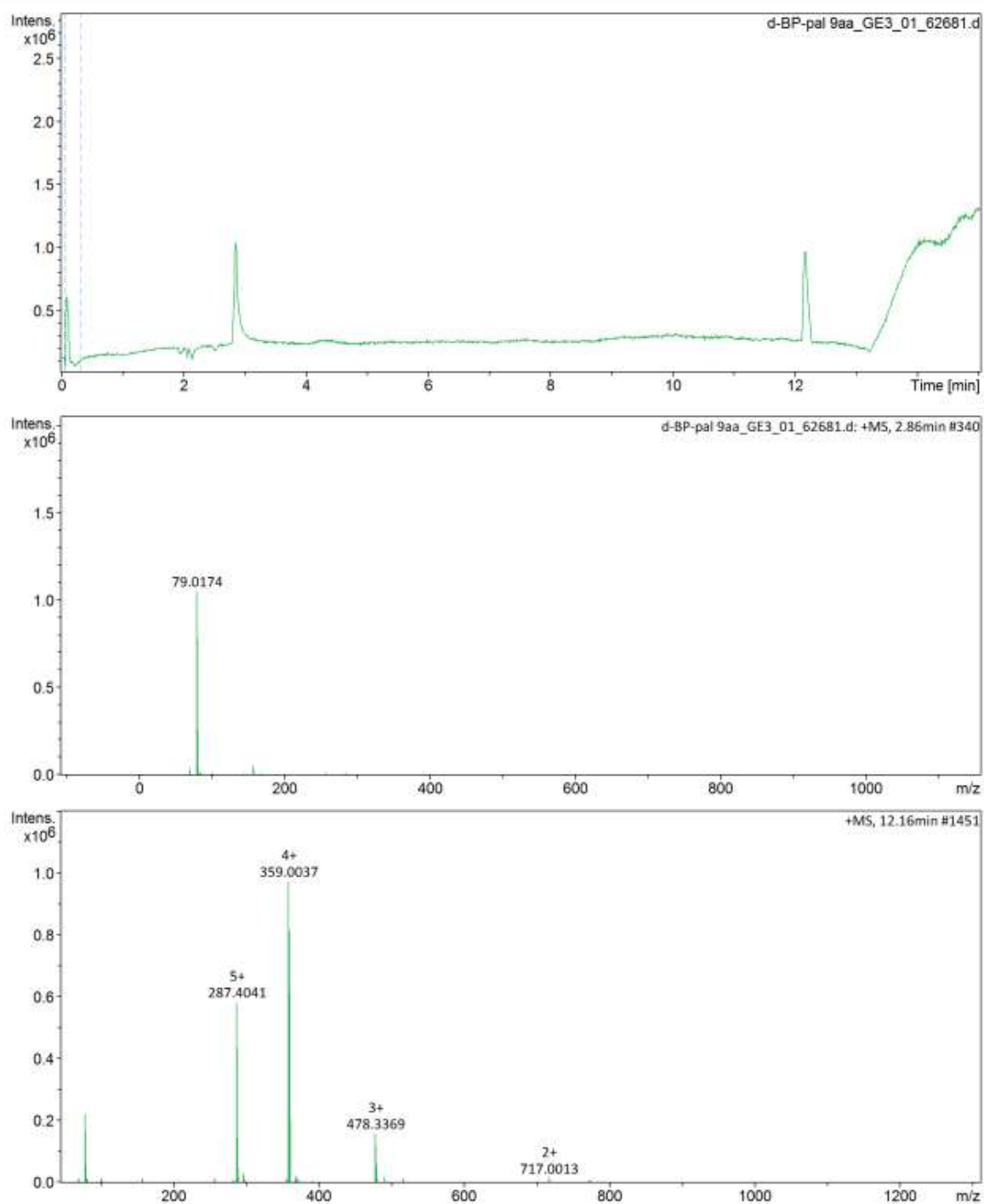


Figure S4.17 LCMS chromatogram of the D-BP-pal peptide after purification.

9.3 Chapter 5

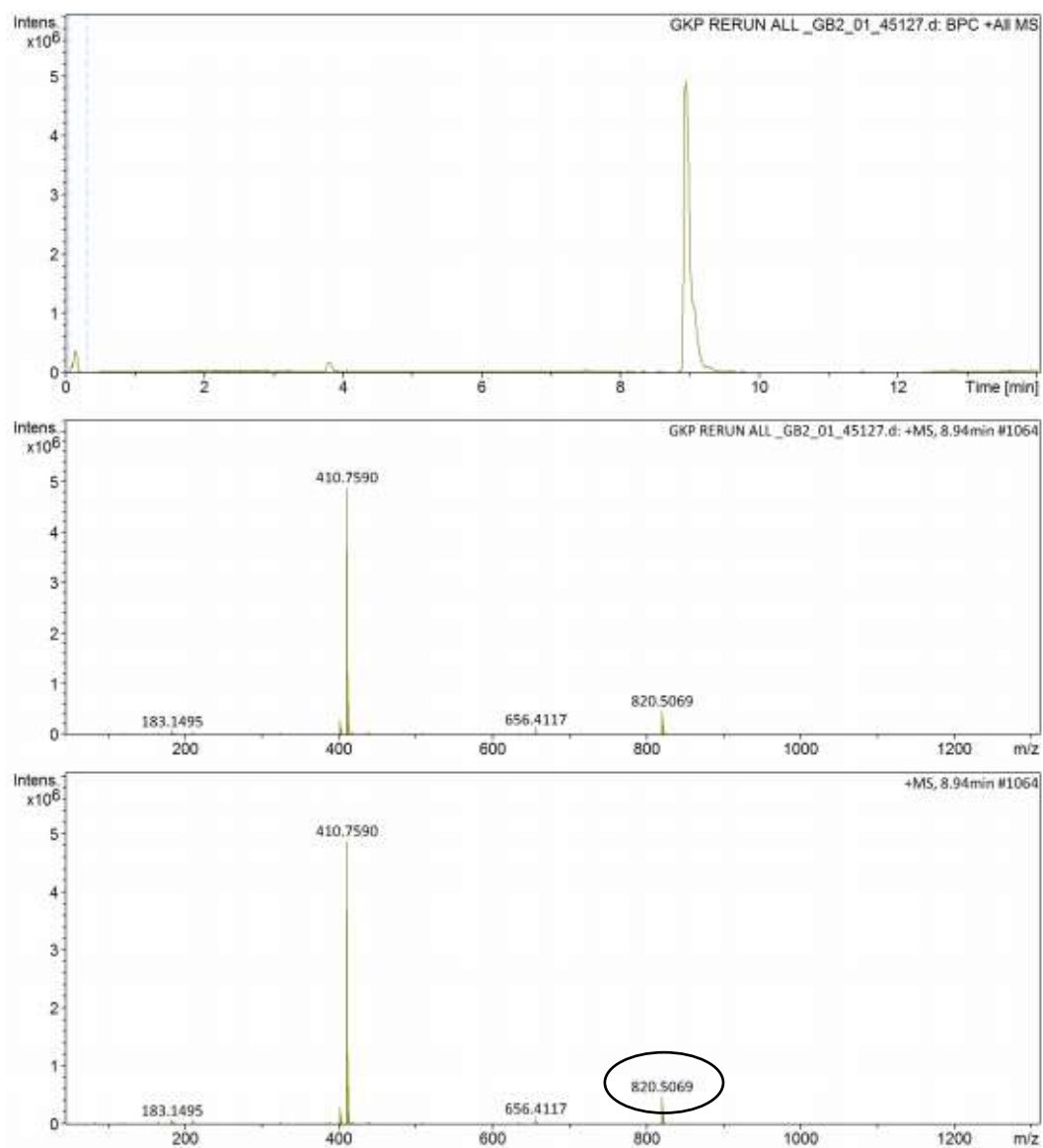


Figure S5.1. LC-MS spectrum of the GKPIILFF peptide with the found mass of $[M+H]^+$ 820,5030 m/z, the main peak coeluted with a mass of 656.4117 $[M+H]^+$

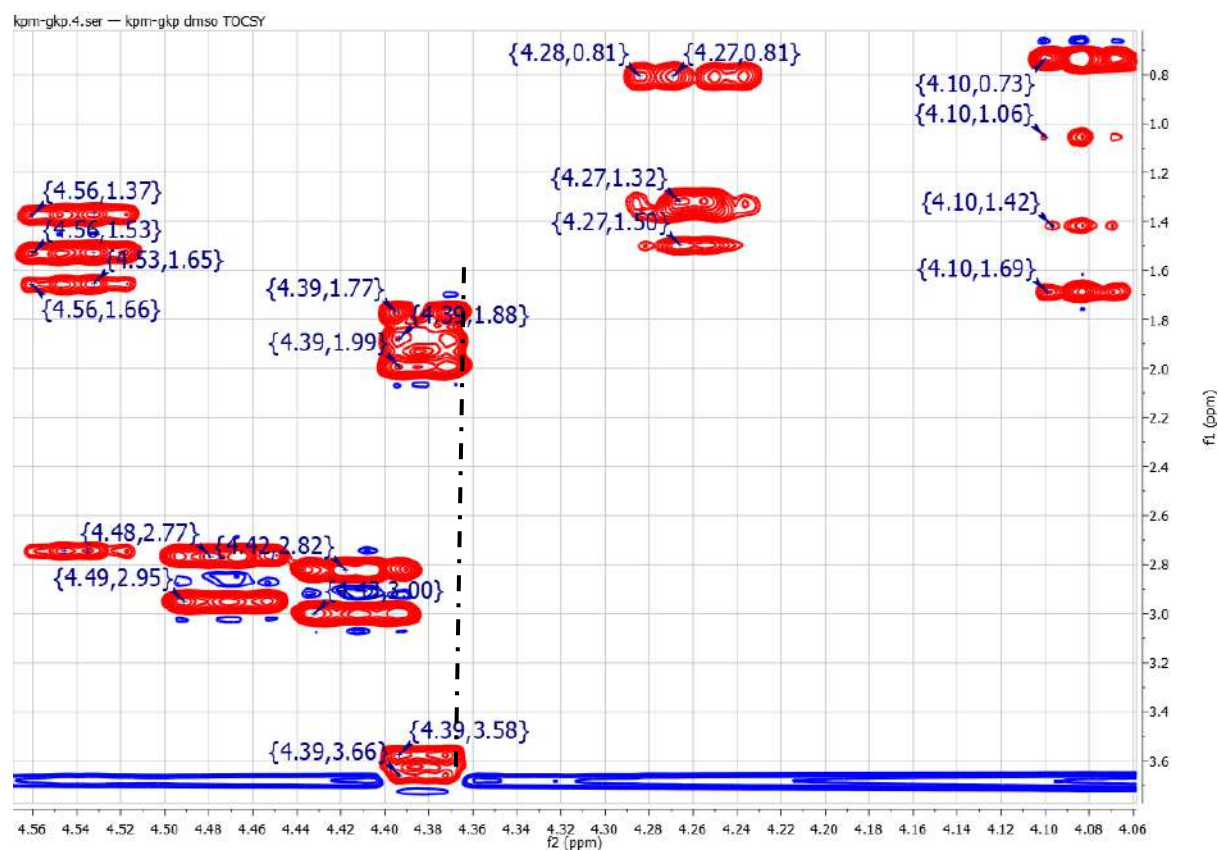


Figure S5.2. Expanded TOCSY NMR spectrum of the GKPILFF peptide showing the proline spin system seen as a dotted line which doesn't appear in the amide region due to the lack of an NH amide proton.

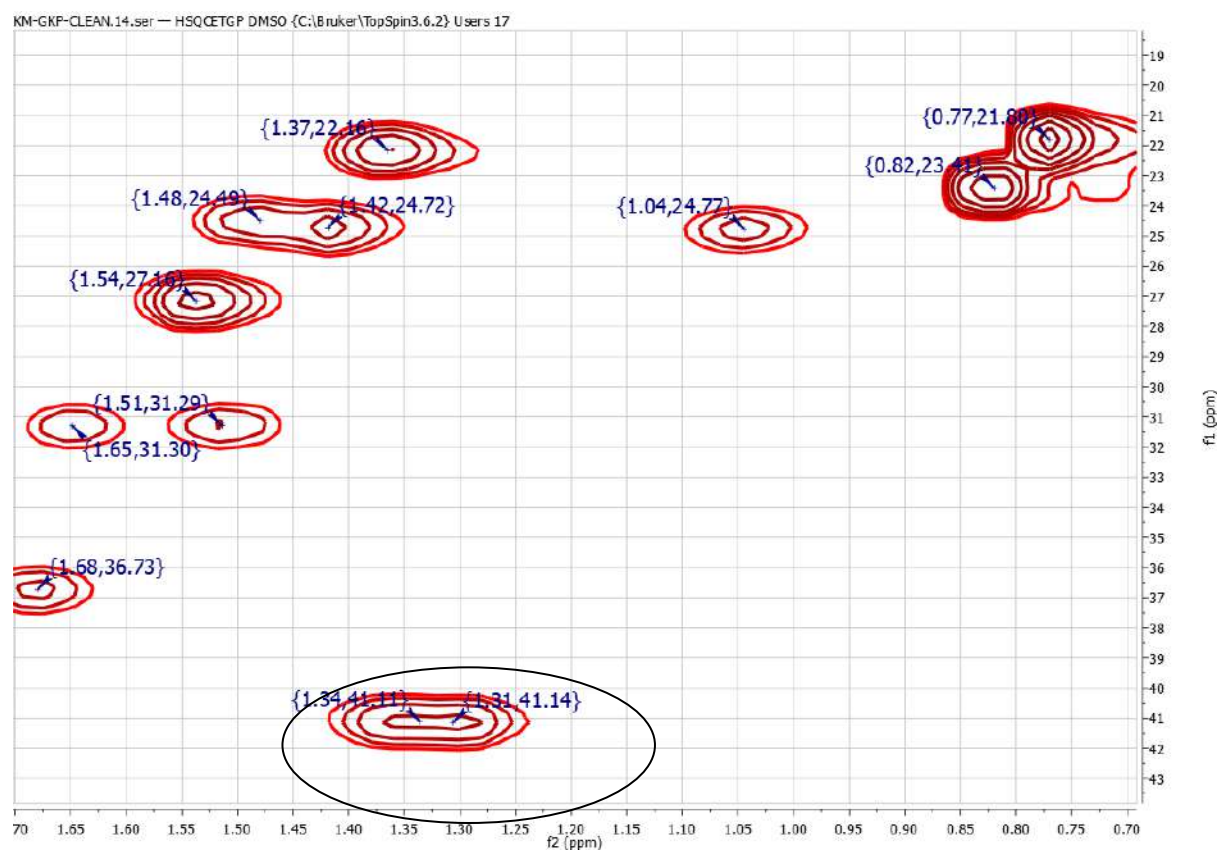


Figure S5.3. Expanded HSQC NMR spectrum of the GKPIILFF peptide showing the leucine CH_2 group which was distinguishable from isoleucine due to its COSY correlation to the alpha proton at 4.26

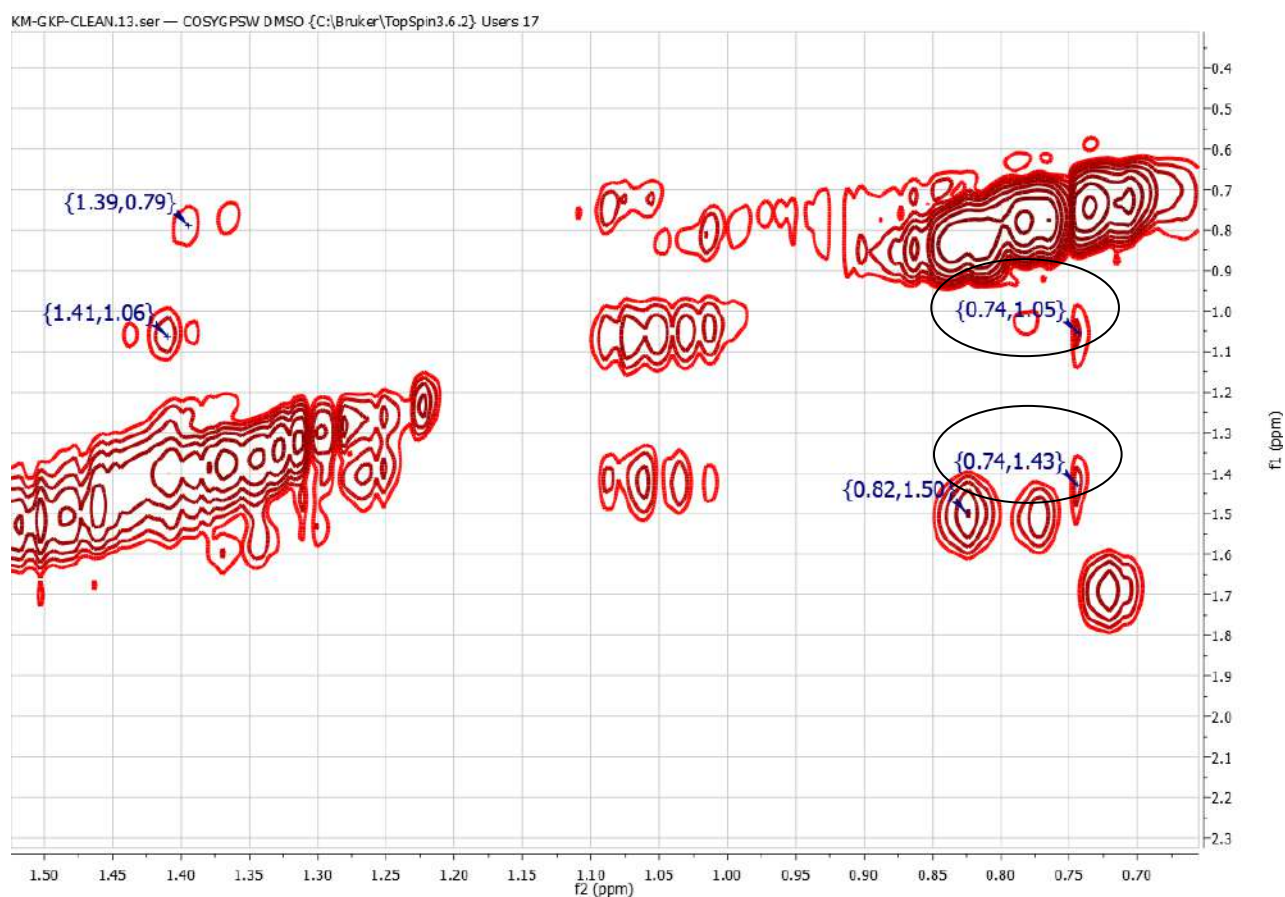


Figure S5.4. Expanded COSY NMR spectrum of the GKPILFF peptide showing the distinct isoleucine methyl protons at 0.74 ppm correlating to the CH₂ protons at 1.05 and 1.43 ppm.

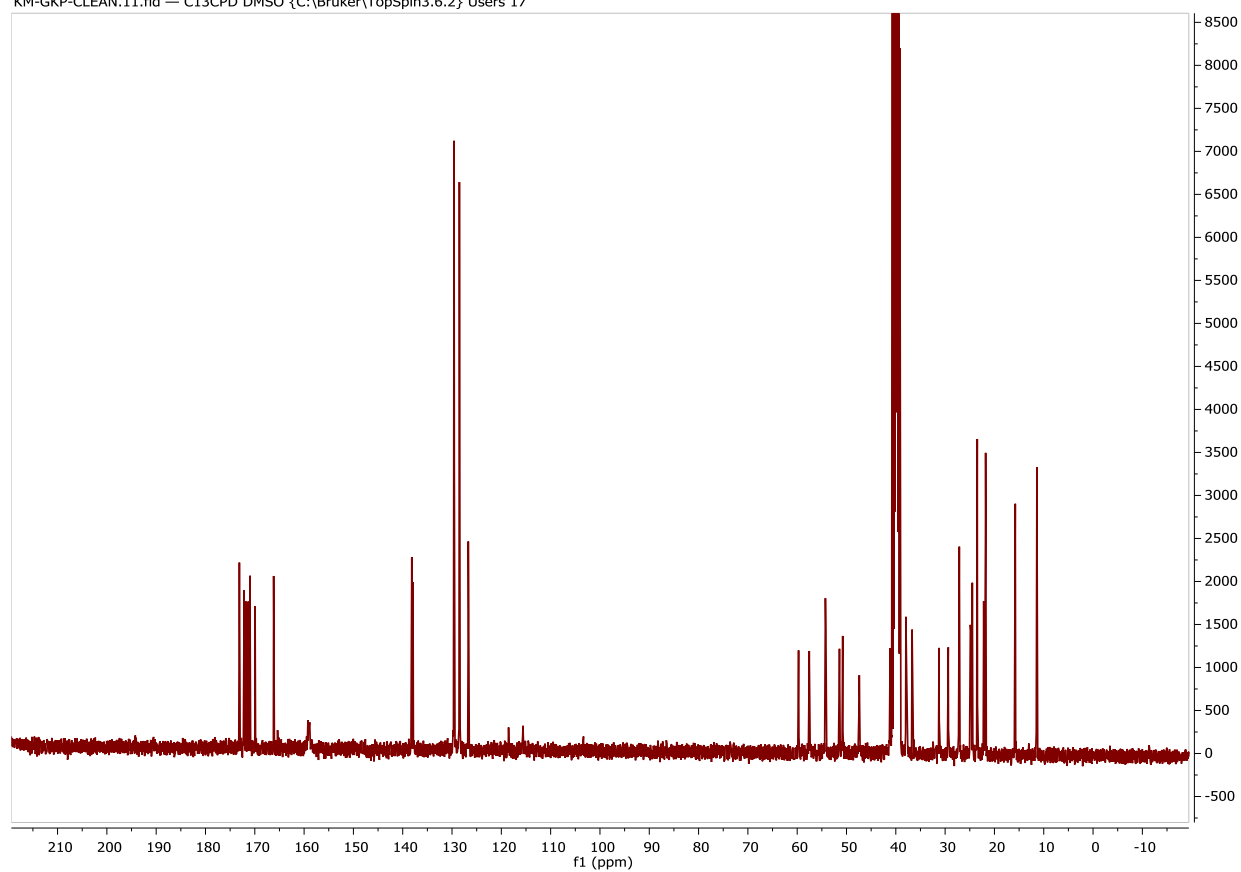


Figure S5.5. Full ^{13}C NMR spectrum of the GKPILFF peptide.

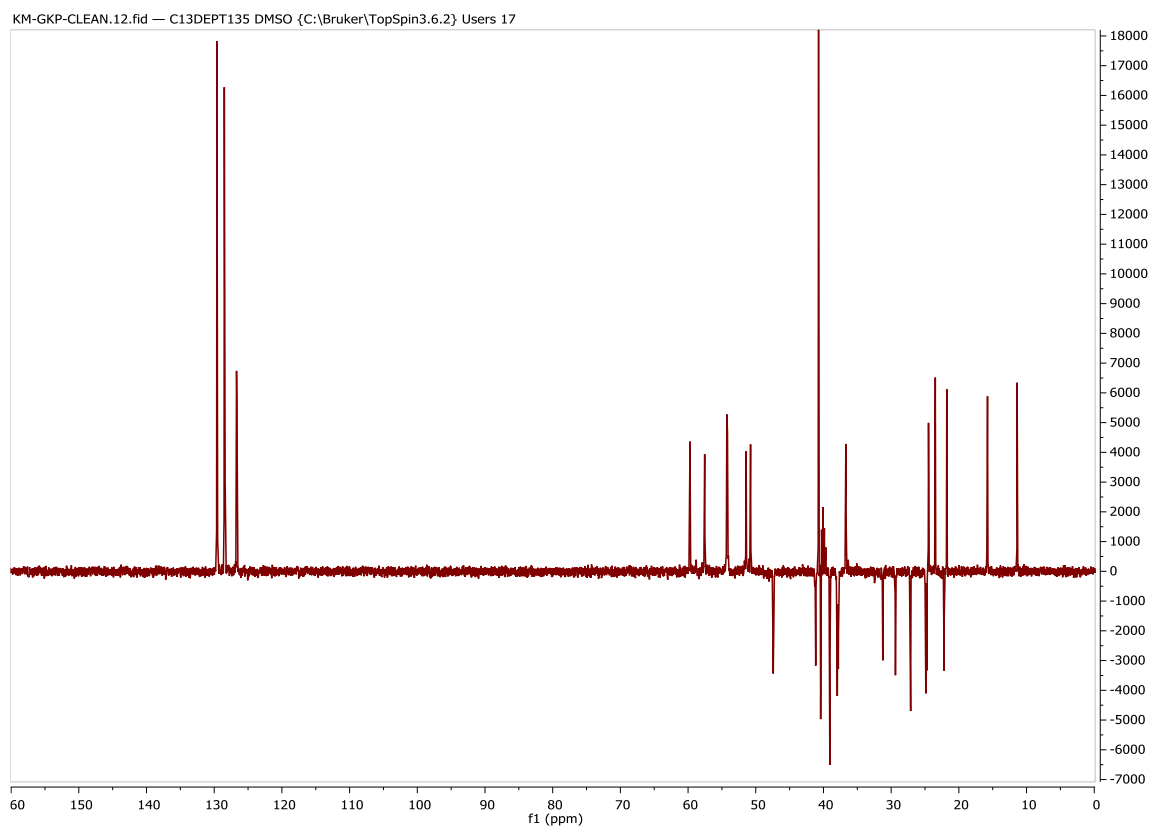


Figure S5.6. Full DEPT NMR spectrum of the GKPILFF peptide.