

**Ph.D. Title: Cyclodextrin Based Polymers for
Advanced Gene and Drug Delivery Applications**

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Ph.D. Degree in Chemical and Material Sciences

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Acknowledgments

I am honoured that this Ph.D. study is carried out from 2019 to 2022 in the group of Polymeric Materials (MP), Department of Chemistry, University of Turin, Italy. It was a great pleasure working in this research group.

Undertaking this Ph.D. has been a truly life-challenging experience both professionally and particularly personally for me, and it would not have been possible to overcome without the support of many people. I would like to express my deepest appreciation to all of those who brought this Ph.D. journey to completion. I am deeply indebted to each person who has provided me, directly or indirectly, extensive and warmly personal and professional guidance and taught me a great deal about both scientific research and life in general. They have shown me, by their examples, how a brilliant scientist and person should be providing unending inspiration. Thank you for understanding the hurdles, for your kindness, and for always being there.

I can never repay the debt I owe you.

Acknowledgments

I would like to express my deepest appreciation, acknowledge my indebtedness and render my warmest thanks to my Ph.D. supervisors, Professor. Francesco Trotta and Dr. Adrián Matencio, and also my fellow supervisor Professor. Parvin Zakeri-Milani, who gave me the golden opportunity to do this interesting work and achieve a higher level of education. their generous guidance, support, persistent help, profound belief in my work, and expert advice have been invaluable throughout all stages of the work.

I would wish to express my gratitude to the doctoral committee Prof. Antonino Mazzaglia, Prof. Claudia Conte, and Dr. Fabrizio Caldera, for manifesting their distinguished skills through the extended discussions, and valuable suggestions which greatly contributed to the improvement of the thesis.

I would like to extend my deepest and specially gratitude to my colleague and friend Dr. Mohammad Mahmoudian, Ph.D. of Nano-Pharmaceutics of Tabriz university of medical sciences; for supporting, encouraging, and for making me feel integrated easily during my Ph.D. journey, for his constant and invaluable support, guidance, mentoring, helpful advice, and feedback on our project's methodology.

Acknowledgments

I am sincerely grateful to Professor. Ken Ken J Ishii and Dr. Tommoya Hayashi from the, University of Tokyo, Japan, for providing valuable feedback for the immunological applications.

I would like to genuinely acknowledge the assistance, the effort, and the big doses of patience of Dr. Fabrizio Caldera, Assistant Professor, for his constant and invaluable support, helpful advice, and feedback starting from my Ph.D. journey, which have been especially beneficial to my professional development experiences.

Special thanks to Dr. Claudio Cecone, Post Doc, for all the priceless support, advice, and assistance received through the collaborative work undertaken, Dr. Alberto Rubin Pedrazzo, Post Doc, and Dr. Gjylje Hoti, Post Doc who gave me a huge hand to deal with the practicalities of working, and develop my technology skills. Many thanks to all for their time, for creating a cordial working environment, for patient support, encouragement, valuable ideas shared, and for all the opportunities to further my research. Their suggestions urged me to do better and to pass the struggles of learning more about polymers. It is immeasurable how much I have learned from them.

Thank you to all our colleagues for their companionship with whom we shared the best moments.

Acknowledgments

I wish to thank to Dr. Mehdi Talebi, Ph.D. of Medical Immunology from department of applied cell sciences, school of advances medical sciences, Tabriz university of medical sciences, Iran, for all of his constant and invaluable supports to carry out the flowcytometry experiments.

Nobody has been more crucial to me in the following of this Ph.D. journey than the members of my family who gave me all they had. I would like to express my deep and sincere gratitude to my family, especially my wife (Soheyla Gholami) and my parents (Abdulkarim and Zarrin), whose unparalleled love, help, support, and guidance are with me in whatever I pursue, and have made me who I am. Thank you very much for always encouraging, believing in me, and pushing me to follow my dreams. My sincere thanks are extended to all my relatives.

DEDICATION

Your words were the sparks that lit up my future. This journey would not have been possible if not for your sacrifice.

*"All my work is dedicated to you, my beloved wife, **SOHEYLA**"*

Abstract

Different problems associated with our current lifestyle needs novel solutions. The increase of life expectancy also gets higher numbers of cancer patients, or the eventual neurodegeneration of population. So that, the demand for material innovations in modern healthcare technology is increasing exponentially as the knowledge of disease pathologies progresses and evolves with years of scientific research. The same diseases that have existed for centuries are better understood today, giving birth to the concept of nanomedicine in different kinds of diseases. For that reason, in this thesis, we have synthesised and characterized different types of CD based polymers as a non-toxic, biodegradable, and biocompatible carriers to highlight the important design parameters that are necessary for the development of safe and effective materials, for using in drug, nutraceuticals, and gene delivery applications as a single therapeutic agent.

Research chapter 2: Synthesis and characterization of cyclodextrin based polymers

We have synthesized different types of β -CD based polymers, and then, we used various characterization approaches including, size exclusion

chromatography, DSC, SEM, DLS, TGA, and FTIR to approve the complex formation of cyclodextrin based polymers. Our results demonstrated that the synthesis of hyperbranched polymers and nanosponges is perfectly reproducible and accurate, giving the same type of material.

Research chapter 3: Biological Applications of β CDNSs and PPoly

Section 1: β CDNS-CDI and PMDA polymers for peptide delivery, Nisin-Z as an example

Proteins and peptides, a macromolecule, have been used widely against bacteria and cancer cells growth because of their high biocompatibility, high specificity, and low toxicity as new therapeutically agents. Curiously, the antimicrobial peptides not only present antibacterial effects but also, they have shown promising anticancer capacity. In this regard, Nisin Z as an antimicrobial peptide (AMP) model is an accepted food additive because of its anti-bacterial ability. Furthermore, this drug would be very useful for intestinal cancers, but the peptide nature would make easier its degradation by digestion procedures, so, its usage is mainly limited by the poor stability and short duration of its antimicrobial and anti-cancer activity. To address these hurdles, nano-carrier-based drug delivery systems

can be developed. For that reason, during this part of the thesis, we have used the β -cyclodextrin (CD)-based NSs(β CDNSs) by reacting cyclodextrin (CD) units with carbonyl-diimidazole (CDI), and pyromellitic anhydride (PMDA) as suitable cross-linking agents for loading Nisin Z to increase its antimicrobial and anti-cancer activity. Firstly, the antimicrobial effect of Nisin-Z free and complexes with β NSs-CDI and PMDA were determined against the Gram-positive (*Staphylococcus aureus*) and -negative (*Escherichia coli*) bacteria. The minimum inhibitory of nisin Z loaded on β NSs formulations was evaluated with agar well diffusion plates. Then, the bactericide concentrations of nisin Z loaded on CD-NS formulations were determined by using microdilution brain heart infusion (BHI) and tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The minimum and bactericide inhibitory values of the Nisin complex with β NSs were potentially decreased against both bacteria, compared with the nisin-free sample, while the nisin complex with β -CD showed lower antibacterial activity. The antimicrobial effect was also demonstrated by free NSs. Furthermore, the total viable counts (TVCs) antibacterial experiment indicated that the combination of nisin Z in both β NSs-PMDA and CDI, especially CDI, can provide a

better conservative effect on cooked chicken meat. Generally, the present study outcomes suggest that the cross-linked β -CD-based NSs can present their own antimicrobial potency or serve as promising carriers to deliver and enhance the antibacterial action of nisin Z.

In the next phase, we aimed to investigate the protective and anti-cancer enhancement effects of Nisin-Z loaded on both β NSs-PMDA and CDI against colon cancer cells (HT-29) and breast (MCF-7) cancer cell lines. The physicochemical properties, loading efficiency, and release kinetics of Nisin complex with nanosponges were studied. Then, tricin-SDS-PAGE electrophoresis was used to understand the effect of NSs on stability of Nisin-Z in the presence of gastric peptidase, pepsin. In addition, the cytotoxicity and cell membrane damage of Nisin Z were evaluated by using the MTT and LDH assay, which was complemented via Annexin-V/ Propidium Iodide (PI) by using flowcytometry. β NSs were able to encapsulated the Nisin-Z with an encapsulation efficiency around 90%. Also, a protective effect of Nisin-Z complexed with β NSs was observed in presence of pepsin. An increase in the percentage of apoptotic cells was seen when the cancer cells were exposed to Nisin Z complexed with nanosponges. Interestingly, Nisin Z free and loaded

on β NSs-PMDA and CDI was more selectively toxic towards HT-29 cells than MCF-7 cancer cells. These results indicated that nanosponges might be good candidates to protect peptides and deliver drugs against intestinal cancers.

After the promising effect of β NSs on enhancing the anticancer capacity of Nisin against in vitro cell lines, we have decided to use those β NSs load Nisin-Z to treat the melanoma cancer animal model. Interestingly, the results showed that Nisin encapsulated in both β NSs (PMDA and CDI-NSs) behaves as an effective antitumor agent by increasing the cytotoxicity, and apoptosis against melanoma cancer cell lines (B16-F10). In addition, Nisin loaded on PMDA-NSs significantly inhibited the in-vivo growth of melanoma cancer in a mouse model compared to free Nisin-Z. Indeed, weight and volume of melanoma tumors were outstandingly decreased in the group treated with Nisin-PMDA-NSs compared to free Nisin. On the other hand, the anti-cancer effects of Nisin related to its apoptosis and anti-oxidant activity were remarkably increased in complex with PMDA-NSs. Moreover, Nisin in nano-formulation led to considerably decrease of CD31, an angiogenesis factor, expression in tumor tissues. Our findings suggest

that incorporating nisin into nanosponges as a safe carrier may allow improving Nisin's anti-tumor effect in melanoma cancer animal models.

Section 2: β CNS- CDI in comparison with HP- β CD for nutraceutical delivery

Although there are several works about the increase of solubility of drugs and nutraceutical with CD-NSs, no one has studied deeply the possible increase of their bioavailability and capacities. For that reason, as an example of the real properties, the oral administration of KYNA complexed with HP β -CD and β NS-CDI was evaluated in mice to check their effects to possible increase of KYNA bioavailability. The solvent-free technology was used to prepare the complexes in a complete comparison between kneading in ball milling and classical inclusion complex preparation. The solvent-free ones showed higher strength and efficiency with ball milling, considerably reducing time. A 50mg KYNA/kg/day dosage was orally administered in formulations showing a higher bioavailability when the nutraceutical was complexed with β NS-CDI compared to HP β -CD and KYNA free, respectively. Several antioxidant statuses demonstrated a higher global antioxidant level perfectly related to bioavailability. Finally, the formulation of KYNA

reduced the temporal oxidative stress damage in the kidney and liver, making β NS-CDI the best formulation. These results suggest an important future application of cyclodextrin-based nanosponges for the oral delivery of nutraceuticals and their stabilization.

Section 3: Hyper-branched cationic cyclodextrin polymers for pDNA and mRNA delivery

Gene therapy has been considered as a new way to change the vital cellular pathways that led to various diseases, such as cancer and inherited disorders. The genetic data of the so-called backbone sequences of a plasmid may not be essential, but it is at least beneficial for the cells to survive in their current environment. Plasmids have emerged as one of the most crucial tools in genetics, molecular biology, and, more recently, in gene therapy and genetic immunization in medicine over the past few decades. On the other hand, recently, messenger RNA (mRNA) injections have been introduced as one such treatment that provides low mutational risk, good safety, and adaptive modularity to express a variety of diverse therapeutically useful proteins, so that research in molecular medicine now focuses on mRNA. This is especially true when it comes to immunization. Many

Abstract

mRNA vaccines, including those for cancer immunotherapy and those against viral infections, have entered clinical trials. The success of mRNA-based therapy has been demonstrated in numerous applications, including gene editing, protein replacement therapy, cancer immunotherapy, and immunization against the SARS-CoV-2 virus. As a results, many groups started working on plasmid DNA and mRNA for vaccines, cancer immunotherapies, and immune interventions for various diseases. Despite all the advantages of nucleic acid therapy, their use in intact form did not show promising results due to their instability and degradation in existing enzymes. So that, over the past decades, viral vectors have been used as the gold standard vehicle to transfect the genes with the high level of expression. While, the safety issues have been remained as a major concern due to their ability to induce significant immunogenicity. But, recently, instead of viral vectors, the non-viral carriers such as inorganic nanoparticles (NPs), liposomes, and cationic polymeric NPs have attracted the scientific community due to their capacity for large-scale easy production with less immunogenicity and their ability to transfer the large DNA plasmids and RNAs. Meanwhile, their low transfection efficiency has been suggested as the main problem of

these systems. Therefore, in this study we have aimed to address this problem by using the new modified cationic hyper-branched cyclodextrin-based polymers (PPoly) to deliver both pDNA and mRNA. In the first step, we used the 2D and 3D multicellular spheroid model to increase the understanding of the gene delivery process of new modified cationic hyper-branched cyclodextrin-based polymers loaded plasmid encoding Enhanced Green Fluorescent Protein (EGFP). The gel retardation assay illustrated that PPoly loaded pDNA were effectively retarded at N/P ratio 25:1. Furthermore, PPoly loaded pDNA were taken up more than 80% and 20% by cells in 2D and 3D cell culture models, respectively, meanwhile, pDNA cellular uptake at both 2D and 3D cells was better in complexes with PPoly compared to lipofectamine after 24h. Interestingly, the results of the histopathology confirmed the ability of our PPoly to penetrate into and allow the transfection of a 3D spheroid. Furthermore, cytotoxicity (MTT and LDH) assay outcomes showed that our NPs were non-cytotoxic at a defined N/P ratio (25:1), specifically in 3D spheroid culture, and hemolysis results revealed that the complexes of PPoly and pDNA were hemocompatible when exposed to the red blood cells of rats with less than 4% lysis rate. Although the quantification data of cellular uptake illustrated that the

EGFP expression in 3D spheroid cells transfected by PPoly-pDNA complexes at a determined ratio was lower than in 2D cells, this phenomenon may be due to the extracellular matrix (ECM) and high cell density multicellular barriers in the deep regions of the 3D spheroids, which inhibit the gene delivery agents' penetration inside the spheroid cells. Taken together, thanks to our NPs properties; biocompatibility, hemocompatibility and considerable cellular uptake to transfect the pDNA inside the cells, might be considered as an efficient and promising platform for gene delivery in 2D and 3D mediums.

After demonstrating that Ppoly can be effectively complexed with pDNA, and it could improve remarkably the transfection efficiency of pDNA in both 2D and 3D cell line, we decided to evaluate also use of Ppoly to deliver the mRNA due to its different biochemical composition and structure. Therefore, we have used the Ppoly to increase the mRNA delivery in both in-vitro and in-vivo melanoma cancer. Here, we have shown that the transfection efficacy of the mRNA-EGFP-loaded PPoly system markedly was improved compared to lipofectamine and free-mRNA in both 2D and 3D melanoma cancer cells. After that, the mRNA-EGFP was injected intra-tumoral to check its local delivery in induced melanoma cancer mice, the results illustrated time depending

and higher of EGFP expression in complexes with PPoly compared to free-EGFP. Then, the anti-tumor effect of intratumorally injected oval albumin mRNA (mOVA), as a foreign antigen, loaded on Ppoly through inducing the immune system responses against melanoma cancer model was checked; the results showed a significant decrease in both tumor size and weight in group treated with mOVA in complexes with Ppoly compared to other formulations. Finally, we observed that mOVA complexes with Ppoly strategy caused an efficient adaptive immune response by dramatically increasing the majority of leukocyte subtypes and OVA-specific CD8⁺ T cells in both spleen and tumor tissues in vivo, in comparison to untreated groups. Additionally, mOVA in formulation with Ppoly has significantly increased the humoral immune responses in mice with induced melanoma tumors. These facts were taken into account in the strategy, which resulted in an efficient anti-tumor immunity against melanoma cancer and significant inhibition of tumor growth (3 times reduction vs. control). Our findings suggest that the polymeric conjugates bearing OVA-mRNA, a foreign antigen, may be innovative and promising cancer immunotherapeutic agents.

Abstract

To sum up, this Ph.D. research introduces the extensive literature about the synthesis, characterization, and multi-biological applications of the cyclodextrin-based polymers delivery system that will further contribute to the delivery of drug and genes with more potent materials based on these results.

Keywords: Cyclodextrin-polymers, Cyclodextrin-based Nanosponges, Transfection, Plasmid eGFP, Spheroid 3D, DAPI, mRNA-eGFP, mRNA-OVA, nisin Z; Nisin-Z stability; cyclodextrin; pyromellitic dianhydride; carbonyldiimidazole; nanosponges; *Staphylococcus aureus*; *Escherichia coli*; antibacterial, anti-cancer; MCF-7; HT-29, Melanoma animal cancer, Kynurenic acid; nutraceutical.

CURRICULUM VITAE (CV)



PERSONAL DETAILS



16/08/1990



Male



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Delfan, Iran



Persian and English

Educations

- **Ph.D. student at Chemical and Material Sciences, Turin University, Italy**
- **Master of Medical Biotechnology** (2014-2017), Qazvin University of Medical Science, Iran
- **Bachelor's degree in Medical Laboratory of Sciences** (2010-2014), Lorestan University of Medical Science, Iran

Honored and Awards

- Ph.D. full-scholarship of Turin University. 2019
- Ranked Top 1 percent in Iran's nationwide B.Sc. entrance exam in 2010
- The youngest researcher of the Ministry of Health and Medical Education, Iran. 2017

Professional experiences

- ❖ Medical Laboratory technician (2014-2015), Erfan Hospital, Tehran, Iran
- ❖ Medical Laboratory technician (2015-2019), Pasteur Hospital, Qazvin, Iran
- ✓ I was appointed as a lab technician in different sections including Biochemistry, Microbiology, Pathology sample preparation, and Hematology & Blood Banking.

Projects

- Investigation of the novel positive cyclodextrin-based nanosponges' potential for using as a vaccine delivery carrier to mRNA delivery by in-vivo/ in-vitro against melanoma cancer

CURRICULUM VITAE (CV)

- Plasmid-based gene delivery using positive cyclodextrin-based nanosponges in 2D/3D spheroid cell culture models
- Investigation of the pharmacokinetic parameters of orally-delivered Kynurenic acid in mice model
- Investigation of the in vitro/in vitro anti-cancer effects of the nisin free and complexed with the cyclodextrin-based nanosponges
- Anti-bacterial effects of the nisin free and complexed with the cyclodextrin-based nanosponges

Skills

- **Analytical techniques:** Flow cytometry, Fluorescence microscopy, HPLC, UV-spectrophotometry, FT-IR, DSC, and DLS.
- **Animal studies;** Animal handling and anesthesia, Oral gavage for PKs study, Melanoma mice induced cancer model, Rat fibrosis model, mice abdominal surgery and autopsy, Blood collecting, Isolation the single cells suspension from tumor and spleen for checking the immune system responses

CURRICULUM VITAE (CV)

- **Cell culture;** Stem cell isolation and culture, 3D cell culture, conventional 2D culture
- **Medical laboratory experiences:** Real-Time PCR, Western blotting, bioinformatics for designing the primers, ELISA
- **Nonpharmaceutical;** Preparation and characterization of Nano drug delivery systems
- **Gene delivery;** Plasmid, *CRISPER/Cas9*, and mRNA delivery based Cyclodextrin-Nanosponges
- **Protein analysis;** SDS-page electrophoresis, Bradford's assay, Nanodrop
- **Immunofluorescence analysis;** CD marker analysis, Cytokine analysis, Annexin V/PI staining
- **Bacterial assay;** Agar plate, MIC, MBC, CFU
- **General computer knowledge:** RStudio software Primary, FlowJo, Origin Pro, Auto dock Vina, Spss, GraphPad Prism, ImageJ, and Microsoft Office

- **Primary bioinformatic skills including:** Primer design, RNA-Seq analysis with CLC Genomic Workbench 20, analysis of Whole genome Sequencing

Books

Farzam, S.A, **Khazaei Monfared,Y**, Mohamadi, M, Khodabandehloh, E, Pourali, M,Hosseini, S.M, Khodadadi, A,. (2019) Pharmaceutical Biotechnology, Fundamentals and Applications, Knowledge Mehregan. (Translated to Persian language)

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Publications of Ph.D. journey

Yousef Khazaei Monfared, Mohammad Mahmoudian, Fabrizio Caldera, Alberto Rubin Pedrazzo, Parvin Zakeri-Milani, Adrián Matencio and Francesco Trotta (2022). **Nisin Delivery by Nanosponges Increases Its Anticancer Activity Against *In-vivo* Melanoma Model.** *Journal of Drug Delivery Science and Technology.* <https://doi.org/10.1016/j.jddst.2022.104065>

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2. Babak Pakbin, Abdollah Didban, **Yousef Khazaye Monfared**, Razzagh Mahmoudi, Amir Peymani and Mohammad Reza Modabber (2021). Antibiotic susceptibility and genetic relatedness of Shigella species isolated from food and human stool samples in Qazvin, Iran. BMC Research Notes, BMC Res Notes (2021) 14:144 <https://doi.org/10.1186/s13104-021-05554-3>.
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In Preparation

Francesco Trotta, Mohammad Mahmoudian, Parvin Zakeri-Milani, Claudio Cecone, Ken J Ishii, Tomoya Hayashi, Joao Conde, Adrián Matencio, and Yousef Khazaei Monfared* (2023). **Therapeutic anti-tumour immunotherapy vaccine against melanoma via *in vivo* delivery of mRNA hyper-branch cyclodextrin nanoparticles.** **Nature Nanotechnology (Preparation)**

Conference presentations

YUSEF KHAZAEI MONFARED, Adrián Matencio, Niles K. Dhakar, Alberto Rubin Pedrazzo, Fabrizio Caldera, Francesco Trotta, An Increase Of Kyna Solubility And Capacities By Cyclodextrin Based-Nanosponges And Cyclodextrins Monomers, 43rd Annual Meeting Of The Spanish Society Of Biochemistry & Molecular Biology, 2021.

Gjylje Hoti, Fabrizio Caldera, Claudio Cecone, Alberto Rubin Pedrazzo, Adrián Matencio Durán, Silvia Lucia Appleton, **Yousef Khazaei Monfared**, Francesco Trotta, Dextrin-Based Nanosponges As An

Effective Approach For Drug Delivery Applications. 43rd Annual Meeting Of The Spanish Society Of Biochemistry & Molecular Biology, 2021.

Adrián Matencio, Silvia Navarro-Orcajada, Gjylje Hoti, Irene Conesa, Silvia Lucia Appleton, **Yousef Khazaei Monfared**, Fabrizio Caldera, Francisco García Carmona, José Manuel López-Nicolás, Modulation of The Phosphodiesterase Activity And Lifespan In *Saccharomyces Cerevisiae* By Different Stilbenes And Roflumilast. 43rd Annual Meeting of The Spanish Society Of Biochemistry & Molecular Biology, 2021.

Khazaei Monfared. Y., Farzam. SA., Moghbelinejad. S., Modabber.MR., Gholami S. Survey on effect Saffron as an antioxidant on sperm parameters in the men by normal spermatogenesis after freezing and thawing process, International Conference on Pharmaceutical Chemistry, July 16-17, 2018 at Paris, France.

Khazaei Monfared. Y., Sarookhani. MR., Farzam SA., Foroughi. F., miR-135a as a biomarker for predicting Pre-Diabetes and type 2 Diabetes New, International Conference on Pharmaceutical Chemistry, July 16-17, 2018 at Paris, France.

Ph.D. SCHOOLS

- ✓ Green Chemistry Postgraduate Summer School-Ca' Foscari University of Venice, Venice (Italy), 6-10 July 2020.
- ✓ V International Summer School on Cyclodextrins, Advances in cyclodextrin chemistry and applications, Ascea (Italy), 4 – 7 September 2022.

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List of Abbreviations

ATR	Attenuated total reflectance
APC	Antigen presenting cells
AUC	Area under the curve
BHI	Brain heart infusion
Bax	BCL2 associated X protein
BCL2	B-cell lymphoma 2
CDs	Cyclodextrins
α -CD	α -Cyclodextrin
β -CD	β -Cyclodextrin
γ -CD	γ -Cyclodextrin
CE	Complexation efficiency
CDI	1, 1'-carbonyldiimidazole
CDNSs	Cyclodextrin-nanosponges
CHO	Choline chloride
CD	Cluster of differentiation
CTLs	Cytotoxic T lymphocytes
CFU	Colony-forming units
CLSI	Clinical and Laboratory Standards Institute
C-6	Coumarin-6
DSC	Differential scanning calorimetry
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DMF	N, N- dimethylformamide
DPC	Diphenyl Carbonate
DAPI	4', 6-diamidino-2-phenylindole
DC	Dendritic cells
DS	Degree of substitution
D/CD	Drug/Cyclodextrin complex
DL	Drug Loading
EPI	Epichlorohydrin
EGFP	Enhanced Green Fluorescent Protein

List of Abbreviations

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent
ECM	Extracellular matrix
EE	Encapsulation Efficiency
EMSA	Electrophoretic mobility shift assays
FTIR	Fourier-transform infrared
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FACS	Fluorescence-activated Cell Sorting
GSH	Glutathione
GPx	Gutathione peroxidase
GRAS	Generally recognized as safe
HPaCD	Hydroxypropyl α -Cyclodextrin
HP β CD	Hydroxypropyl β -Cyclodextrin
HDI	Hexamethylene diisocyanate
IR	Infrared spectroscopy
IFN	Type I interferon
ISGs	IFN-stimulated genes
IFN- γ	Interferon gamma
IHC	Immunohistochemistry
IgG	Immunoglobulin G
IVT	In vitro transcription
KYNA	Kynurenic acid
Log	Logarithm
LDH	Lactate dehydrogenase
MS	Molar degree of substitution
MTT	3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide
mRNA	Messenger RNA
MDA5	Melanoma differentiation-associated protein 5
MICs	Minimal inhibitory concentrations
MBCs	Minimum bactericidal concentrations
MDA	Malondialdehyde

List of Abbreviations

M β CD	Maltosyl- β -Cyclodextrin
MHC	Major histocompatibility
MHA	Mueller–Hinton agar
N/P	Nitrogen/Phosphate
NBF	Neutral buffered formalin
NADES	Natural deep eutectic solvents
NK	Natural killer cells
OAS	2',5'-oligoadenylate synthetase
OVA	Oval albumin
PdI	Polydispersity index
PVA	Polyvinyl alcohol
pDNA	Plasmid DNA
PBS	phosphate-buffered saline
PPoly	Purified polymer
PKR	Protein kinase receptor
PE	Phycoerythrin
PI	Propidium iodide
PEI	Polyethylenimine
RM β CD	Randomly methylated β -Cyclodextrin
RBCs	Red blood cells
RIG-I	Retinoic acid-inducible gene 1
SBE β CD	Sulfobutylether β -Cyclodextrin sodium salt
S _t	Total solubility of the drug
SEM	Scanning electron microscopy
S.C.	Subcutaneously
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
TDI	Toluene-2, 6-diisocyanate
TGA	Thermogravimetric analysis
TAE	Tris-acetate-EDTA
TLRs	Toll-like receptors
TNF- α	Tumor necrosis factor

List of Abbreviations

TVCs	Total viable counts
TMD	Tumor microvessel density
TAS	Total antioxidant status
UV	Ultraviolet-visible spectroscopy
2D	Two dimensional
3D	Three dimensional



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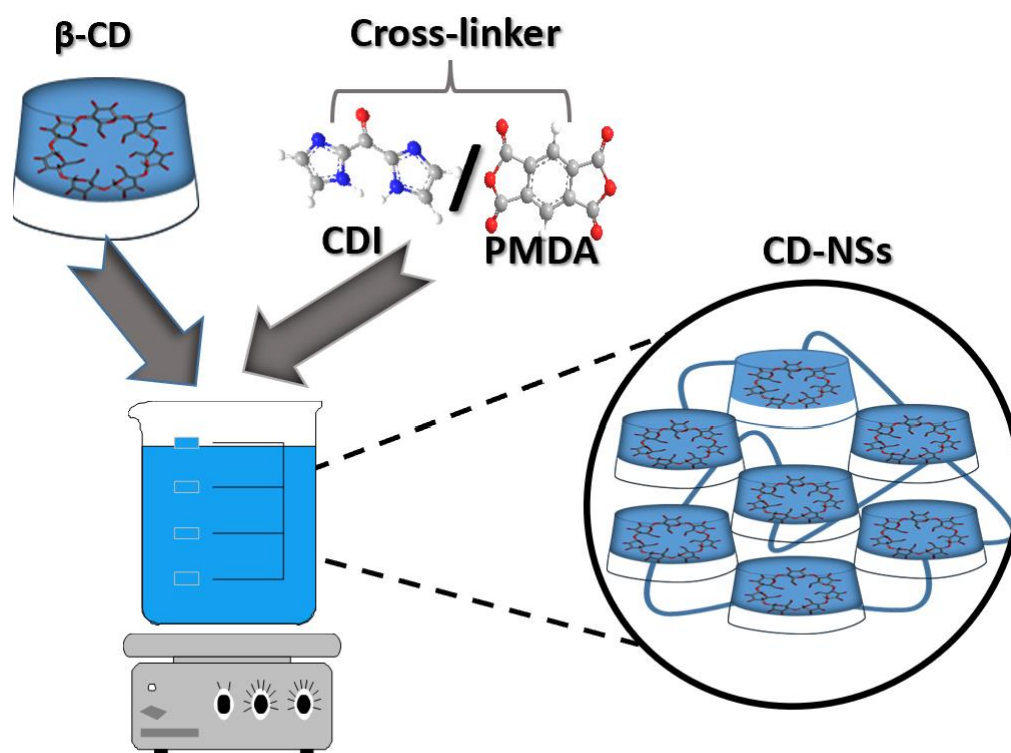
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Chapter 1

Literatures Review

1. Introduction of Cyclodextrin based Polymers



1.1. Cyclodextrins

Cyclodextrins are nanometric biomaterials whose molecular state and supramolecular characteristics are closely correlated[1, 2]. Chemically, CDs are truncated, cone-shaped oligosaccharides made up of α -(1,4)-linked glucose units, obtained by the degradation of starch by the enzyme cyclodextrin glucosyltransferase (CGTase)[3, 4]. The three most prevalent natural cyclodextrins are α , β and γ , each of which contains six, seven, or eight glucopyranose units, respectively (Table.1). They have a distinctive toroidal shape that creates a distinct lipophilic chamber with the shape of a truncated cone[5]. Compounds whose shape and polarity are compatible with that of their cavities can be included in cyclodextrins. Inclusion complexes with some compounds, such as hydrophilic or high-molecular-weight drugs, cannot be formed by natural cyclodextrins. Additionally, β -CD is the cheapest form of CDs, has a limited water solubility (1.85 percent w/v at 25 °C) and is hazardous when administered intravenously[3, 4, 6]. The Food and Drug Administration (FDA) lists cyclodextrins as generally recognized as safe (GRAS) substances (FDA). Studies on toxicity have demonstrated that cyclodextrins taken orally are not hazardous since they are not absorbed by the gastrointestinal tract [7]. The main purpose of the cyclodextrin complexation process is to change a drug's solubility. When a medication forms a compound with cyclodextrin, its solubility substantially alters[8]. Host-guest connection or host-guest complex are other names for inclusion complex. The hydrophobic moiety acts as a guest and the cyclodextrin's internal hydrophobic cavity functions as the host. A hydrophobic moiety of the appropriate size is partially or completely encapsulated by the cavity. Cyclodextrins can include molecules of any size or polarity that are compatible with their lipophilic inner cavity. Certain hydrophilic substances or big molecules cannot be incorporated into native cyclodextrins.

Three distinct eras may be identified in the history of CDs: (1) the invention (1891–1930); (2) the development (1930–1970); and (3) the usage in industry (1970-onwards)[9, 10] (Figure 1).

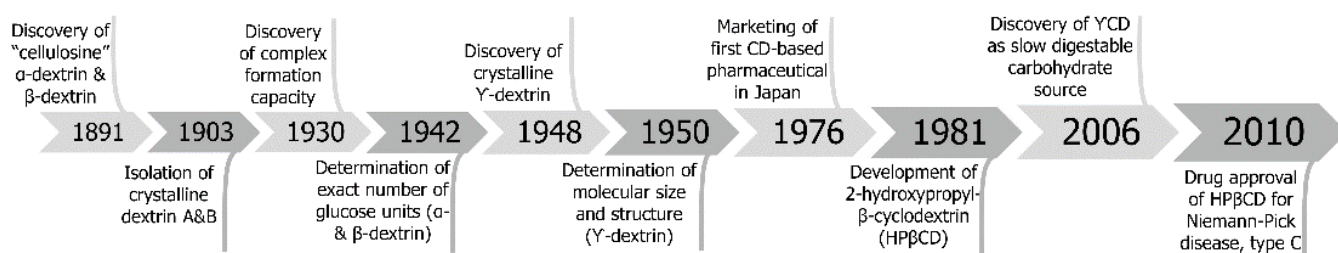


Figure1. Milestones of the research history of CD.

CDs are cyclic oligosaccharides with Low molecular weight (between 973 and 2163 Da) and can be fabricated in three steps: (1) bacterial fermentation and CD glycosyltransferase extraction by a particular microbe, like as *Bacillus macerans*; (2) enzymatic starch degradation via cyclodextrin (CD)-glycosyltransferase; and (3) precipitation of CD through complexation. Due to steric constraints, these cyclic oligomers have six (α-CD), seven (β-CD), eight (γ-CD), and more D-glucose units. These units are connected by -(1,4) glycosidic connections to form rings, as seen in Figure 2.

β-cyclodextrin (β-CD) has the lowest water solubility among all CDs, because of intramolecular hydrogen interactions that develop between the C2-OH group and the C3-OH group of nearby glucose units[11, 12], blocking hydration of the CD molecule. In the crystal state of β-CD, the binding of the CD molecules is relatively strong and exhibits a relatively high crystal lattice energy[13]. In pure aqueous solution, the half-life ($t_{1/2}$) for

the ring-opening of CD is thought to be 15 hours at 70 °C and a pH of 1.1. While α CD's half-life is 1.5 times more stable than γ -CD's, β -CD's half-life is 1.5 times less stable [4].

Table 1. Characteristics of the natural cyclodextrins α -CD, β -CD, and γ -CD.

Property	α -CD	β -CD	γ -CD
Glucose units	6	7	8
Molar mass (g/mol)	972	1135	1297
Height (Å)	7.9	7.9	7.9
Internal diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3
External diameter (Å)	14.6	15.4	17.5
Cavity volume (Å ³)	174	262	427
Water solubility (g per 100 cm ³) at 25 °C	14.5	1.85	23.2
Shape of crystals	Hexagonal lattice	Monocyclic parallelograms	Quadratic prism
Density, [g/cm ³]	1.48	1.44	1.52
pKa by potentiometry (25 °C)	12.3	12.2	12.1
Diffusion constant at 40 °C (m/s)	3.4	3.2	3.0
Hydrolysis by α -amylase	Negligible	Slow	Fast

1.1.1. Cyclodextrin derivatives

The CDs' primary and secondary hydroxyl groups can be randomly substituted to achieve the enhancements in the CDs' solubility. CD derivatives are produced when methyl, ethyl, carboxymethyl, hydroxyethyl, hydroxypropyl, sulfobutyl, or saccharide groups are substituted for CDs. Methoxy functionalities and other hydrophobic moieties may potentially aid in these advancements. CD derivatives of importance to the pharmaceutical industry include the hydroxypropyl derivatives of α -, β - and γ -CD (HP α CD, HP β CD, and HP γ -CD), randomly methylated CD (RM-CD), sulfobutylether CD (SBE β CD), and the so-called branched CDs like maltosyl-CD (M β CD). Figure 3 presents the distribution of some common CDs that can be found in marketed pharmaceutical products, and Table 2 presents their characteristics.

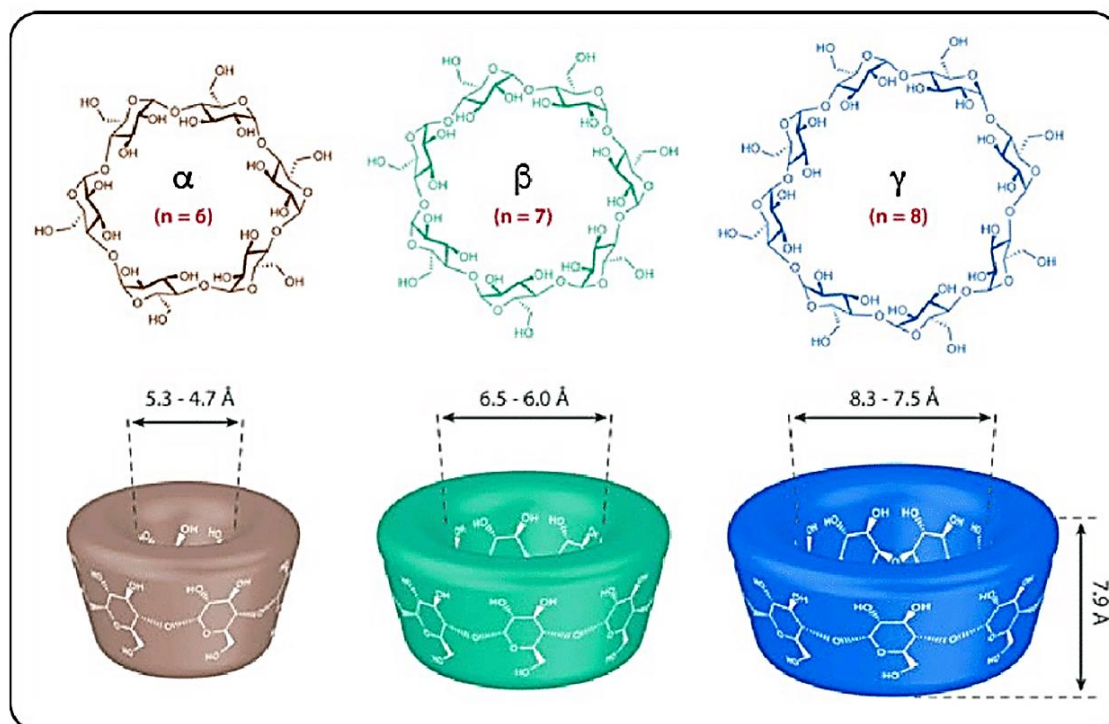


Figure 2. Chemical structure of the three main types of cyclodextrins (α , β , and γ).

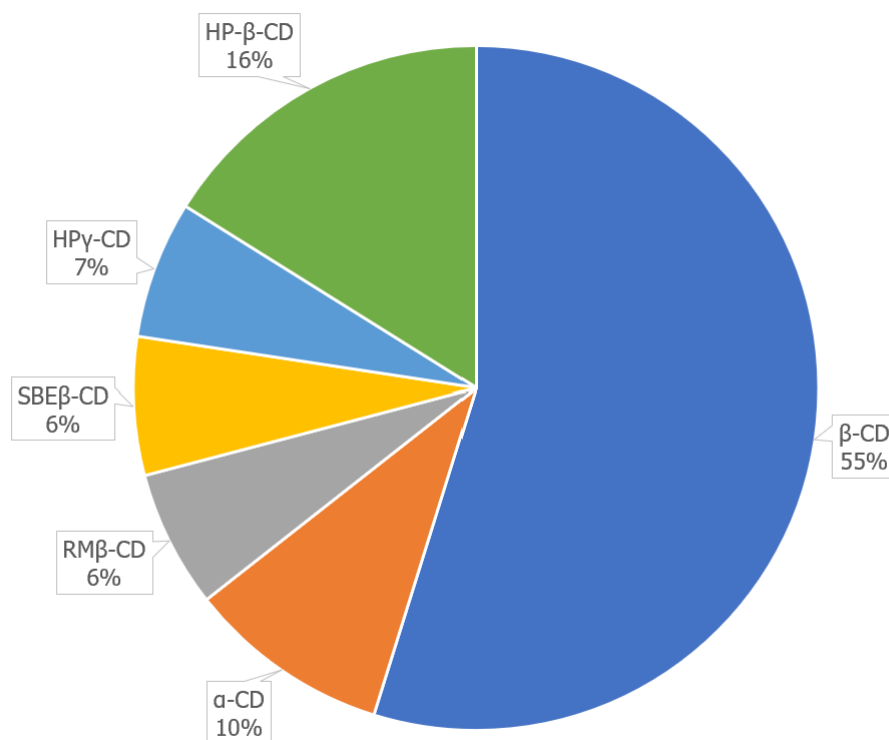


Figure 3. The distribution of CDs found in marketed pharmaceutical products[14].

Table 2. Characteristics of some common CDs found in marketed pharmaceutical products.

Cyclodextrin	Trade name and manufacturer	MS	MW (Da)	Solubility (mg/mL)	LogK _{o/w}
HP α CD	Cavasol W6 HP (Wacker, Germany)	0.65	1199	>500	<-10
HP β CD	Kleptose HPB (Roquette, France)	0.65	1400	>600	-8.5
HPYCD	Cavasol W8 HP (Wacker, Germany)	0.6	1576	>600	<-10
RM β CD	Kleptose Crysmeb (Roquette, France)	0.57	1191	200	
M β CD	Ensuiiko Sugar Refining Co., Japan	0.14	1459	>500	<-10
SBE β CD	Captisol (CyDex Pharmaceuticals, USA)	0.9	2163	>500	<-10

All of the factors including; the structure of the appended substituent, its position inside the CD molecule, and the quantity of substituents per CD molecule can affect the physicochemical characteristics of the CD derivatives. The term "molar degree of substitution" refers to the typical number of substituents that have interacted with one glucopyranose repeat unit (MS). The range of the MS value is 0 (no substitution) to >3 (reaction of two or more substituents to form oligomeric or polymeric side chains).

As a result, a polymeric side chain can be created when the electrophile reacts with the hydroxyl groups of the substituents. The degree of substitution (DS) in the study of carbohydrates is the quantity of substituted hydroxyl groups per anhydroglucose unit, with values ranging from 0 (no substitution) to 3 (when three hydroxyl groups are substituted). Contrarily, the DS in CD chemistry is a representation of the average amount of substituents in a CD molecule[13].

Cyclodextrin complexes

The lack of covalent bonds and the presence of a driving force that releases enthalpy-rich water molecules from the cavity section make up the complexation's mechanism. Non-inclusion complexes are created when the hydroxyl groups located on the outside of

the CD molecule establish Van der Waals, hydrophobic, and hydrogen bond interactions with the target drug. CDs have been demonstrated to participate in inclusion complexes, with the exception of the many kinds of non-inclusion complexes containing organic salts and polymers that are water soluble. The drug/CD inclusion combination can improve the drug's physical and chemical stability, water solubility, and capacity to cross biological membranes. Some hydrophobic CDs derivatives restrict the dissolution speed of water-soluble drugs, much as CDs and their derivatives are employed to increase the solubility or dissolution rate of weakly soluble drugs[9, 12, 15-17]. Hydrophobic Van der Waals type interactions will keep hydrophobic guests inside the CD cavity while the creation of hydrogen bridges between polar guests and the primary hydroxyls simultaneously establishes polar connections[4, 18, 19].

The CD complex (D_mCD_n) formation is a reversible process:



where m drug molecules (D) associate with n CD molecules to form a complex of m: n stoichiometry (Figure 9). The stability constant of the complex, or the binding constant can be written as follows:

$$K_{m:n} = \frac{[D_mCD_n]}{[D]^m[CD]^n} \quad \text{Equation 2}$$

The driving mechanisms for complex formation include electrostatic interactions, Van der Waals contributions, hydrogen bonds, the release of structural strain, and charge-transfer interactions. The water molecules in the cavity have lower hydrogen bonds while having high energy or enthalpy content. Despite the fact that the removal of water molecules

from the cavity is associated by a negative enthalpy change, the total process's free energy change is not always negative. In an aqueous medium, the water molecules that inhabit the slightly apolar CD cavity are energetically undesirable (apolar-polar interaction). As a result, "guest molecules" that are less polar than water can easily replace the cavity (Figure 4). The dissolved CD serves as the "host" molecule, whilst the "driving force" is the appropriate guest molecule replacing the high-enthalpy water molecules. Small circles, which stand in for the water molecules, are attracted to the host (lipophilic cavity of the CD) and the guest (hydrophobic p-xylene), respectively.

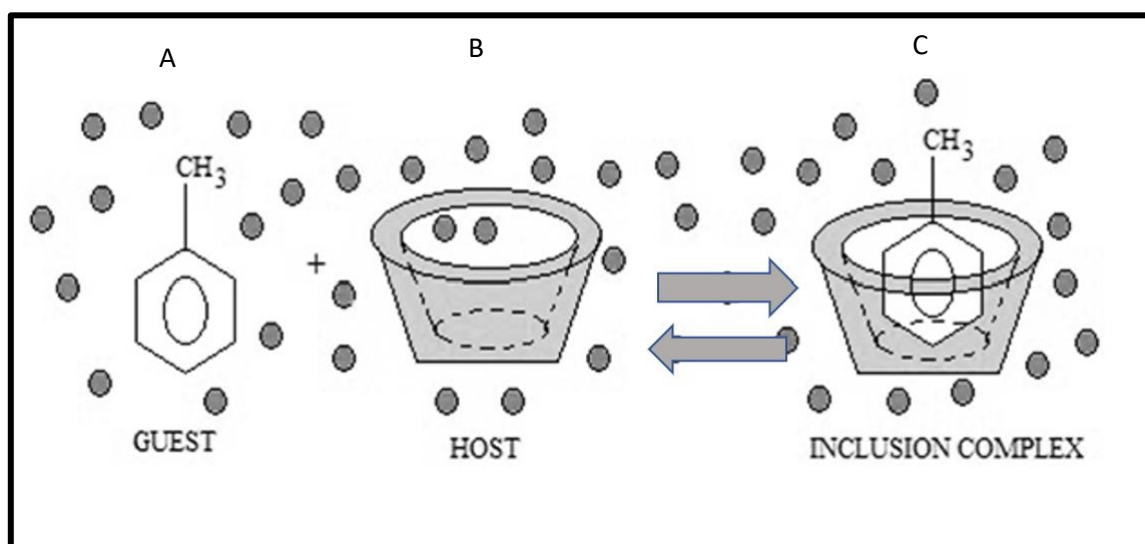


Figure 4. Schematic representation of the driving forces to promote the inclusion complex formation of CD as a host with the guest[20].

Different strategies to form complexes

Complexes containing CDs can be produced in solid, semi-solid, or liquid phases. Depending on the types of the guest and the type of CD used, there are several ways to create cyclodextrin-guest complexes. The kneading approach is thought to be appropriate and straightforward for weakly water-soluble guests because the guest is gradually dissolved during the development of the complex. First, the liquid or dissolved solid is put

to a slurry of CD and mixed in an extruder or a mortar (on a laboratory scale) (on large scale). The paste is dried, and the resulting solid is rinsed with a little solvent to get rid of the loose particles that have adsorb on the surface of the CD, and then it is dried in a vacuum. This method's drawback is that it's unsuitable for large-scale preparation. Co-precipitation is an additional technique for non-water-soluble compounds. The guest is dissolved in a variety of organic solvents before being mixed with the appropriate quantity of CD aqueous solution. After the solution has cooled, the complex crystals form, are washed with an organic solvent, and dried at 50 °C. The solid inclusion complex is recovered from the saturated aqueous solution using the co-precipitation technique. It is based on phase solubility and unfit for large-scale preparations. The amounts of CD and guest utilized can be calculated using the Bs-type phase-solubility diagrams. CD and the guests are dissolved in heated water, allowed to cool gently, and then the resulting powder is filtered out and dried. By sealing in a container, the physical mixture of the active substance and the CD that has absorbed a specific amount of water vapor, a crystalline inclusion compound is produced. The method can be employed for thermostable volatiles because it is carried out at temperatures between 43 and 142 °C under nitrogen gas pressure. For guests that are water-soluble or thermolabile, the freeze-drying or lyophilization method is appropriate. The CD and the guest molecule are dissolved in water while being stirred in the necessary proportions, and the solution is then freeze-dried. The solution (CD and drug) is first frozen, and then dried under decreased pressure to remove the solvent. The resulting powder is dried in a vacuum before being cleaned with an organic solvent. The inclusion complex has a high yield and is easily scalable. The freeze-drying method is frequently used to generate CD inclusion complexes, particularly hydroxypropyl-CD inclusion complexes. Another method for

making the CD and guest molecule complexes is the spray-dryer. The intake temperature and sample feeding rate are controlled while using the spray dryer. Since temperatures between 50 and 70 °C are employed to dry the mixture of CD and guest molecules, the molecules used must be thermostable. We can use a microwave oven to create the drug and CD complexes. This new technique for industrial-scale preparation uses microwave irradiation. The better yield of the product and the quicker reaction times (between one and two minutes at 60 °C) are its main benefits. Whatman filter paper is used to separate the product from the remaining uncomplexed free drug and CD before it is dried at 40 °C under vacuum. Numerous approaches are applied to prove the formation of inclusion complexes and to investigate the interaction between CD and a guest molecule, including visible and ultraviolet (UV) spectroscopy, phase solubility, differential scanning calorimetry (DSC), infrared spectroscopy, vacuum methods, X-ray diffraction, chromatography, mass spectrometry, nuclear magnetic resonance spectroscopy, fluorescence spectroscopy, and optical methods [9, 21, 22]. Additionally, they have a number of restrictions, including the impossibility to include certain hydrophilic chemicals or high molecular weight drugs[23], low water solubility, and toxicity in the case of β -CD when injected intravenously[24-26]. Consequently, specialized applications call for the chemical modification of CD structures to get over the aforementioned restrictions [5, 27]. Create proper CD-based delivery systems that can enhance the qualities of a drug or nutraceutical is appealing for various uses, especially in the pharmacy.

1.2. Cyclodextrin-based polymers

Chemical functionalisation is a popular strategy for enhancing parent CDs' performance[28]. The reactive hydroxyl groups (2-, -3, and 6-OH) in CDs could be changed by substituting either the hydrogen atom or the hydroxyl group with a number

of other substitution groups. The most reactive groups are 2,6-OH. Two or more CD units that are covalently bonded together are formed as CD-based polymers (Figure 5). The manufacture of CD-based polymer can increase the use of CDs in pharmaceutical development. The fundamental synthesis techniques are condensation, dehydration, and deprotonation. An anion that exhibits SN2-type polymerizations is created when an acidic OH group reacts with a strong base (e.g., the reaction of the CD-ring with epichlorohydrin). Through the dehydration process, CDs interact with diol or diacid in sulphuric acid solution to make polyethers and polyesters. Condensation is the result of CDs reacting with a bi-functional linker (e.g., diisocyanate), CD polymers can be water-soluble or insoluble depending on the reaction and substituents. This has a far higher solubility than the original CD, particularly in the case of polymers based on β -CD. The reactivity of the cross-linking agent, reaction duration, and substituents all affect solubility [20]. The most popular cross-linkers are citric acid, epichlorohydrin, and pyromellitic dianhydride [29]. There are several ways to make soluble CD polymers, including grafting CDs onto already synthesised polymers, polycondensing difunctionalized CD monomers generated by selectively altering the CD secondary hydroxy groups, and preventing gel formation in a sol-gel cross-linking reaction. In contrast to less polar solvents like acetone, ethanol, ethyl acetate, and ethyl ether, the soluble polymers are found to be insoluble in some aprotic organic solvents like dimethyl sulfoxide (DMSO) and N, N-dimethylformamide (DMF). The soluble polymer has a molecular weight between 2000 and 30,000 Da [29]. When more CD rings are joined together, the insoluble polymers are created. This is called nanosponges (NSs) and feature a 3D network structure that is heavily cross-linked. These polymers are very hydrophilic and exhibit substantial aqueous system swelling [30, 31].

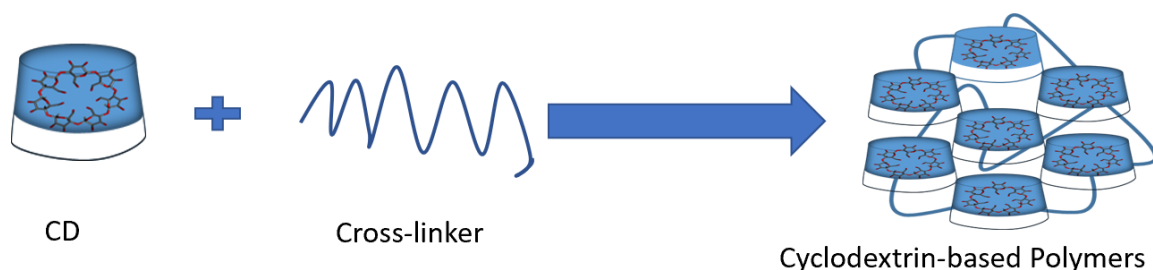


Figure 5. Schematic formation of CD-based polymers.

1.2.1. The rationale for choosing cyclodextrin-based polymers

Cyclodextrins (CD), the monomer unit, is a cyclic oligosaccharides containing, glucopyranoside monomeric units linked via α -(1, 4)-glycosidic bonds with the central lipophilic cavity and outer hydrophilic surface [32], their application in the pharmaceutical and nutraceutical industries and similar fields has been significantly raised[33-35]. In addition, the natural CDs have been considered as “Generally recognized as safe” (GRAS) [36]. However, they could not be directly interacted with the molecules due to their weak interactions with these agents [37, 38]. Thus, modifying the CD structure would be necessary to increase their capacities and abilities for the pharmaceutical application. To follow this purpose, in this thesis, we have synthesized the CD based polymers including; β NS-CDI, β SN-PMDA, and Ppoly to compare and test their reproducibility and better capacities for our selected molecules, in these cases the nucleic acid, peptide, and nutraceutical agents.

1.3. Nanosponges

Numerous chemical modifications of cyclodextrins have been investigated in an effort to get around their drawbacks and enhance their technical properties. Making cross-linked cyclodextrin-based polymers to create insoluble multifunctional cyclodextrin derivatives is an alternative strategy [39-41]. Native cyclodextrins can be used to create these polymers by reacting them with a cross-linking agent, which, once the reaction is complete, exerts its own properties and affects the behaviour of the cyclodextrin unit. Sub-microscopic particles with cavities a few nanometers broad make up the class of hyper cross-linked polymer-based colloidal structures known as nanosponges[27, 42]. At the moment, CDs are widely employed in a variety of items, including some medical ones, textiles, clothing, and various cosmetics [43]. One of the suggested method in literature for synthesising cyclodextrin polymers is utilising cross-linking agents [27]. Multiple cyclodextrins cross-link with the linkers to form CD-NS, which combines them. They are called "cyclodextrin nanosponge (CD-NS)" because their inner central chamber functions as a sponge's pore and can contain things [44, 45]. The name "cyclodextrin nanosponges" was initially coined by De Quan Li and Min Ma in 1998 to describe the insoluble cyclodextrin polymers produced by parent cyclodextrin reactions with cross-linkers such as epichlorohydrin, dialdehydes, diacyl chlorides, epoxides, etc[46]. Through inclusion and non-inclusion complexation, CD-NS accommodates poorly soluble compounds with extremely high efficiency[47, 48]. The capacity to include a wide range of chemicals is made possible by the existence of hydrophilic channels in the porous structure of CD-NS and lipophilic cavities in the cyclodextrin monomers [49]. In addition, different ratios of CD to cross-linker can be used to create polymers with different drug loading capacities

and desirable release profiles[50, 51]. When dispersed in water, NS have the unusual ability to create nanosuspension. While giving a particular truncated cone shape, monomeric cyclodextrin in chair conformation is nevertheless unable to form inclusion complexes with several hydrophilic or high molecular weight drugs[42]. They are highly reactive substances with two electrophilic sites that can interact with the cyclodextrins' nucleophilic hydroxyl group. Thus, cross-linkers aid in cyclodextrin's creation of a nanosponge[11, 48, 52]. The cyclodextrin polymer's solubility behaviour is influenced by the cross-linkers' own characteristics. It is possible to generate cyclodextrin polymers that are either water soluble or water insoluble by carefully regulating the reaction conditions[16].

1.3.1. Synthesis of CD-NSs

Numerous techniques, including the melting method, solvent method, ultrasound-assisted synthesis, microwave-assisted synthesis, and emulsion solvent diffusion method are used to create NSs [64, 88].

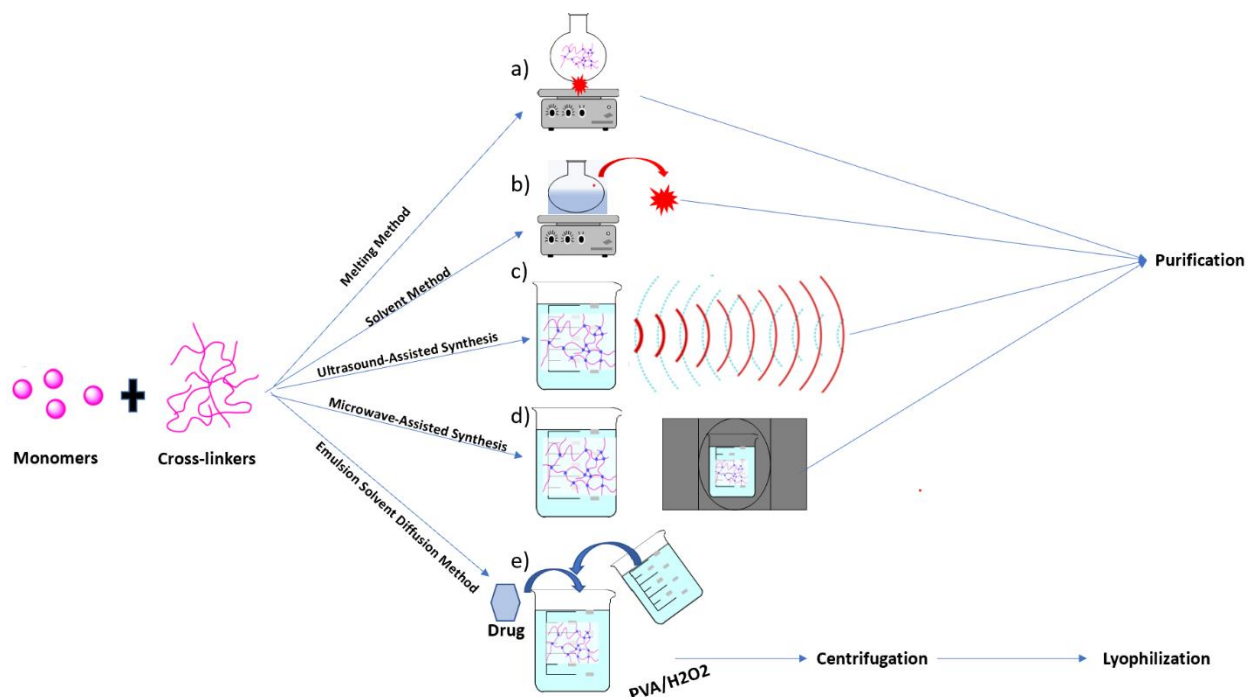


Figure 6. The methods of the NSs preparation[53].

1.3.1.1. Melting method

The crosslinker and monomer are heated between 90 and 100 °C together during the melting process (Figure 6 a), and all materials are thoroughly homogenised. The resulting mass is repeatedly washed with an appropriate solvent to remove the unreacted excipients and byproducts after cooling at room temperature. For further characterisation and assessment, the produced polymers are kept in storage at 25 °C[54].

1.3.1.2. Solvent method

The solvent technique, shown in Figure 6 b, avoids the melting stage by combining the polymer with an appropriate polar aprotic solvent, such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO), and then adding the cross-linker. The crosslinker/monomer molar ratio is modified. The reaction occurs at a specific temperature (between 10 °C and

the solvent's reflux temperature), for a specific amount of time (1-48h). The output is then recovered by adding it to an excessive volume of distilled water, mixing the product and water, and filtering the mixture while under vacuum. If necessary, Soxhlet extraction or Speed Extractor E-914 can be used to fully purify the NSs. The finished product is dried in the air[55]. With the high-pressure homogenization process, the size of generated NSs can be decreased by homogenising the aqueous suspension of NSs with Ultra Turrax for 10 min at a set speed (rpm) [56]. Another method for reducing particle sizes is the use of a ball mill [57].

1.3.1.3. Ultrasound-assisted synthesis

The ultrasound-assisted synthesis technique (Figure 6 c) allows the polymer to be cross-linked without the addition of any solvent. In this method, a suitable molar ratio of monomer and cross-linker was mixed using ultrasonic waves. This combination is placed in an ultrasonic bath at 90 °C for five hours. The mixture is chilled after the ultrasonication procedure. Comparable to the melt and solvent procedure, the solid mass is severely fractured and washed with excessive water to remove unreacted polymer and chemicals[58].

1.3.1.4. Microwave-assisted synthesis

For the production of CD-NSs, microwave irradiation uses a basic technique (Figure 6 d)). The monomer and cross-linker are combined in the proper molar ratio before being exposed to microwave radiation (power -18W) for up to 3.5 minutes, roughly. In the microwave synthesiser, the monomer:crosslinker is heated to 180 °C with a pre-heating time of 5 minutes and a reacting period of 30 minutes. As with the aforementioned procedures, the product is quickly cooled to 60 °C and repeatedly washed and filtered

[59]. The resultant powder is then ground in a mortar and dried in an oven overnight at 60 °C. When compared to the traditional heating approach, the reaction time is shorter, and the synthesised NSs exhibit consistent crystallinity and homogeneous particle sizes[54].

1.3.1.5. Emulsion solvent diffusion method

The two immiscible phases that result in the development of the emulsion are the principles of the emulsion solvent diffusion method (Figure 6 e). The first is the internal phase, which consists of a drug and polymer solution in a suitable common volatile solvent, and the second is the exterior phase, which consists of an aqueous emulsifier solution, such as polyvinyl alcohol (PVA) in water. Dropwise additions of the internal phase are made while the external phase is continuously stirred, and stirring is continued until the solvent for the internal phase has evaporated (overnight at 700 rpm under atmospheric conditions). To prevent PVA from adhering to the surface of the NSs, the mixture is centrifuged, and the pellet of drug-loaded polymer is then purified using milli-Q water. The stabilizer-free NSs are initially freeze dried for particle analysis and assessments[60].

1.3.2. Classification of CD-NSs

According to their chemical components and physical characteristics, CD-NSs can be divided into Four generations, which are concisely described below [61].

1.3.2.1. The first generation of CD-NSs

The straightforward interaction between CDs and a crosslinker constitutes the first generation of CD-NSs (Figure 7 a)). The first generation could be further split into

urethane (or carbamate), carbonate, ester, and ether CD-NSs depending on the chemical composition of the functional group connecting the CD to the cross-linker.

To create urethane (or carbamate) CD-NSs, diisocyanates like hexamethylene diisocyanate (HDI) and toluene-2, 6-diisocyanate (TDI) are employed as crosslinking agents. The rigid structure, excellent resilience to chemical degradation, small range of swelling in organic solvent, and low BET surface area ($1\text{--}2\text{ m}^2/\text{g}$) compared with activated carbon ($600\text{--}700\text{ m}^2/\text{g}$) are the characteristics of the nano-porous polymer.

In order to create carbonate CD-NSs, active carbonyl compounds like 1, 1'-carbonyldiimidazole (CDI), triphosgene, and diphenyl carbonate (DPC) are needed. These compounds have a low BET surface area (around $2\text{ m}^2/\text{g}$), short cross-linking bridges, good stability to acidic, neutral, and slightly alkaline solutions, and lowered swelling ability. An amorphous or crystalline cross-linked polymer is produced, depending on the reaction technique used. In order to create ether CD-NSs, cross-linking agents with epoxide groups are utilised, such as epichlorohydrin (EP), bisphenol A diglycidyl ether, ethylene glycol, and diglycidyl ether. These cross-linking agents are notable for their strong chemical resistance and regulated swelling capacity. $10.9\text{ m}^2/\text{g}$ was the BET surface area of β -CD with EP. Pyromellitic dianhydride (PMDA), ethylenediamine-tetraacetic dianhydride (EDTA dianhydride), butanetetracarboxylic dianhydride, and citric acid are some examples of the dianhydrides or di/polycarboxylic acids used to make ester CD-based NSs. Polyester NSs, in contrast to polycarbonate and polyurethane NSs, typically have a remarkable capacity to absorb water (up to 25 times/g dry sample) and create stable hydrogels. Similar to the case with mechanical properties, the degree of cross-linking generally affects the CD-NSs' propensity to swell. The ability to swell is typically inversely correlated with the concentration of cross-linking: the greater the water

intake, the lower the degree of cross-linking. Chemical stability is significantly influenced by the material's structure. Compared to polycarbonate and polyurethane NSs, ester NSs are more easily exposed to hydrolysis in aqueous conditions. The material is suitable for use as an ionic exchange resin because it has free carboxyl groups, which are moieties that can be used to absorb cations. This makes the structure very fascinating. Low surface area ($0.29 \text{ m}^2/\text{g}$) is produced by ester NSs based on PMDA [46, 62-65].

1.3.2.2. *The second generation of CD-NSs*

The functionalized NSs of the second generation (Figure 7 d) are characterised by unique characteristics including electric charge and tagging with fluorescence active moieties, which were initially investigated in 2005. There are three methods for adding particular moieties to CD polymers: post-cross-linking functionalization of the NS, pre-cross-linking functionalization of CDs, or simultaneous addition of the functionalizing agent and the cross-linking phase. While the other two produce a homogeneous concentration of chemical functions to be distributed throughout the entire NS particle, the first technique results in a change to the particle surface. In a polar aprotic solvent (DMSO) at 90°C for 3 hours, CD-NSs and fluorescein isothiocyanate are combined to create the fluorescent derivative. While NSs is reacted with succinic anhydride at 90°C to produce new carboxylated NSs, which has its surface charge modified. The produced NSs are amorphous, with an average diameter of around 400 nm, a narrow polydispersity index (0.11-0.13), and a negative surface charge (-35, or -39 mV) [62, 66]. Moreover, the carbonate -CD NSs is biofunctionalized by an improved one-step chemical reaction using cholesterol hydrogen succinate on its surface (CHS). In dimethylformamide, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC, 1.5 moles equivalent to CHS) and 4-dimethylamino pyridine (DMAP, 1 mole equivalent to CHS), respectively, activate the CHS

(DMF). The reaction is carried out at 45 °C for 24 hours after the addition of the -CD NSs. Following this time, the product is cooled to room temperature and the sediment is cleaned with ethanol and water to get rid of the cholesterol and unreacted catalysts. To show the presence of cholesterol in the polymeric structure, the average zeta potential of CD-NSs-CHS is more negative (-18 ± 2 mV) than the average zeta potential of CD-NSs (-5.03 ± 2 mV), whereas CD-NSs-CHS has an average particle size of 390 ± 20 nm. The characterisation methods validated CHS's [67] biofunctionalization of NSs.

1.3.2.3. The third generation of CD-NSs

The third generation of CD-NSs is reported to include stimuli-sensitive CD polymers (Figure 7 b). These polymers may adapt to changes outside of themselves by changing their structure, permeability, or colour. Temperature, pH, electromagnetic radiation, and redox potential can all induce NSs. Since the synthesis of the pH-sensitive materials from α -CD using a one-pot acetonating technique in 2013, the emergence of stimuli-sensitive NSs has been observed. They undergo additional processing to become nanoparticles (NPs) with a manageable size dispersion. The hydrolysis period of NPs can be precisely adjusted by materials with a variety of acetal types that are easily manipulated by acetylation time. By reacting 2-hydroxyethyl disulfide with the β -CD and PMDA, 2-hydroxyethyl disulfide is converted into Glutathione/pH-responsive CD NSs (GSH/pH-NSs). GSH/pH-NSs had a narrow polydispersity index (0.20), a zeta potential of -30 mV, and an average diameter of about 200 nm [62, 68, 69].

1.3.2.4. The fourth generation of CD-NSs

The fourth generation of NSs includes molecularly imprinted polymers (MIPs) (Figure 7 c). They are very selective, affine, and capable of recognising particular compounds. The development of specific contacts between a functional monomer, a cross-linking agent, and a template molecule forms the basis for their synthesis [62]. One could classify the NSs functionalized with biological ligands as a new fifth generation. Functional groups located on the surfaces of NSs make it possible to use them for the grafting of biological ligands that can bind to particular receptors on the surface of target cells[70].

1.3.3. Characterization of CD-NSs

With CD NSs becoming more and more important, numerous characterisation methods have been employed. The spectroscopic methods including, UV-Vis spectrophotometry, nuclear magnetic resonance (NMR), Raman spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, and attenuated total reflection (ATR); etc. Thermal analysis methods including, differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA); X-ray diffraction methods; and microscopy methods include confocal laser scanning microscopy (CLSM) Spectroscopic methods, particularly FTIR, are used to confirm the creation of NSs. NMR is used to investigate the structure of NSs. By combining thermoanalytical methods with XRD, the useful information about the development of NSs and host-guest interactions is obtained. Techniques like SEM and TEM are used to precisely investigate the dimensions, morphology, and form of NS. CLSM examines how fluorescent dyes integrated into NSs are distributed in the cytoplasm and how these dyes function in bodily cells [5, 11, 25, 56, 70-76].

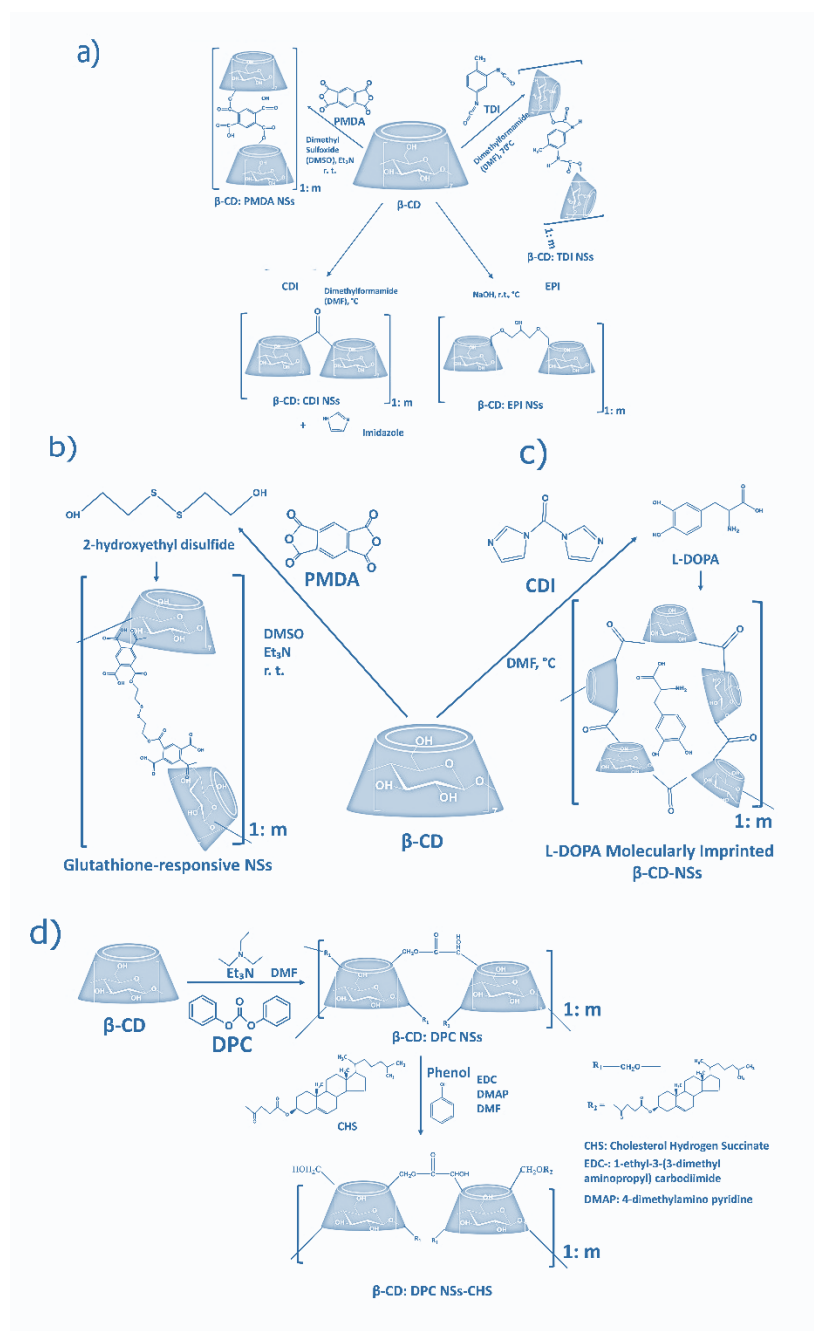


Figure 7. a) The synthesis of the first generation of CD-NSs; b) the synthesis of the third generation of CD-NSs; c) the synthesis of the fourth generation of CD-NSs; d) the synthesis of the second generation of CD-NSs.

1.3.4. Applications of CD-NSs

Controlling synthesis processes is crucial for producing CD-based NSs with the correct properties, especially when the polymer is used in medical devices and other applications. Initial experiments suggested that this technology is more effective than traditional methods at delivering drugs, increasing their solubility, prolonging their release, and enhancing their bioavailability and stability. These characteristics have made NSs to contribute significantly to the treatment of various diseases.

Scientific review has been sparked by NSs' capacity to entrap a variety of molecules in the matrix as a result of their highly porous nanomeric structure. As a result, CD-based NSs are seen as a potential material in a number of industries, including biomedicine, enzymology, the environment, agriculture, catalysis, and pharmaceuticals [77, 78, 99, 109-111]. The CDNSs as a relatively straightforward polymer network made up of crosslinked CDs in the 1960s [107]. From then, the NSs developed throughout time. Their ability to bind to organic materials when investigated for potential separation applications led to the development of stationary phases for nucleic acids and other chemicals in the 1970s. Studies in the 1980s looked at novel polymers and tried to comprehend their characteristics as well as their potential to bind. CD-based polymers were used as food component transporters and debittering agents in the 1990s (e.g., caffeine, vanillin, and theobromine). Additionally, because of their great adsorption, tunability, and low cost, they surpass the restrictions of purifying technologies utilised up until now in the field of water remediation. The functionalization of NS in the modern era increases the elimination of organic contaminants even at low concentrations (a few ppb). CD-based NSs are being studied for the first time as drug delivery platforms. Different types of

drugs can be loaded effectively, and a controlled release is made possible. As they are meant for human use, their safety, minimal toxicity, and biodegradability also become issues. The next years see substantial research in this area of application (2010–2015). The primary application area from 2016 to 2019 was pharmaceuticals [107]. CD-NSs are of interest for drug delivery because they behave as transient drug carriers rather than being absorbed through the skin or the digestive tract.

Drugs must typically dissolve quickly in order for the body to absorb them effectively [77, 78]. Additional uses have been discovered in the catalysis, textile, solid-phase extraction, and environmental fields. Various antibiotics, including rifampin (RM), novobiocin sodium salt (NB), and vancomycin hydrochloride (VM), are administered using CD-based urethane NSs. Using a standard solvent/solution absorption technique, the antibiotics are introduced to the polymer. These antibiotic-loaded gels' antibacterial activity demonstrated a distinct zone of inhibition. An anti-infectious drug delivery system was created in this study [79]. These polymers also include the anti-proliferative chemical mitomycin C (MMC). Due to a lower total exposure to MMC, it is predicted that this therapy has a lower clinical risk to the patient and surgical team than more conventional approaches. For the transport of MMC to the eye, the polymer platform is a feasible approach[80]. In addition to the following compounds: quercetin[81] , curcumin[55], resveratrol [82], oxyresveratrol[83], thyme essential oil (TEO), norfloxacin [73], kynurenic acid[74], tamoxifen[75], rilpivirine [76], acyclovir[66], camptothecin [50], telmisartan [84], babchi oil.

To load anti-inflammatory drugs like naproxen (NAP), nabumetone (NAB), and diclofenac [85], antifungal medications like naftifine (NF) and terbinafine (TB), and anticancer medications like curcumin and 5-fluorouracil[86], CD-based ether NSs are also employed. Insulin[87], imiquimod [IMQ, 1-[2-methylpropyl]-1H-imidazo[4,5-c] quinoline-4-amine], an immune response modifier[88], acetylsalicylic acid [89], doxorubicin[90], meloxicam[90], and rilpivirine are among the drugs that can be loaded with ester CD-based NSs (an antiviral drug)[91]. When compared to CD-inclusion complexes or uncomplexed pharmaceuticals, each of those data show an improvement in the biocompatibility and aqueous solubility of those drugs, as well as a regulated and extended drug release, making NSs a potential nanocarrier technology. The functionalized CD-based NSs with cholesterol[67] and gold nanoparticles (AuNPs) [29] make up other generations of CD-based NSs. Modified NSs are successfully loaded with doxorubicin, an anticancer medication [67], phenylethylamine (PhEA), an antidepressant, and 2-amino-4-(4-chlorophenyl)-thiazole (AT), an antimicrobial and anti-inflammatory agent[29]. Drug solubilization levels, complexation effectiveness, and solubility and bioavailability all show improvements. Although CD-based NSs are capable of substantial drug loading, pharmacological effectiveness remains a difficult challenge because to their non-selectivity. In order to improve the tumor-specific delivery of resveratrol[92], doxorubicin, anticancer medicines[93], and DB103, the optimal drug candidate for drug-eluting stents (DES) [94], a stimuli-responsive drug delivery system was developed. Additionally, molecularly imprinted polymers (MIPs) are thought to be a potential delivery mechanism for the delayed release and protection of glucose, which causes diabetes [95], and L-DOPA, a non-proteinogenic amino acid that is commonly used to treat Parkinson's disease (PD) [96]. In comparison to their NIPs-based counterparts, the MIPs-based NSs shown

more significance in specificity and greater binding for the templates. Combination therapy using CD-based NSs is a viable platform for therapeutic applications [53].

1.4. Encapsulation and controlled release capacity

The limitations of the abovementioned nutraceuticals have long been addressed by encapsulation technology[97] . Important factors including the stability of the active component and the matrix, the intrinsic physicochemical properties of the active and matrix elements, the interactions between the active component and the matrix, etc. must be taken into account during encapsulation [97]. Before being administered into a system, bioactive molecules such isoprenoid derivatives, phenolic compounds, fatty acids, structural lipids, carbohydrates, derivatives of amino acids, microorganisms, and minerals must be encapsulated. The steps in the encapsulation process include the inclusion of the bioactive compounds in a solid or liquid matrix, the dispersion or spraying of liquid in the case of a solid matrix solution, and the stabilisation of the system through a physical, chemical, or physicochemical process. The bioactive compounds' protection from harmful conditions aids in enhancing their bioavailability [98]. While the steps of the bioactive released include surface release, diffusion through the swelling matrix, matrix erosion, matrix degradation, hydrostatic pressure, fissure development, shear-mediated changes in shape, etc.[99]. Nanocarriers that offer continuous or controlled release of a drug with a low dosing rate are preferable. The diffusion of a therapeutic agent via a carrier matrix can affect the release kinetics. The composition (drug, polymer, and additives), their ratio, physical and/or chemical interactions among the components, and the preparation techniques all have an impact on how well drugs release from carriers [100].

1.5. Scope and Organization

The main objective of this thesis was using the cyclodextrin based polymers for advanced gene and drug delivery applications.

In particular, the goals of the present thesis can be summarized in the following points:

- ❖ To synthesis different types of β CDNSs and cationic hyperbranched cyclodextrin (CD)-based polymer (Ppoly), and characterisation by using TGA, DSC, SEM, FTIR, and etc methods (Chapter II).
- ❖ To fully understand the effect of the cyclodextrin (CD)-based nanosponges (NSs) to increase the stability and improvement of Nisin, a peptide, anti-bacterial activity as well as its both in-vitro and in-vivo anti-cancer effects against different cancer cells and melanoma animal model (Chapter IIII).
- ❖ To fully understand the effect of the solvent free prepared cyclodextrin (CD)-based nanosponges (NSs) in complexes with Kynurenic Acid to increase its oral bioavailability in animal model (Chapter III).
- ❖ To fully understand the effect of Ppoly to increase the transfection efficiency of the Plasmid DNA (pDNA) in different cancer cells both 2D and 3D. Furthermore, the cellular uptake and cancer immunotherapy improvement effect of Ppoly for messenger RNAs (mRNA) against melanoma animal model was checked (Chapter III).

Chapter 1 Literature Review

Chapter 2 Material and Methods

Chapter 3 Biological Applications

Chapter 4

General Conclusions

Section 3.1 β CDNS-CDI and PMDA for peptide delivery: Nisin-Z as an example

First Project:
Cyclodextrin-Based Nanosponges as Perse Antimicrobial Agents Increase the Activity of Natural Antimicrobial Peptide Nisin

Second Project:
Stabilization and Anticancer Enhancing Activity of the Peptide Nisin by Cyclodextrin-Based Nanosponges against Colon and Breast Cancer Cells

Third Project:
Nisin Delivery by Nanosponges Increases Its Anticancer Activity Against In-vivo Melanoma Model

Section 3.2 β NS-CDI in comparison with HP- β CD for nutraceutical delivery

Section 3.3 Cationic Hyperbranched Polymers for pDNA and mRNA Delivery

First Project:
Hyper-branched cationic cyclodextrin polymers for improving the plasmid transfections in 2D and 3D spheroid cells

Second Project:
Intratumorally delivery of mRNA loaded on positive charge Hyper-branch Cyclodextrin polymer induced therapeutic anti-tumor immunity against melanoma animal model

Chapter 2

Material and Methods

2.1. Material and Reagents

All chemical reagents were supplied of Sigma-Aldrich without additional purification. The human colorectal carcinoma cells HT-29, breast cancer (MCF-7), melanoma cancer (B16-F10), and Fibroblast cell lines were provided from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). β -cyclodextrins (β CD) were provided by Roquette Freres (Lestrem, France), while choline chloride (CHO), carbonyldiimidazole (CDI), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Darmstadt, Germany), and 2-Hydroxypropyl-Bcd (CIDs 44134771, DS 3) were kindly provided by Roquette Freres, (Lestrem–France). Lysozyme from chicken egg white (62970), albumin from porcine serum (A1830), and bovine serum albumin (A3912) were purchased from Sigma-Aldrich (Darmstadt, Germany) and stored in a refrigerator. Coomassie brilliant blue (R-250) was obtained from Bio-Rad. Furthermore, the OVA modified and EGFP (5moU) mRNAs as well as DreamFect™ (DF40500) were provided from OZ-Biosciences (France). Nisin Z (2.5% w/w) and pepsin were obtained from Sigma Aldrich (Milan, Italy). *E. coli* (ATCC 25992) and *S. aureus* (ATCC 25923) were prepared from a Persian collection of bacteria in the Iranian Research Organization for Science and Technology (Tehran, Iran). Pepsin (P0525000), MTT (M2003), and Cytotoxicity Detection Kit PLUS (LDH) were obtained from Sigma Aldrich (Milan, Italy). FITC Annexin-V apoptosis kit I (BD Pharmigen™, BD

Biosciences, and San Jose, CA, USA, 556547) and fetal bovine serum (FBS) were purchased of Cegrogen-Biotech (Germany, A0100-3010). Pen-Strep (10,000 Unit/mL penicillin and 10,000 unit/mL streptomycin) and 0.25% trypsin-EDTA were purchased from Bioidea (Tehran, Iran). RPMI was prepared from GIBCO Laboratories (Grand Island, NY, USA, 11530586). Coumarin-6 and Oxaliplatin were provided by Sigma Aldrich (Milan, Italy). Kynurenic acid (KYNA) were purchased from Sigma-Aldrich (Milan, Italy). Animal food was supplied by Javaneh Khorasan Co, Iran. All chemicals and reagents used were of analytical grade unless otherwise specified.

2.2. Synthesis and characterization of CD based polymers

2.2.2. Polymers synthesis

2.2.2.1. CDI cross-linked NS polymer synthesis

17.42 g of anhydrous β -CD (15.35 mmol) was added to 100 mL DMF in round bottom flask to attain complete dissolution by monitoring clear solution. Then, 19.91 g of CDI (122.8 mmol) was added as a cross-linking agent and the solution allowed to react at 100°C. After the completion of condensation reaction, the solid block of cross-linked cyclodextrin was roughly ground in mill or unreacted reagents were completely removed by Soxhlet extraction with ethanol [37,38]. Furthermore, nanosponges were

purified in ethanol by Soxhlet extraction for 24 h (Figure 8A). The solid obtained was stored in a desiccator at 25°C for further usage.

2.2.2.2. PMDA cross-linked NS polymer synthesis

11.35 g of anhydrous β -CD (10.00 mmol) and 17.45 g of PMDA (80.0 mmol) were dissolved in 100 mL of DMSO containing 2.7 mL TEA (19.4 mmol) and were allowed to react at room temperature for 3 h. Once the reaction was over, the solid obtained was ground in a mortar and Soxhlet extracted with acetone for 24 h (Figure 8B) [38].

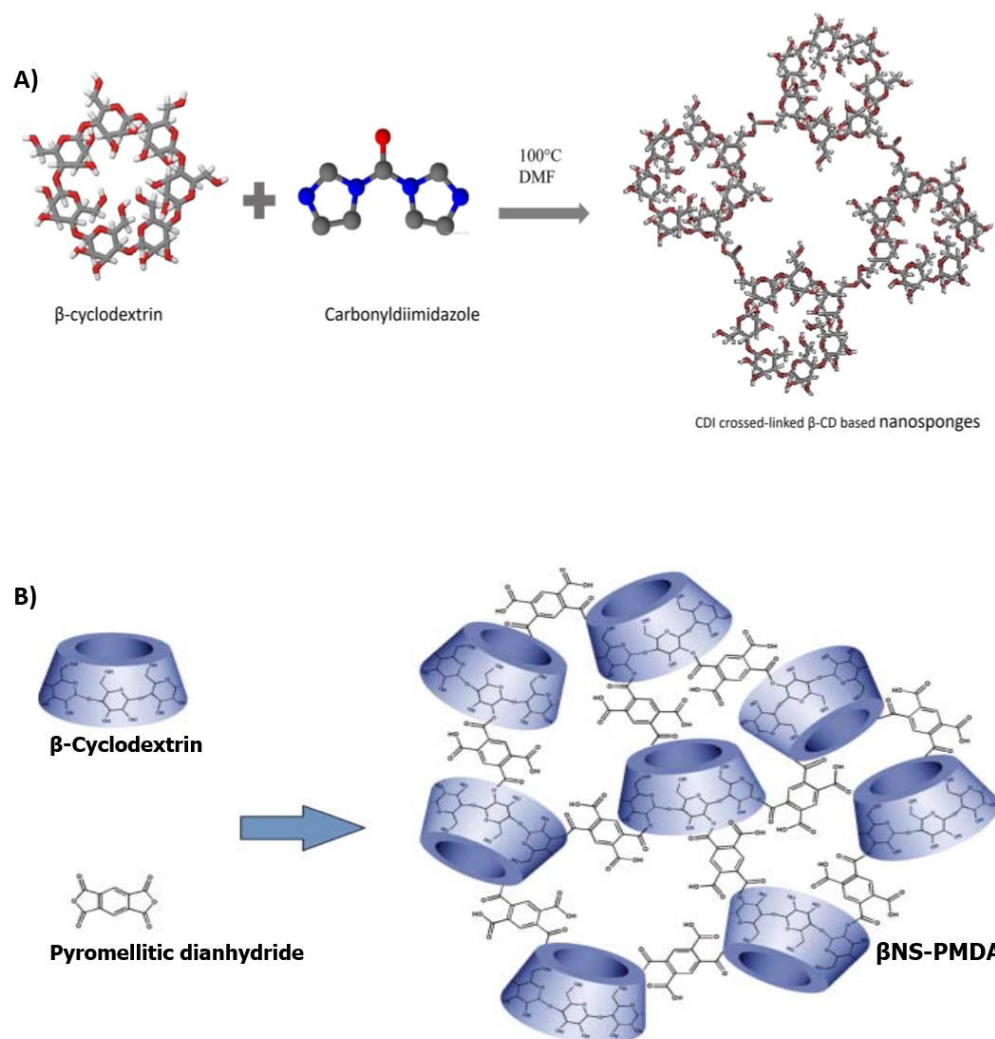


Figure 8. The schematic synthesis of PMDA/CDI-NSs [111].

2.2.2.3. Ball Mill synthesis of Synthesis of carbonate β CD Nanosponges (β NS-CDI 1:4)

The cross-linked β CD Nanosponges were prepared using the ball mill (Planetary Ball Mill: PM200 High Speed Planetary Ball Mill, Retsch) in a one-step reaction without solvents, respecting the 1:4 molar ratio between anhydrous cyclodextrin and carbonyl diimidazole: 3.75 g of β -cyclodextrins were placed inside a 50 mL jar containing 10 zirconia balls. The amount of CDI added in each batch to maintain the 1:4 molar ratio was 2.25 g. After 3h of sun wheel rotation at 600 rpm, changing the direction from clockwise to anti-clockwise every 15 minutes, the reaction was completed. The measured external temperature of the jars was around 60°C. After the synthesis a finely ground white/yellowish powder is obtained; subsequently, the powder is dispersed in water and washed several times with deionized water and acetone. All syntheses were extracted with Pressurized Solvent Extraction (PSE, SpeedExtractor E-914 from Buchi), to remove the residual imidazole in the NS structure and unreacted material (Figure 9).

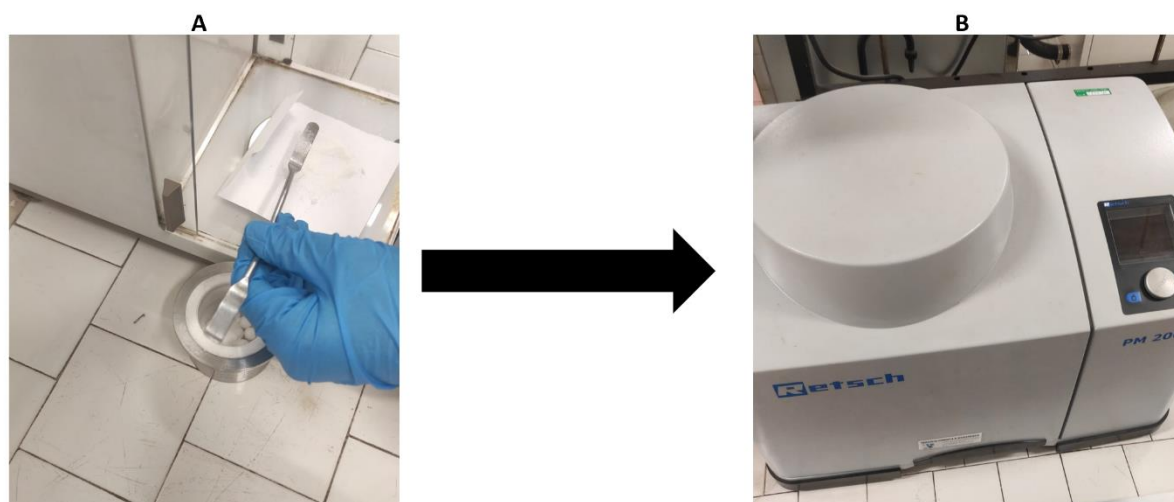


Figure 9. The cross-linked β CD Nanosponges preparation method by using the ball mill. **A;** The β -cyclodextrins were placed inside a 50 mL jar containing 10 zirconia balls and the amount of CDI added in each batch to maintain the ratio. **B;** The prepared samples were put inside the Planetary Ball Mill: PM200 High Speed Planetary Ball Mill, Retsch, instrument.

2.2.3. Characterizations

2.2.3.2. Complex size and zeta-potential measurement

Particle size and zeta-potential measurement was performed on a 90 plus particle sizer (Malvern Instruments, Malvern, UK). A total of 5 mg of β -CD, plain CDNSs, Nisin-free, KYNA, KYNA and Nisin loaded on CDNSs, and their physical mixtures were suspended in distilled water (5 mL) to measure their size and polydispersity index (PdI), and they

were sonicated for 15 min to avoid the presence of aggregates. The particle size was measured at 25 °C in a glass cuvette.

2.2.3.3. Scanning electron microscopy (SEM)

To obtain the optical and structural properties of the plain drugs, CDNS, loaded drugs on CDNSs, and their physical mixture complexes, SEM was used to visualize the complexes. The complexes were observed using SEM visualization, the gold-coated complexes were observed using a Cambridge Stereoscan 260 SEM (Cambridge, UK) at 20 kV.

2.2.3.4. Fourier transformed infrared (FTIR)

The plain drugs, CDNS, loaded drugs on CDNSs, and their physical mixture complexes were analyzed by using Fourier transform infrared (FTIR) spectroscopic studies (Bruker, Tensor 27, Germany instrument), in the region of 400–4000 cm^{-1} , with a resolution of 4 cm^{-1} , to find the existence of interactions between nisin Z and β -CD-NS systems. KBr was mixed with the samples (the ratio of KBr to sample is 100:1); then, the KBr pellet was prepared by applying sufficiently high pressure to a homogeneous mixture of the KBr and sample until the pellet turned transparent.

2.2.3.5. Thermogravimetric Analysis (TGA)

Thermogravimetric Analysis (TGA) were carried out on a TA Instruments Hi-res Q500 Thermogravimetric Analyzer, (New Castle, DE, USA). TG Analysis parameters: nitrogen flow, heating rate 10 °C/min, RT to 700 °C.

2.2.4. Synthesis of hyperbranched cationic cyclodextrin-based polymer

The synthesis of the polymer was carried out by dissolving, at room temperature, 1.00 g ($8.81\text{E-}4$ mol) of anhydrous β CD in 7.5 mL of DMSO. After complete solubilization, 1.11 g ($7.93\text{E-}3$ mol) of choline chloride was added, followed by 1.14 g ($7.05\text{E-}3$ mol) of carbonyl diimidazole. After a transparent liquid was obtained, the solution was kept under stirring at 90 °C for 120 minutes; an increase of the viscosity was observed during that time, as a proof of the occurring of the polymerization reaction. Afterwards, the product was precipitated and washed with acetone, then recovered by vacuum filtration. Later, the dry product was solubilized in distilled water and purified using ultrafiltration technique (cut-off 5k Da), to separate the polymer from unreacted reactants, byproducts, and solvent residues. The product was then recovered from the ultrafiltration cell and subsequently freeze dried, obtaining a white powder as the product. Considering the weight of the final product with respect to the theoretical weight, equal to the sum of β CD, CHO, and CDI, the mass balance resulted approximately 85%. This part was done in collaboration with Dr. Claudio Ceccone to prepare and synthesis the nanoparticles.

2.2.5. Characterizations methods

2.2.5.1. Size exclusion chromatography and Elemental analysis characterization

Size exclusion chromatography analyses were performed using a PL aquagel-OH MIXED-M 8 μm (Agilent, CA, USA) column installed on a Perkin-Elmer Flexar chromatograph. The mobile phase was a 0.1 M sodium nitrate water solution at a flow rate of 0.3 mL/min. Refractive index was used as detector, while the duration of a single run was set at 15 minutes. The samples chemical composition was studied using a Thermo Fisher FlashEA 1112 Series elemental analyzer (Waltham, MA, USA).

2.2.5.2. Complex size and zeta-potential measurement

Particle size and zeta-potential measurement was performed on a 90 plus particle sizer (Malvern Instruments, Malvern, UK). DNA/ mRNA-polymer complexes with different N/P ratios (average number of nitrogen atoms on the cyclodextrin core/number of phosphate groups of pDNA/ RNA) were formed in 0.5 ml Eppendorf tubes by adding 10 μl of plasmid DNA/ mRNA solution (containing 1 μg of pmaxGFP Pdna/ mRNA in filtered distilled water) and 10 μl of polymer solution (containing varying amounts of polymer in filtered distilled water). Then, they were left for 30 min at room temperature to form the complexes, and were then diluted to 1 ml with filtered distilled

water. The particle size was measured at 25 °C in a glass cuvette.

2.2.5.3. Scanning electron microscopy (SEM)

To obtain the optical and structural properties of the Ppoly- free, DNA and mRNA loaded Ppoly, SEM was used to visualize the complexes. The complexes were observed using SEM visualization, the gold-coated complexes were observed using a Cambridge Stereoscan 260 SEM (Cambridge, UK) at 20 kV.

2.2.5.4. Fourier transformed infrared (FTIR)

Ppoly- free, DNA and mRNA loaded Ppoly were analyzed by using Fourier transform infrared (FTIR) spectroscopic studies (Bruker, Tensor 27, Germany instrument), in the region of 400–4000 cm^{-1} , with a resolution of 4 cm^{-1} , to find the existence of interactions between nucleic acids and Ppoly systems. KBr was mixed with the samples (the ratio of KBr to sample is 100:1); then, the KBr pellet was prepared by applying sufficiently high pressure to a homogeneous mixture of the KBr and sample until the pellet turned transparent.

2.2.5.5. Thermogravimetric Analysis (TGA)

Thermogravimetric Analysis (TGA) were carried out on a TA Instruments Hi-res Q500 Thermogravimetric Analyzer, (New Castle, DE, USA). TG Analysis parameters: nitrogen flow, heating rate 10 °C/min, RT to 700 °C.

2.3. Biological methods

2.3.1. Determination of Nisin Content in β -CDNSs

To break the complex, a determined number of Nisin-CDNSs complexes were dispersed in Milli Q water and sonicated for 10 min. The protein concentration in the solution was determined by the Bradford reagent, which is due to the formation of a complex between the dye, Brilliant Blue G, and the proteins in the solution. The protein–dye complex has a maximum absorption at 595 nm. Bradford reagent was used to detect low concentrations of protein molecules in solutions due to its high sensitivity [39]. The encapsulation efficiency (EE) and Drug Loading (%DL) of the drug was calculated according to the following formulas:

$$\text{Drug Loading (\%DL)} = \frac{\text{Total drug (mg)} - \text{Free drug (mg)}}{\text{Total amount of NPs (mg)}} \times 100$$

$$\text{Encapsulation Efficiency (\% EE)} = \frac{\text{Total drug (mg)} - \text{Free drug (mg)}}{\text{Total drug (mg)}} \times 100$$

2.3.2. In Vitro Studies of Nisin Release

Nisin Z release profile was evaluated by suspending 2 mL of a 25 mg/mL solution of Nisin Z and CDNSs suspension, including the same quantity of entrapped Nisin Z into 20 mL of PBS solution (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na₂HPO₄, and 0.0018 M KH₂PO₄, pH 7.4) for 48 h at 37 °C. The suspensions were stirred and then sonicated

for 5 min and kept at incubator (37 °C) under shaking at 150 rpm. At the appointed times, 0.5 mL of sample were collected from the suspension and transferred into the Amicon® tube (Ultra-30 kDa molecular weight cut-off membrane, Millipore, Germany) and centrifuged at 3500× g for 10 min [10]. Bradford's method was implied for quantifying the amount of Nisin in the buffer, as earlier explained. All experiments were done in triplicate. In contrast to the procedure in our previous article, in which the release profile of nisin Z was examined at physiological pH [33], in this study, the release was examined at an acidic pH (pH = 4) over 48 h at 37 °C. Then, the nisin-Z content in the buffer solution was determined by the Bradford assay, as previously presented [38].

2.3.3. Preparation of complexes and characterization

Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*S. aureus*) were grown, sub-cultured, and maintained in Mueller–Hinton agar and stored at 4 °C. In this experiment, a single colony of each organism was inoculated into 10 mL of brain heart infusion (BHI) broth, and it was then incubated in an incubator (37 °C and 200 rpm) for 24 h. The optical density of the overnight culture was adjusted to that of a 0.5 McFarland standard (1.5×10^8 colony-forming units (CFU)/mL). Through the dilution of the 0.5 McFarland standard with BHI, the final working concentration (1×10^6 CFU/mL) was obtained. Antibacterial activities of the free-nisin-Z and samples

complexed with β -CD and NSs at different concentrations were determined by 2-fold dilution of nisin Z, ranging from 5000 to 625 $\mu\text{g/mL}$, and a suitable quantity of NSs according to the drug loading; in the case of β -CD, the same quantity as NSs were added. Native CDs and NSs were evaluated using carrier quantity. The formulations were as follows: (i) 625 $\mu\text{g/mL}$: 625 $\mu\text{g/mL}$ nisin + 2500 $\mu\text{g/mL}$ of NS or β -CD; (ii) 1250 $\mu\text{g/mL}$: 1250 $\mu\text{g/mL}$ nisin + 5000 $\mu\text{g/mL}$ of NS or β -CD; (iii) 2500 $\mu\text{g/mL}$: 2500 $\mu\text{g/mL}$ nisin + 10,000 $\mu\text{g/mL}$ of NSs or β -CD and; (iv) 5000 $\mu\text{g/mL}$: 5000 $\mu\text{g/mL}$ nisin + 20,000 $\mu\text{g/mL}$ of NS or β -CD; furthermore, the same quantity of NSs and nisin Z complexed with β -CD were used to evaluate antimicrobial effect. Antimicrobial tests were carried out against *S. aureus* and *E. coli* following the Clinical and Laboratory Standards Institute (CLSI) guidelines as previously described, with some modifications [39].

2.3.4. Antimicrobial Evaluation in Solid Culture Medium

An agar well diffusion plate was used to determine the inhibition zone of the nisin-free sample and the one complexed with formulations in Mueller–Hinton agar (MHA) medium. A 20 mL of the agar medium was added to each Petri dish (95 mm $\times$ 15 mm) and allowed to solidify. Then, the mediums were inoculated with 20 μL of brain heart infusion (BHI) broth containing bacterial cultures. Further, 75 μL of antimicrobial agents were added to the holes made by sterile pipette tips, with a diameter of 7.0

mm. The calculation of the diameter of the inhibition zone determined the antimicrobial properties of formulations after 24 h incubation of plates at 37 °C.

2.3.5. Antimicrobial Evaluation in Liquid Culture Medium

A modified microdilution brain heart infusion (BHI) broth test was used to show bacterial growth (Optical Density 600 nm) at different time intervals by using 96-well microplates. To determine the minimal inhibitory concentrations (MICs), a visually modified broth microdilution method [40] with yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used [39]. The living and dead cells were considered by the observation of purple- and yellow-colored wells. As regards negative and positive control groups, a medium free of bacteria and a medium consisting of bacteria cells without antibiotics or antimicrobial peptides were used, respectively. MICs were considered as the concentrations of the first yellow wells observed in each treatment after being contacted for 24 h with selected formulations. Furthermore, the minimum bactericidal concentrations (MBCs) were determined by spreading aliquots (50 µL) of each well that illustrated MIC value on MHA plates, which were incubated for 24 h at 37 °C. The MBC of selected formulations for each strain was determined as the lowest concentration at which no colony formation appeared on MHA plates.

2.3.6. Capacity of Bacteria to Use β -CD and NSs as Carbon Sources

Drug-free β -CD and nanosponges with indicated concentrations were mixed as a component of agar medium in a minimal media [41] containing NH_4Cl as nitrogen source and cultivating both positive and negative bacteria on the surface of agar medium to understand the capacity of bacterial to use the β -CD and nanosponges as carbon source.

2.3.7. Total Viable Counts (TVCs)

To determine the antibacterial effect of the nisin-free sample and the sample complexed with the formulations on the chicken cooked meat, the total viable count (TVC) test was used. The fresh chicken meat was kept at 0 °C for 1 h before use, and then similar pieces weighing about 10 g were obtained. Then, samples were washed with deionized water and cooked in boiling water for 20 min. The total viable counts (TVCs) of cooked chicken samples were determined by the aerobic plate count method as previously reported, with some modifications [42]. Firstly, the antimicrobial solutions were freshly prepared including (C) samples treated with distilled water; (N) samples treated with 0.05% nisin; (N-P) samples treated with 0.05% nisin and 0.15% pepsin; (N- β) samples treated with 0.05% nisin and 0.1% β -CD; (N- β -P) samples treated with 0.05% nisin, 0.1% β -CD, and 0.15% pepsin; (CDI-N) 0.05% nisin and 0.1% CDI-NSs; (CDI-N-P) 0.05% nisin, 0.1% CDI-NS, and 0.15% pepsin; (PMDA-N) 0.05% nisin and 0.1% PMDA-NSs; (PMDA-N-P) 0.05% nisin, 0.1%, PMDA-NS, and

0.15% pepsin. The intervals times—namely, 0, 3, 7, 14, and 30 days—were set following the previous protocol [42]. Plate count agar was evaluated after 24 h of storage at 37 °C for TVC determination, and the logarithms of the number of colony-forming units (CFU/mL) were used to report the results.

2.3.8. Cell Culturing Conditions

HT-29 (Passage Number: 4) and MCF-7 (Passage Number: 8) cells were obtained from Pasture Institute National Cell Bank of Tehran, Iran. The cells were cultivated in RPMI 1640 supplemented with 10% FBS and 1% Penicillin/Streptomycin solution. The flask was incubated in a humidified incubator at 37 °C containing 5% CO₂. The media was exchanged every other day, and the cells were detached and harvested after trypsinization at 80~90% confluence in each passage.

2.3.9. Cellular Uptake Study

Cellular uptake was performed to evaluate the effectiveness of CDNS internalization within cancer cells. Nano-carrier was labeled by Coumarin-6 (C-6) as a fluorescent molecule model. The Amicon® tube (Ultra-30 kDa molecular weight cut-off membrane, Millipore, Germany) was used to purify free C-6 from nanoparticles. The cellular uptake of coumarin-6-encapsulated with PMDA-NSs and CDI-NSs in cancer cells was qualitatively and quantitatively studied. The cancer cells were seeded in 6-

well plate at a density of 5×10^5 cell/well, incubated overnight, and then treated with coumarin-6-loaded CDNSs for 4 h, thereafter, the cells were observed by Cytation 5 Cell Imaging Multi-Mode Reader (San Jose, CA USA, Gen5™ software). Quantitative cellular uptake of CDNSs in cancer cells were determined using flow cytometry. The cells at a density of 200×10^4 cell/well were seeded in 12-well plate and incubated for 24 h to obtain an 80% confluency. The cells were treated with CDNS containing C-6 for 4 h and kept in an incubator with 5% CO₂ at 37 °C. After that, the cells trypsinized and washed with PBS to analyze the uptake potential of CDNSs with Fluorescence-activated Cell Sorting (FACS) caliber flow cytometer (BD Pharmingen™, BD Biosciences, and San Jose, CA, USA) based on the fluorescent intensity.

2.3.9. In Vitro Cytotoxicity Assays

For toxicity assays, cells were seeded at a density of 25,000 cells per well in 96-well plates. A 250, 125, and 62 µg/mL solution of Nisin Z and CDNSs suspension containing the same amount of entrapped Nisin Z were freshly prepared prior to each experiment. All experiments were performed in serum-free RPMI1640 medium. MTT and LDH assays were used to determine the cytotoxicity of the Nisin free and in complexes. Finally, the flow cytometric assay was tested to define the apoptosis and necrosis of cancer cells. All experiments were at least performed in duplicate and repeated independently.

2.3.10. MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl Tetrazolium Bromide) Assay

The 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay was used to determine in vitro cell viability. HT-29 and MCF-7 cells were seeded in 96-well plates and incubated until cells were 80% confluent, followed by exposure to Nisin Z (250, 125, and 62 µg/mL), CDNSs suspension containing the same amount of entrapped Nisin Z and CDNSs free of drug for 24 h. Vehicle control groups were also included. Following exposure, growth medium was removed, cells carefully rinsed with 1X phosphate buffered saline (PBS), and 100 µL fresh serum-free medium containing 0.5 mg/mL MTT solution added. Cells were then incubated for 4 h at 37 °C, after which the MTT was removed and replaced with 100 µL dimethyl sulfoxide (DMSO). After 1 h of incubation at 37 °C, cell viability was determined using a micro-plate ELISA reader (Biotech, San Jose, CA, USA, Gen5™ software). Absorbance was measured at a wavelength of 560 nm and background at a wavelength of 630 nm with DMSO measured as a blank. Blank and background measurements were subtracted and cell viability is expressed as a percentage relative to the untreated control, which was set as 100% viable. The cell viability was calculated based on the optical density (OD) values of the treated and un-treated cells using the following equation:

$$\text{Cell Viability \%} = (\text{OD treated} - \text{OD blank}) / (\text{OD untreated} - \text{OD Blank}) \times 100 \quad (3)$$

2.3.11. Lactate Dehydrogenase (LDH) Assay

Cell membrane integrity was evaluated using the Plus Cytotoxicity Detection Kit (method) according to the manufacturer's protocol with some modifications. Briefly, HT-29 and MCF-7 cells were seeded in a 96-well plate and incubated until cells were 80% confluent. Cells were thereafter exposed to Nisin Z (250, 125, and 62 $\mu\text{g/mL}$), CDNSs suspension containing the same amount of entrapped Nisin Z and CDNSs free of drug for 24 h. A total of 100 μL of freshly prepared Reaction Mixture was added into each well and incubated 10 min at 25 $^{\circ}\text{C}$ under dark. A total of 50 μL Stop Solution was then added into each well, thereafter, the plate was shaken for 10 sec for quantifying the LDH leakage. Cells were contacted to 0.01% Triton-X 100 as a positive control. The absorbance at 492 nm was measured by micro-plate ELISA reader (Biotech, USA, Gen5™ software). Results are exhibited relative to the LDH release of control negative and positive groups with 0 and 100 percentage, respectively.

2.3.12. Preparation of Nisin-Loaded β -CDNSs and Coumarin-6 CDNSs

Nisin Z loaded CDNSs 1:4 was prepared by freeze-drying method. Briefly, a weighed amount of CDNSs 1:4 was suspended in 20 mL of Milli Q water under stirring, then the calculated amount of Nisin in a weight ratio of 1:4 (Drug: CDNS) was added. The mixture was sonicated for 15 min and kept for 24 h under stirring. To remove uncomplexed drug suspensions, it was centrifuged at 2000 rpm for 15 min. The

precipitated was dried in an oven at 60°C in vacuumed pressure. The dried powder was grinded and stored in a desiccator. In addition, CD-NSs with fluorescent dyes were built in a similar way by adding the fluorescent marker 6-coumarin (0.1 mg/mL) to the aqueous nanosuspension of CDNSs (10 mg/mL) in the dark condition.

2.3.13. Determine the Apoptosis Assay

The FITC Annexin-V apoptosis kit I (BD Pharmingen™, BD Biosciences, and San Jose, CA, USA, 556547) has been applied to determine the apoptosis percentage. Loss of cell membrane integrity and apoptosis were defined by green fluorescence released from FITC Annexin-V, while late apoptotic or necrotic cells were identified by Propidium iodide (PI) staining. The assay was performed according to the manufacturer's instructions, with some changes. In brief, HT-29 and MCF-7 cells were seeded in a 12-well plate and grown to 80% confluence. Cells were then exposed to 250 µg/mL concentration of Nisin Z and CDNSs suspension containing the same amount of entrapped Nisin Z. After 24 h, cells were two times washed with 400 µL of fresh PBS and suspended in 1 X binding buffer. Then, 5 µL of FITC Annexin V and 5 µL of propidium iodide (PI) were added to each sample and the cells were mixed slowly and incubated in the dark at room temperature for 20 min. After incubation, 100 µL 1 X binding buffer was added to each tube and then analyzed using the BD FACSVerse™ and BD FACSuite™ Software (BD Biosciences, San Jose, CA, USA).

Oxaliplatin (OxPt) and unstaining cells were analyzed as positive and negative controls, respectively. At least 10,000 cellular events were analyzed for each sample. A logarithmic amplification scale was employed and events plotted on forward scatter (FSC), side scatter (SSC), green fluorescence (FL1), and red fluorescence (FL2). Subsequent data analysis was performed using FlowJo X (10.0.7r2) single cell analysis software (FlowJo-V10, LLC). All experiments were at least performed in triplicate and independently repeated.

2.3.14. Tricine-SDS-PAGE

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine- SDS-PAGE) was done according to a previous report (25). For sample preparation, 10 μ L of a 2X gel loading buffer (0.25 M Tris-HCl, pH 6.8, 5% glycerol, 5% 2-mercaptoethanol, 3% SDS, and 0.2 mg.mL⁻¹ bromophenol blue) was added to 10 μ L of samples containing Nisin Z free and PMDA/CDI-NSs containing the same amount of Nisin, then, samples were heated to 95°C for 10 min. Samples were then loaded on a tricine gel consisting of a stacking gel containing 4% acrylamide, a spacer gel containing 10% acrylamide, and a separation gel containing 16.5% acrylamide. The gel was run at 30 V for 1 h and then at 80 V for 3 h. Coomassie Blue staining (G250) was used to visualize the peptide. Spectra multicolor low range protein ladder was

used. Thermo scientific spectra low range protein ladder was used, ranging from 1.7 to 40 kDa.

2.3.15. Experimental design and tumor induction

Six to eight-week-old female C57BL6/J mice were obtained from Pasteur institution, Tehran, Iran. The mice were injected subcutaneously (S.C.) with B16-F10 cells (1×10^6 cells/ml) to induce the melanoma cancer [101]. All mice were bred under pathogen-free conditions in the animal facility of the department of Pharmacy and were treated in accordance with the Tabriz university of medical science ethical committee. In totally 25 mice were randomly divided into 2 main groups: control and test groups that were further segregated into four subgroups: untreated tumor group, Nisin free, PMDA-NS + Nisin and PMDA-NS free, five mice were included for each group. When tumors appeared, mice were given 0.1 mL of Nisin free (150 mg/kg), PMDA-NS + Nisin (complex contains 150 mg/kg Nisin) at ratio 1:4(drug/CDNSs), and PMDA-NS free every two days, as well as the same quantity of physiological solution (NaCl 0.9 percent) for the control group. A digital caliper was used to measure the tumor size every two days, and mice were euthanized after 10 days. Tumor size was quantified daily in two dimensions using calipers and an average between each 2 days was reported in the graph. The mice were anesthetized and blood samples were taken from the heart for anti-oxidant and biochemistry tests after 10 days. The volume of

the tumors is calculated as follows: length x width² x 0.52[102]. At the end of treatment period, the melanoma tumors were dissected from the mice and weighed.

2.3.16. Histopathological studies

Neutral formaldehyde (10 percent) was used to fix the removed tumors. The samples were dehydrated using various concentrations of ethanol (30, 50, 70, 90, and 100%), after which the tissues were cleaned in benzene and embedded in low melting-point paraffin wax. After that, 5 µm thick sections were mechanically cut into sections and sequentially placed on clean glass slides. These slides were then stained with Hematoxylin and Eosin for examination under a light microscope (LEICA DM 3000).

2.3.17. Tumor microvessel density (TMD)

Immunohistochemistry staining of CD31 antibody was measured to calculate the tumor microvessel density (TMD), followed by counting the individual microvessels in three fields of each tumor section selected in the areas of most intense staining upon slide scanning (Panoramic midi II, 3D Histech, Budapest, Hungary), as previously described [103, 104]. Both staining clusters and single endothelial cells were considered.

2.3.18. Immunohistochemical staining for Bax and Bcl-2

In summary, the 5 µm sections were deparaffinized, rehydrated, and subjected to antigen retrieval using a variety of procedures[105]. The samples were washed in PBS before being incubated in a 10% goat serum blocking solution. The samples were

incubated overnight at a temperature of 40°C with the primary antibody (1:500; antiBax and AntiBcl2 antibody; Abcam, Cambridge, UK). After washing, sections were kept for 90 minutes at 37 ° C with the secondary antibody (1:1500; goat antirabbit IgG; Abcam). Finally, the slides were viewed using a Leica DM500 Biological Microscope after the samples were washed.

2.3.19. Anti-oxidant agents and biochemical parameters

Blood samples were collected from groups of mice after 10 days of treatment by subcutaneous injection of various formulations. Then, the values of oxidant agents including, total antioxidant status (TAS), superoxide dismutase (SOD), glutathione peroxidase (GPx) and Malondialdehyde (MDA) were determined in blood samples of different groups. The diagnostic kits manufactured by Randox Laboratories Ltd., Crumlin, County Antrim, UK were used to determine the TAS (mmol of TAS/g of hemoglobin) [106], SOD (U of SOD/g of hemoglobin) [107] and GPx (U of GPx/g of hemoglobin) [108], respectively, and MDA level was measured according to the method of Yoshioka, et al. and was expressed as MDA mol/L [109]. Other biochemical parameters were measured by using fully automated clinical chemistry spectrophotometer SPECORD M40 (Carl Zeiss, Jena, Germany) analyzer.

2.3.20. Preparation of KNYA/HP β -CD and KNYA/ β NS-CDI 1:4 inclusion complexes with kneading method and conventional approach

KYNA with HP β -CD and β NS-CDI 1:4 inclusion complexes were prepared as follows using the previously studies values of loading and strength[74, 110]: The conventional were prepared using 55 mg of pure KYNA with 240 mg of HP β -CD and β NS-CDI 1:4 in 50 mL of distilled H₂O. After 24h of mixing the solution was lyophilized and stored in darkness. On the other hand, the kneading protocol required 800 mg of pure KYNA was added into a 50 mL ball mill jar, with 3500 mg of HP β -CD and β NS-CDI 1:4 and 100 μ L of distilled H₂O. The mixtures were kneaded for 30 min at 300 rpm, changing the direction from clockwise to anti-clockwise every 5 minutes, with a 2-minute pause between each direction change. Approximately 12 mL (volume, measured with graduated cylinder) of sintered zirconium oxide balls, 5 mm of diameter, were present in each jar. Quantities of kneaded reactants were optimized for this specific volume of balls and, consequently free volume in the jars.

2.3.21. In vivo experimental design

The *in vivo* experiment was performed on 115 male BALB/C mice bought in the Tabriz university of medical sciences (animal center) for oral supplementation; their body weights ranged from 21-23 g after two weeks of quarantine. Animals were housed in standard cages in a temperature-controlled room. Animals were fed standard laboratory mice diet with free access to tap water and kept under constant environmental conditions (22 $\pm$ 3 $^{\circ}$ C; 50 $\pm$ 5% relative humidity and 12 hr of light/dark

cycle) and diet was containing the commonly used materials like vitamins and proteins. Mice were randomly and equally divided into five groups. The first group received free KYNA orally at dosage of 50mg KYNA/Kg/day. The second and third groups received the free HP β -CD and BNS-CDIs (mass of carrier), respectively, and the fourth and fifth groups received the KYNA in complex with HP β -CD and BNS-CDIs at dosage contain the same amount of free KYNA, respectively. The quantities were: Free KYNA, dosage of 1.1 mg/mice (50mg/kg); free HP β -CD and BNS-CDIs dosage 4.4 mg/mice(200mg/kg) and finally complexes 5.5 mg/mice (250mg/kg). All the samples were resuspended in PBS to be administered. The negative control group was considered as received the sterile PBS orally. Animals were fasted overnight prior to the experiment, being free to eat after supplementation until overnight, where they were fasted again. The supplement was administered by oral gavage using a stomach tube. The whole blood (the blood contained in EDTA as anti-coagulant) and serum sample (separated supernatant of the clotted blood) were collected after euthanasia by cardiac puncture (25G needle), from all five groups of mice after one-week oral administration (daily one dosage) of various formulations to check the biochemical parameters changes and anti-oxidant agents status as well as the cytotoxicity effect by histopathology examination. At the second step, for the bioavailability evaluation six time points were selected for KYNA free and complexes with BNS-CDI and HPB-CD, and then for each time points, four mice were used. After that, the blood was

collected at 5, 15, 30, 60, 120 and 240 min after complexes oral administration and blood samples were collected and centrifuged to collect the plasma, and then they were stored at - 80 C until laboratory analyses. The experiments were approved by Tabriz university of medical sciences, Pharmacy department, Iran Ethical Code: IR.TBZMED.AEC.1401.022.

2.3.22. KYNA analysis in plasma by HPLC

The plasma samples were prepared using a rapid protein precipitation method ²⁹. Briefly, the plasma was diluted with acetonitrile at 1:3 ratio, and then vortexed and centrifuged for 10 min at 3500 rpm. Fifty microliters of the supernatant were then transferred into HPLC syringe for the subsequent instrumental analysis. The quantitative determination of KYNA was performed by an HPLC system (Agilent 1100, USA) equipped with a UV detector (Flexar UV/Vis LC spectrophotometer) using a Phenomenex C18 analytical column. The mobile phase consisted of a mixture of 0.14% (v/v) acetic acid in water-acetonitrile (90:10 v/v) and pH was adjusted at 6.2, filtered and ultrasonically degassed prior to use. The mobile phase was pumped through the column at a flow rate of 1 ml/min and the samples (20 µL) were analyzed at 330 nm using a UV detector[74].

2.3.23. Antioxidant and biochemical analysis

The serum sample of targeted groups were used to determine the status of oxidant agents including; total antioxidant status (TAS), superoxide dismutase (SOD), glutathione peroxidase (GPx) and Malondialdehyde (MDA). The diagnostic kits manufactured by Randox Laboratories Ltd., Crumlin, County Antrim, UK were used to determine the TAS (mmol of TAS/g of hemoglobin), SOD (U of SOD/g of hemoglobin) and GPx (U of GPx/g of hemoglobin), respectively, and MDA level was measured according to the method of Yoshioka, et al. and was expressed as MDA mol/l. Other biochemical parameters were measured by using fully automated clinical chemistry spectrophotometer SPECORD M40 (Carl Zeiss, Jena, Germany) analyzer.

2.3.24. Plasmid encoding eGFP preparation

The pmax plasmid (pmaxGFP) encoding enhanced green fluorescent protein was kindly gifted of Dr.Heidari, Medical Biotechnology department (TUOMS), Iran. The plasmid pmaxGFP was grown by being transformed in *E. coli*/DH5 α strain cultures for overnight in 2 L shake-flasks containing 250 mL of LB (Luria-Bertani) medium and antibiotics (30 mg/mL of Gentamicin). Then, the purified plasmids were obtained by using a GenElutet Plasmid Miniprep EndoFree plasmid purification Kit (Sigma-Aldrich, Milan, Italy). The purified plasmid was diluted using tris-EDTA (TE) buffer solution and stored at -20 °C. The purity and concentration of the plasmid were determined by NanoDrop

spectrophotometer (ThermoFisher, Waltham, Massachusetts, USA) absorbance at 260/280 nm.

2.3.25. Polymer–DNA complex formation and gel retardation assay

The complex between cationic-CD polymers with pmaxGFP were made before transfection experiments. Different N/P ratios (average number of nitrogen atoms on the cyclodextrin core/number of phosphate groups of pDNA) of polymer/DNA complexes were formed (0.5:1, 1:1, 6:1, 12:1 and 25:1), by mixing the 10 µl of plasmid DNA solution (containing 1 µg of pmaxGFP pDNA in filtered distilled water) and 10 µl of polymer solution (containing varying amounts of polymer in filtered distilled water). To form the complexes, they were mixed and then vortexed for 10 s and incubated at room temperature for 30 min. Finally, obtained solutions were mixed with loading buffer and loaded onto 1% agarose gel for gel electrophoresis conducted in 1× TAE buffer (40 mmol/L Tris acetate and 1 mmol/L EDTA) at 90 V for 45 min. DNA bands were visualized by a UV trans-illuminator (Tamilnadu, India). The N/P ratios were evaluated by using following formulation[111].

$$n = \frac{N}{P}$$

$$C = \frac{n \times P \times M}{X}$$

Where N was the concentration of nitrogen in cationic compounds (nmol/mL), P was the concentration of phosphorus pDNA (nmol/mL), X was the number of nitrogen atoms in cationic

compounds, M was the molecular weight of cationic compounds(g/mol), and C was concentration of cationic compounds (ng/mL).

2.3.26. Determination of pDNA/mRNA Encapsulation Efficiency

To check the encapsulation efficiency of nanoparticle for pDNA and mRNA the ultracentrifugation method was used to determine the difference between the total amount of pDNA/mRNA added in the nanoparticle preparation buffer and the amount of free pDNA/mRNA remaining in the aqueous suspension by ultraviolet spectrophotometry at 260 nm. The concentration of pDNA/mRNA was determined by using $1(\text{OD}_{260 \text{ nm}}) = 50 \mu\text{g}$ of DNA [112]. The pDNA/mRNA encapsulation efficiency (EE) of the process were calculated from equations as indicated below:

$$EE\% = \frac{[(a-b) \times \frac{d}{c} - (a-b)]}{a} \times 100 \quad (1)$$

a: total amount of pDNA/mRNA, b: free amount of pDNA/mRNA, c: nanoparticle weight, and d: total amount of polymer.

2.3.27. Cell culture protocol for 2D cell culture

HT-29 (Passage Number: 3) cell line was obtained from Pasture Institute National Cell Bank of Tehran, Iran. The cells were cultivated in RPMI 1640 supplemented with 10% FBS (FBS, Gibco, USA) and 1% Penicillin/Streptomycin solution (Biochrom GbmH, Berlin, Germany). The flask was incubated in a humidified incubator at 37 °C

containing 5% CO₂. Media was exchanged daily, and for each passage the cells were detached and harvested after trypsinization at 80~90% confluence.

2.3.28. Cell culture protocol for 3D spheroid

The multicellular 3D cancer cell spheroids were made by using the conventional hanging drop method [113]. Briefly, 1.5×10^4 HT-29, 7.5×10^3 HUVEC and 7.5×10^3 Fibroblast cells were re-suspended in 20 μ l of culture medium containing 1% FBS and placed at the inner surface of culture plate lids. The lid and drop containing cells were inverted over the culture plates. To prevent the evaporation of hanging drops the plate were filled with phosphate-buffered saline. The plates were kept at 37 °C under a humidified condition with 5% CO₂. After 4 days when thick and compact micro aggregates with dark edges were generated, the spheroids were transferred into each well of 96-well plates containing 200 μ l of culture medium with 1–2% FBS.

2.3.29. MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide) assay

The MTT assay was used to determine in vitro cell viability according to previous report with slightly modifications [114]. HT-29 cells were seeded in 96-well plates and incubated until cells were 80% confluent, followed by exposure to different N/P ratios of polymer/DNA complexes (1:0.5, 1:1, 1:6, 1:12 and 1:25), by mixing the 10 μ l of plasmid DNA solution (containing 1 μ g of pmaxGFP DNA in filtered distilled water) and

10 μ l of polymer solution (containing varying amounts of polymer in filtered distilled water), and free plasmid DNA for 24 h.

2.3.30. Lactate dehydrogenase (LDH) assay in 2D and 3D cell cultures

Cell membrane integrity was evaluated using the Plus Cytotoxicity Detection Kit (method) according to the manufacturer's protocol with some modifications. Briefly, HT-29 monolayer was seeded in a 96-well plate and incubated until cells were 80% confluent, and one of multilayer 3D spheroid cells were seeded per each well. Cells were thereafter exposed to different N/P ratios (12:1, 25:1, 50:1 and 100:1) containing the same amount of CD-polymer free of plasmid for 24 h. The release of LDH to the supernatant culture medium was collected to determine the occurrence of cell membrane injury. The micro-plate ELISA reader (Biotech, USA, Gen5™ software) at absorbance at 492 nm was used to determine the concentrations of LDH.

2.3.31. Measuring the DNA-polymer transfection using fluorescence microscopy in 2D and 3D

Trypsinized cells were seeded in a 6-well plate with a density 1×10^6 cells per well (2D cell culture model) and 3×10^4 cells per 20 μ L drop in 96-well plates (3D cell culture model) in RPMI medium supplemented with 10% FBS. After 24 h, the culture media was replaced with serum-supplemented media containing 1 and 2 μ g pDNA-eGFP free and complexes with CD-NS at N/P ratio of 25 for 2D and 3D cells, respectively. The

cells were incubated with the complexes and naked DNA for 2, 4, 6 and 24 hours in 2D cell culture model, and, the 3D cells were incubated for 6 and 24 hours. Then, cells were washed twice in PBS. For fluorescence microscopy observation (Cytation 5 Cell Imaging Multi-Mode Reader) cells were fixed by 4% paraformaldehyde followed by nuclear staining with 4', 6-diamidino-2-phenylindole (DAPI) at room temperature for 30 min. in addition, the DreamFect™ Transfection Reagent (Lipofectamine) was used at ratio 3:1 (DreamFect/pEGFP) according to the manufacturer's protocol as a positive control for 24 h on both 2D and 3D cell culture.

2.3.32. Measuring the DNA-polymer uptake using flow cytometry analysis

For this purpose, the monolayer 2D and multilayer spheroids cells were seeded in 12-well plate and incubated for 24 h. After 24 h, the culture media was replaced with serum-supplemented media containing 1 and 2 µg pDNA-eGFP free and complexes with CD-NS at N/P ratio of 25:1 for 2D and 3D cells, respectively. The cells were incubated with the complexes and naked DNA for 2, 4, 6 and 24 hours in 2D cell culture model, and, the 3D cells were incubated for 6 and 24 hours. Then, the 2D and spheroids were washed twice with PBS before being disaggregated by incubating in the Trypsin–EDTA solution. The cells were analyzed by the BD FACS Calibur system to calculate the fluoresce intensity of each group and compared to the non-treated control cells. The raw data were analyzed using FlowJo software (ver. 7.6.1).

Additionally, the DreamFect™ transfection reagent (Lipofectamine) has been used as a positive control for 24 hours in both 2D and 3D cell culture at a ratio of 3:1 (Lipofectamine/pEGFP).

2.3.33. Histopathology method for evaluation DNA-polymer penetration in 3D spheroid cells

Prior to spheroid cell fixation, the cells were incubated with 2 µg pDNA-eGFP in complexes with CD-NS at N/P ratio of 25:1 for 48 h. Then the cells were washed twice with PBS and followed stained by nuclear staining with 4', 6-diamidino-2-phenylindole (DAPI) at room temperature for 30 min. Following procedure was used to preparation the spheroid samples. Multilayer cancerous spheroid suspensions in media were collected from plates, placed into a micro-centrifuge tube, and spun at 1,000 rpm for 2 min to form a pellet. After discarding supernatant phosphate-buffered saline (PBS) was used to wash the participated pellet and centrifuged at 1,000 rpm for 2 min to reform a pellet. The pellet was fixed in 10% neutral buffered formalin (NBF) for 10 min, followed by removal of fixative and replacement with PBS, and stored at 4°C until the time of sample preparation for processing and paraffin embedding. At processing and embedding, the spheroid samples inside the micro-centrifuge tubes with PBS were centrifuged at 1,000 rpm for 5 min. The PBS was removed, and a 1% agarose solution was pipetted into the micro-centrifuge tube containing the pellet, gently dispersed

throughout the agar, and allowed to solidify for at least 1 hr at room temperature. Then, the intact spheroid pellets in agarose were taken from the micro-centrifuge tubes. The suspended pellets were sliced longitudinally and placed into a tissue cassette cut side down. The pellets were processed on a tissue processor and infiltrated with 70% and 80% ethanol for 10 min each, followed by 95% ethanol for 15 min and three changes of 100% ethanol for 10 min each. Next, the pellets were processed in three changes of xylene, 10 min, followed by three baths of paraffin: 15 min in the first bath and 25 min in each of the remaining two paraffin baths and then placed (embedded) in a mold with paraffin. Sections at 5 mm diameter were cut and mounted onto hydrophilic glass slides. Finally, the hydrophilic glass slides were used to understand the penetration of pDNA-eGFP in complexes with cationic CD-polymer in 3D spheroid cells by fluorescence microscopy observation.

2.3.34. Hemocompatibility

Hemolysis activity was tested based on previous study procedure (Zhou et al., 2018). The male Wistar rats were used to collect their blood cells in tubes containing the ethylenediamine tetra acetic acid (EDTA) as an anti-coagulated agent. Red blood cells (RBCs) from serum were isolated by centrifugation (1000 rpm, 5 min). After the supernatant was removed, the pellet was washed several times with phosphate-buffered saline (PBS) (pH = 7.4) to obtain a final concentration of 2% (v/v). 1 mL of RBCs suspension

was combined with 1 mL of CD-polymer at the concentration range of 125, 250, 500 and 1000 µg/mL, and the polyplex complex at different N/P ratio (0:1, 1:1, 6:1, 12.5:1, and 25:1).

2.3.35. Cell culture protocol for 2D and 3D spheroid cell culture

B16-F10 cells (passage number: 4) were obtained from National Cell Bank of Pasteur Institute, Tehran, Iran. Cell growth conditions were provided as in our previous study to create 3D cancer cell spheroids using 3×10^4 B16-F10 cells through the traditional hanging drop method[113].

2.3.36. MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide) assay

The cytotoxicity effect of free-Ppoly at different concentrations was assessed with using the MTT assay according to previous report with slightly modifications[114]. Furthermore, the cytotoxicity effect of different ratios 1:1, 1:5, and 1:10 of free Ppoly and complexes with mRNA was evaluated compared to lipofectamine at 3, 6 and 12 µL concentrations in free and complex with mRNA after 24 h.

2.3.37. Measuring the EGFP (mRNA)-polymer transfection using fluorescence microscopy in 2D and 3D

For the 2D cell culture model and the 3D cell culture model, trypsinized B16-F10 cells were seeded in RPMI medium with 10% FBS at a density of 1×10^6 cells per well and

3×10^4 cells per 20 μ L drop, respectively, in 12-well plates. After 24 hours, the culture medium was changed to serum-free medium containing 1 and 2 μ g of free mRNA-eGFP and complexed with PPoly at a N/P ratio of 10 for 4 hours, respectively, for 2D and 3D cells. Then the cells were incubated for 20 hours in media containing 10% FBS. Next, PBS was used to wash the cells twice. Finally, the cells were fixed with 4 percent paraformaldehyde for fluorescence microscopy observation (Cytation 5 Cell Imaging Multi-Mode Reader), and then nuclear staining with 4', 6-diamidino-2-phenylindole (DAPI) was performed at room temperature for 30 minutes. Additionally, the manufacturer's instructions were followed using the DreamFect™ Transfection Reagent (Lipofectamine) as a positive control for 24 hours on both 2D and 3D cell culture. Furthermore, the same procedure was used to quantify the cellular uptake of EGFP-mRNA in free and complexes with PPoly at ratio 1:10, and Lipofectamine has been used as a positive control for 24 hours in both 2D and 3D cell culture at a ratio of 3:1 (Lipofectamine/EGFP) by using flow cytometry. The BD FACS Calibur system was used to assess the cells and determine the fluorescence intensity of each group, which was then compared to the untreated control cells. The software FlowJo was used to examine the raw data (ver. 7.6.1).

2.3.38. In vivo biodistribution of the EGFP-mRNA complexes with PPoly

The Tabriz University of Medical Sciences carried out the live animal experiments in accordance with all applicable laws and institutional regulations. Female C57BL6/J

mice that had 5.5 weeks old and tumors were established by injecting a suspension of B16-F10 cells (1×10^5 cells/ml) subcutaneously (S.C.) to form the melanoma cancer[101]. 100 μ L of EGFP-mRNA (10 μ g) and in complex with PPoly (contain the same quantity of free mRNA) at ratio 1:10 were injected intratumorally when tumours reached a volume of about 400–500 mm³. The vivo imaging system (Kodak live animal imaging) was used to determine the quantity of fluorescent intensity at a predetermined time points.

2.3.39. Experimental design and tumor induction

Six to eight-week-old female C57BL6/J mice were obtained from Animal institution, Zanjan, Iran. B16-F10 cells (1×10^5 cells/ml) were administered subcutaneously (S.C.) into the mice to establish melanoma cancer [101]. All mice were housed in the animal facility of the department of Pharmacy under pathogen-free circumstances and were cared for in line with the ethical guidelines defined by the Tabriz University of Medical Science. sixteen mice were divided into four main groups at random: the untreated tumors, free-OVA mRNA, free-Ppoly, and OVA- mRNA in complexes with PPoly groups. When tumors appeared, mice have received four dosages of free-OVA mRNA (7 μ g), and in complexes with PPoly at N/P at ratio 1:10 every four days, as properly as the same volume of PBS for the control group by using intra-tumoral injection. After that, mice were sacrificed after 32 days and the tumor size was measured every day with a digital caliper. At the end of the treatment period, the melanoma tumors were

removed from the mice and weighed. Thereafter, the tumors' width, length, and height were measured using calipers, and the volume of the tumors was determined using the formula: $\text{length} \times \text{width}^2 \times 0.52$ [102].

2.3.40. Detection of in vivo immune system cell responses in tumor and spleen by flow cytometry

Following the prior procedure, mouse tumors and spleens were aseptically removed after anesthesia and used to make single cell suspensions using 70 μm filters (BD Biosciences)[102, 115]. A sharp scalpel was then used to cut the tumours and spleens in pieces. Each tumour and spleen tissue were quickly placed in 10ml of the dissociation buffer (100 U/ml of collagenase IV and 100 $\mu\text{g}/\text{ml}$ DNase I in RPMI media with 10% FBS), then stored at 37 °C in an incubator for 30 to 40 minutes. Once the 70 μm strainer was wet after adding 1 ml of cold medium to a 50 ml falcon tube, the spleen or tumors were placed on it. The organs were then lightly mashed before being passed through strainers to create single cell suspensions. The splenocytes and tumor cells were then collected by washing the strainer with 4 ml of cold medium. The media was aspirated off after the cells were centrifuged at 250 g for 5 min at room temperature. Finally, 1 ml of media was used to resuspend the cell pellet and the purified cells were carefully collected and the resulting washed one time with FACS

buffer. cells were then counted to take 2×10^6 cells per FACS tube for the respective sample.

2.3.41. Antibodies and flow cytometry

The single separated of spleen and tumors of treated groups were incubated with antibodies in staining buffer (0.1 M PBS, 1 percent FCS, and 0.1 percent sodium azide) for 30 minutes on ice in order to stain cell surface markers. The cells were stained with CD4 (GK1.5, PE), CD19 (eBio1D3 (1D3), PE), CD11b (M1/70, PE), and NK1.1 (PK136, FITC). Furthermore, the cells were incubated with tetramer (SIINFEKL-H2Kb APC) and CD8a monoclonal antibody (53-6.7) (BD Biosciences) to determine tetramer+ CD8+ T cells by flow cytometry assay. The cell percentages were evaluated using FlowJo, and the graphs were made using GraphPad Prism.

2.3.42. Determination of antigen-specific antibodies in serum

By using an enzyme-linked immunosorbent (ELISA) test, levels of TNF- α , INF- γ and OVA-specific antibodies, including two subtypes of IgG, IgG1 and IgG2a, were measured. On day 32, serum samples were taken and kept at 4 °C. For, OVA-specific antibodies procedure was following, OVA proteins were applied to 96-well polystyrene microplates and incubated for an overnight period at 4 °C. After serially diluting serum samples two times in blocking buffer, they were added to the washed microplates. The prediluted horseradish peroxidase-conjugated goat antibodies to mouse IgG, IgG1, or IgG2a (Abcam, USA) were added to the microplates after 2-h incubation at

37 °C. Tetramethylbenzidine substrate was added after such an additional two hours of incubation, and the chromogenic process was subsequently inhibited by 2MH₂SO₄. After that, a microplate reader assessed each well's optical absorbance at 450 nm (Tecan, Switzerland). Furthermore, for assessment of the adjuvanticity of Ppoly experiments were conducted according to the appropriate laws and with the approval of Animal Experiment Committee of the Institute of Medical Science, The University of Tokyo. Six-week-old female C57BL6/J mice were obtained from CLEA, Japan. OVA protein (10 µg) were mixed with Ppoly in 20 µL of PBS. Then, the mice were injected the sample solutions by the intramuscular route on day 0 and 14. On day 28, the serum was collected from the immunized mice. OVA-specific antibodies were measured as mentioned above by using horseradish peroxidase-conjugated anti-mouse IgG, IgG1 or IgG2c antibody (Southern Biotech, USA). Titers of OVA-specific antibodies were determined by log-linear interpolation of the serum dilution value corresponding to cut-off absorbance (OD₄₅₀ of 0.2).

2.3.43. Statistical analysis

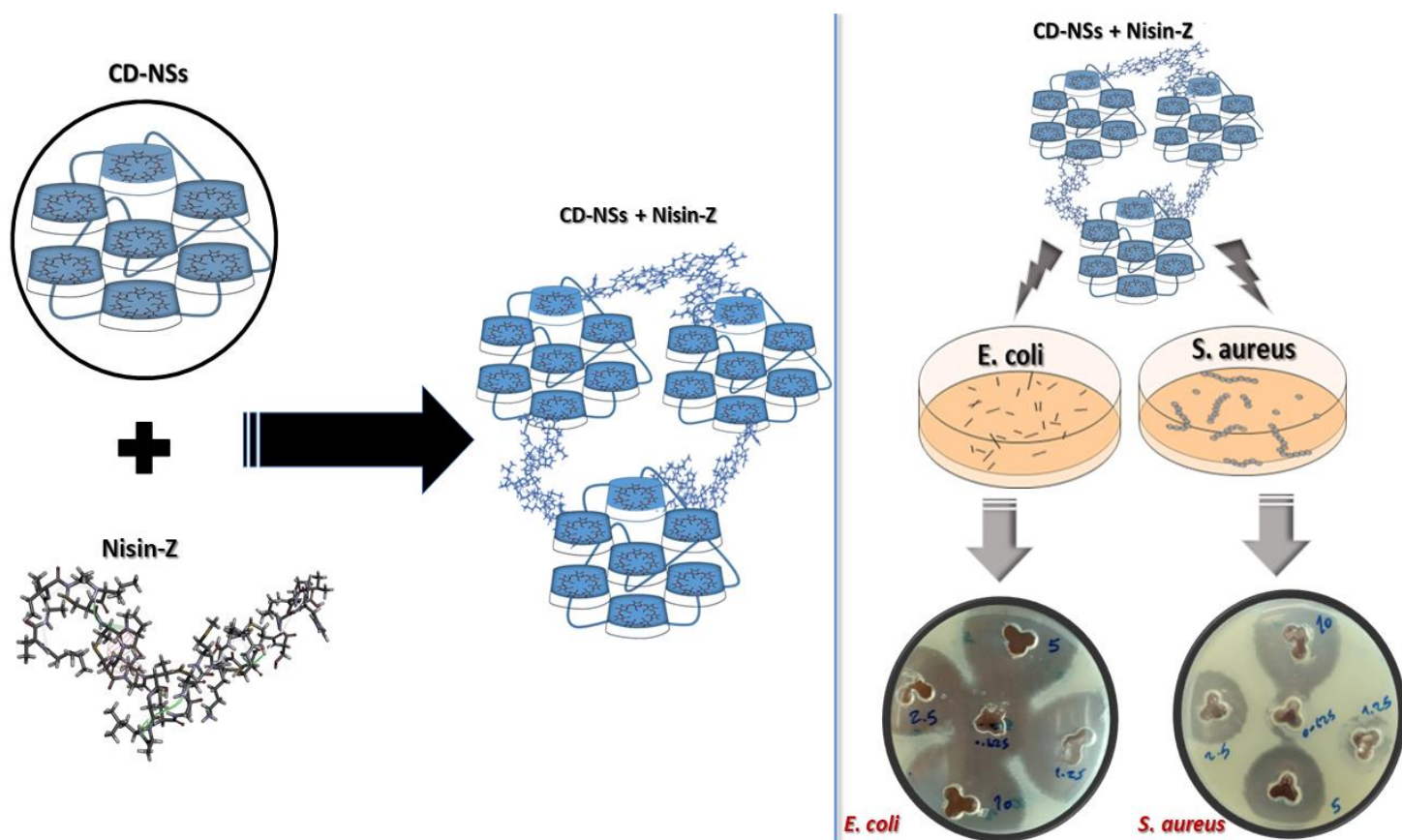
Statistical analysis was conducted using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA). Data were analyzed using one-way ANOVA Analysis of Variance, then the Dunnett's multiple comparisons test was used. All the samples were presented as mean ± standard deviation (SD) for n = 5 or 3. The level of significance was calculated by p-value. Statistically, P value <0.05 was considered significant.

Chapter 3

Biological Applications of β CDNS and Hyperbranched Cyclodextrin polymers (PPoly)

Section 1: β CDNS-CDI and PMDA for peptide delivery: Nisin-Z as an example

First Project: *Cyclodextrin-Based Nanosponges as Perse Antimicrobial Agents Increase the Activity of Natural Antimicrobial Peptide Nisin*



Graphical Abstract

Aim of study:

At present, antibiotic resistance is considered a real problem. Therefore, for decades scientists have been looking for novel strategies to treat bacterial infections. Nisin Z, an antimicrobial peptide (AMP), can be considered an option, but its usage is mainly limited by the poor stability and short duration of its antimicrobial activity. In this context, cyclodextrin (CD)-based nanosponges (NSs), synthesized using carbonyldiimidazole (CDI) and pyromellitic dianhydride (PMDA), were chosen for Nisin Z loading. To determine the minimum inhibitory of nisin Z loaded on CD-NS formulations, agar well diffusion plates were used. Then, the bactericide concentrations of nisin Z loaded on CD-NS formulations were determined against Gram-positive (*Staphylococcus aureus*) and -negative (*Escherichia coli*) bacteria, using microdilution brain heart infusion (BHI) and tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

3.2.1.1. Introduction

Management of bacterial infections has emerged as a phenomenon that weakens our ability to fight them because they constantly develop a new set of mechanisms that enable them to resist the inhibitory action of antibiotics[116]. The main mechanism of antibiotics inhibition against microbes' growth is related to the prevention of the protein or cell wall synthesis in case of infectious conditions, but the most visible limitation for well-established therapeutic antibiotics is related to bacteria resistance, in addition to their inefficient delivery at the specific site [117]. Therefore, the most recommended approach implies higher doses or combinational drug therapy to increase drug efficiency[118, 119], which sometimes present adverse effects. It is clear that the timing of antibiotic resistance is important, and addressing how to better slow drug resistance requires an interdisciplinary approach. The traditional medical subject boundaries no longer act as barriers to sharing ideas and concepts in the broad field of biology. Novel strategies, such as the use of novel excipients with intrinsic antimicrobial activity, provide a preferable and strategic alternative to finding new therapeutic approaches that escape or prevent resistance.

For example, one of the most commonly used antimicrobial peptides (AMPs) in the food industry is nisin Z, which is made by *Lactococcus lactis* strains and contains 34 amino acids (Figure 11)[118]. It has a 3.5 kDa molecular weight, with a broad

spectrum of antibacterial activity against Gram-positive bacteria. Owing to its low level of toxicity for humans, nisin Z has been considered as a “generally recognized as safe” (GRAS) peptide in the food industry by the FAO/WHO[120, 121]. The main mechanisms of nisin Z to kill bacteria are based on the formation of pores in the membrane, and the inhibition of cell wall biosynthesis by binding to lipid II, a part of the membrane-anchored cell wall of bacteria that influences the cell-wall synthesis. This facilitates the transmembrane orientation of nisin[122, 123]. Therefore, the use of nisin as a popular and natural antimicrobial agent in dairy, fruit juices, meat, and plant products has increased due to its capacity against various types of microorganisms[124].

Nisin has achieved substantial prominence in the food business as a substitute bio preservative since its discovery. The use of nisin has started to diversify, however, to encompass a wide range of unrelated applications, such as those relating to the biomedical industry, as a result of the substance's shown safety during the previous 40 years (Figure 11).

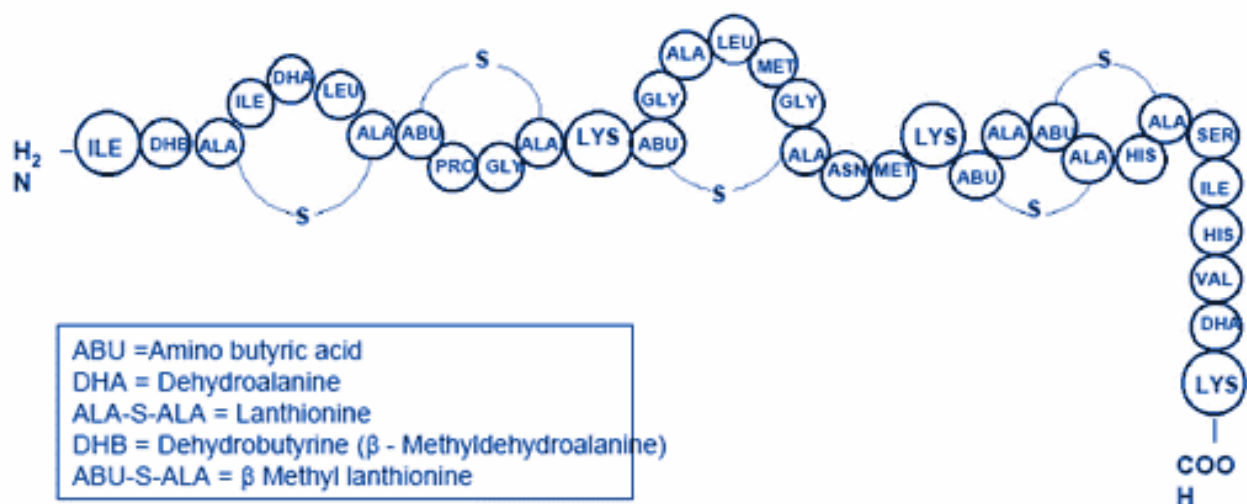


Figure 10. Peptide Structure of Nisin-Z. Modified amino acids indicated of amino acid substitutions for natural variants.

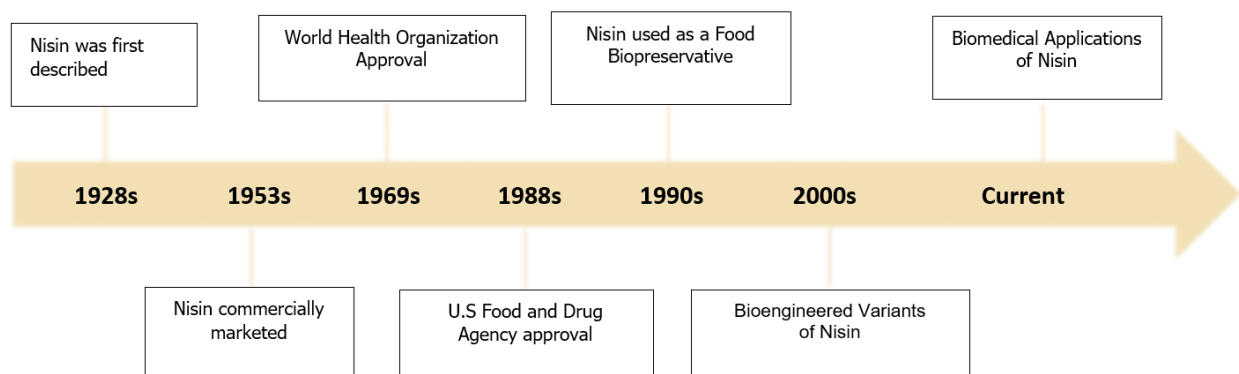


Figure 11. Timeline of nisin development

Nevertheless, the uncontrolled antibacterial function during food storage, sensitivity to environmental stresses, sensitivity to proteolysis, and adverse interactions with food components, are the most common nisin-Z challenges that prevent their potential therapeutical applications[124]. To overcome these challenges, several nano-delivery systems based on proteins, polysaccharides, and lipids have been proposed, allowing the sustained release of nisin Z. The effect of nanoencapsulation systems to increase the release and bioavailability of targeted compounds has been reported [120, 124, 125].

The biopolymers, due to their biodegradability, biocompatibility, and nontoxicity, have attracted considerable attention as a delivery system for nisin Z and other bioactive agents, in food packaging, to decrease unpleasant taste and increase their shelf life [121, 126, 127]. Among the most common biopolymers that have been used for delivering bioactive agents are cyclodextrins (CDs), because of their low cost and high biocompatibility [32, 36, 128]. Natural CDs have been considered as “generally recognized as safe” (GRAS) in food products such as food additives and supplements [36, 129]; moreover, CD-based polymers can provide promising food applications [34]. Meanwhile, recently, the prevention of Gram-positive and -negative bacterial growth [130], and the induction of *Mycobacterium tuberculosis* (Mtb) infection in mice [131] by β -CD-based NSs has been investigated. In addition, β -CD, PEI, and

phosphonitrile chloride trimmer (PNC) are assembled to produce antibacterial nanoparticles (Eb-CDNs) that can be utilized as effective agents for wastewater treatment. Interestingly, they have shown remarkable antibacterial activity (99.9%) for eliminating the harmful pathogens (*E. coli* and *S. aureus*) that can be found in waste water [132].

Subsequently, the aim of this study was the evaluation of the antimicrobial activity of nisin-Z-free formulation, as well as nisin loaded on native β -CD and on PMDA and CDI-based NSs. The PMDA- and CDI-based NS formulations can be further considered as promising strategies against bacteria such as *Escherichia coli* (*E. coli*), and *Staphylococcus aureus* (*S. aureus*).

To summarize the above-mentioned statements, the objectives of this study are as follows:

1. The enhancement of the antibacterial effect of nisin Z complexed with CD-based NSs and β -CD;
2. The understanding of the antibacterial activity of CD-NSs and β -CD;
3. The evaluation of the influence of CD-NSs and β -CD for enhancing the conservative effect of nisin Z on cooked chicken meat and preventing the decomposition of nisin in present pepsin.

3.1.1.3. Results and Discussion

3.1.1.3.1. Characterization and In Vitro Release

Firstly, a DSC was carried out to understand the complex formation. The changes in the thermogram would indicate the formation of the inclusion complex. As Figure 12A shows, the variations of both CD-NS around 80–100 °C due to intrinsic water evaporation, might justify the formation of the complex, although it is not clear. On the other hand, the SEM images showed that Nisin-Z should be included in the CD-NSs due to the morphological changes, from irregular structure to regular sphere (Figure 12B–E). However, the confirmation was found with the increase in Z-average and the variation in the Z-potential (Table 3). Despite of the considerable PDI due to the polymeric nature of the material, the complexation of Nisin created a bigger particle than free NSs and the cationic charge of Nisin would decrease the Z potential of free NSs, as reported the experimental data and bibliography for other proteins and NSs [133]. These points justify the formation of the complex between Nisin and CD-NSs due to the intrinsic charge of Nisin. Indeed, good encapsulation efficiency of 90% was found under optimized conditions. The results of SEM study relatively confirmed the complex formation between nisin Z and both nanosponges while providing the clearest evidence regarding DLS results and the effect of both NSs to change the release profile of nisin Z, as is supported by different publications [87, 134]. In this

study, a DSC method was carried out to determine the complex formation, with unsuccessful results; for this reason, we have used the FTIR as another validation test, but the results were unclear (Figure 13A). The profiles suggested evidence of complexation in the case of β -CD around 1250–1750, where the shape of the β -CD peaks changed considerably, possibly due to the presence of nisin. On the other hand, the spectra in presence of NSs did not show differences between complex and free NSs, possibly due to the larger structure of NSs. Finally, the release profile of Nisin from both β CDNSs was checked at pH 7.4 and 4 as an acidic pH, where food-related bacteria tend to exist. The results showed a clear effect of complexation in presence of NSs, with no statistical differences between both NSs. The pH effect was strongly related to the release capacities. We have found that at pH 4 the release was slightly higher, even with the nisin-free formulation (Figure 13B) comparing to pH 7.4 (Figure 13C). This is because the intrinsic solubility of nisin is higher at acidic pH than that in physiological conditions [135]. These results corroborate and support the idea of the formation of complexes between β CDNS and Nisin, as well as their capacity to control release.

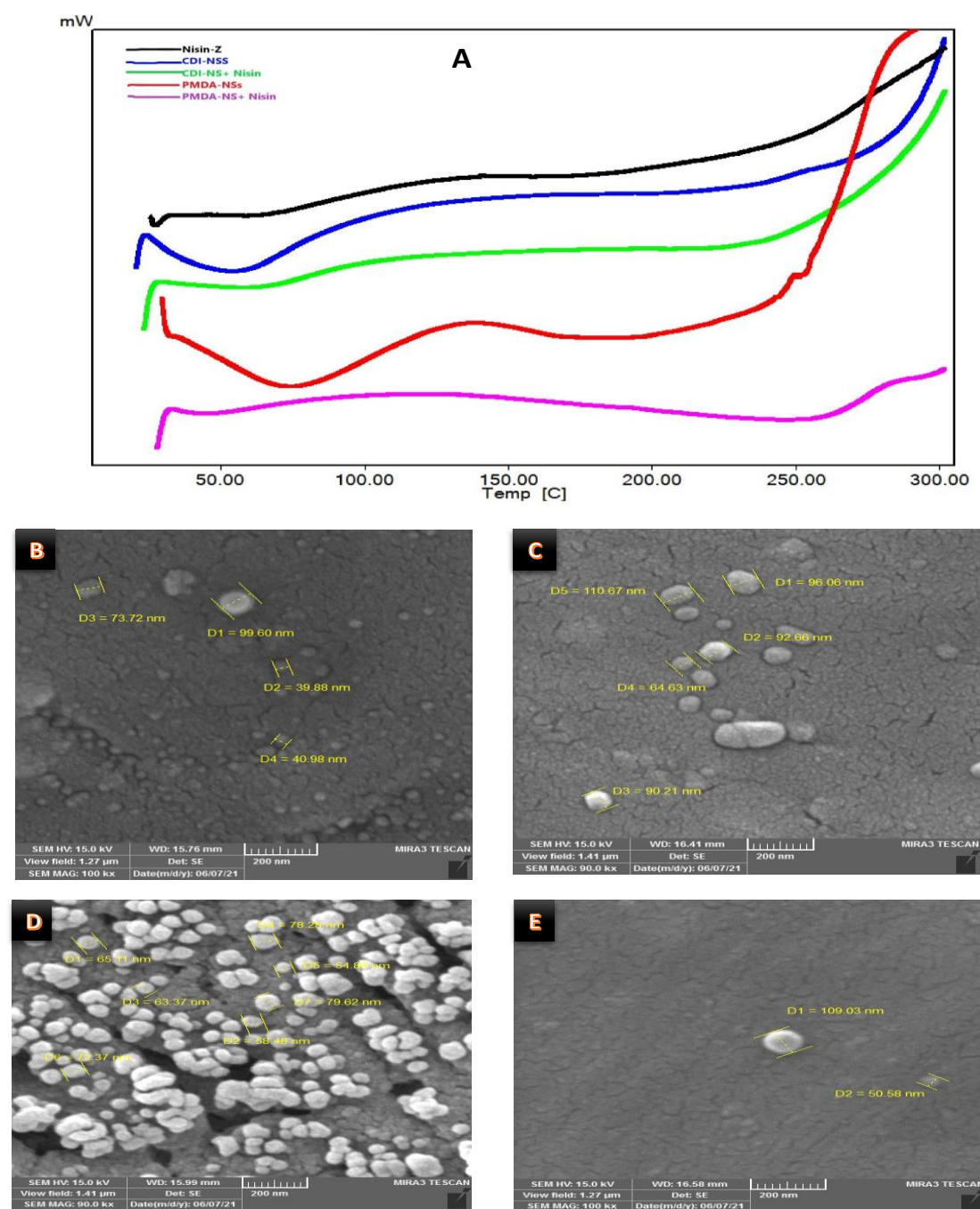


Figure 12. Physicochemical characterization of Nisin-Z and complexes. (A) DSC thermograms of Nisin, Nisin/PMDA-NSs, and Nisin/CDI-NSs, SEM images; (B) CDI-NSs, (C) Nisin/CDI-NSs, (D) PMDA-NSs, (E) Nisin/PMDA-NSs.

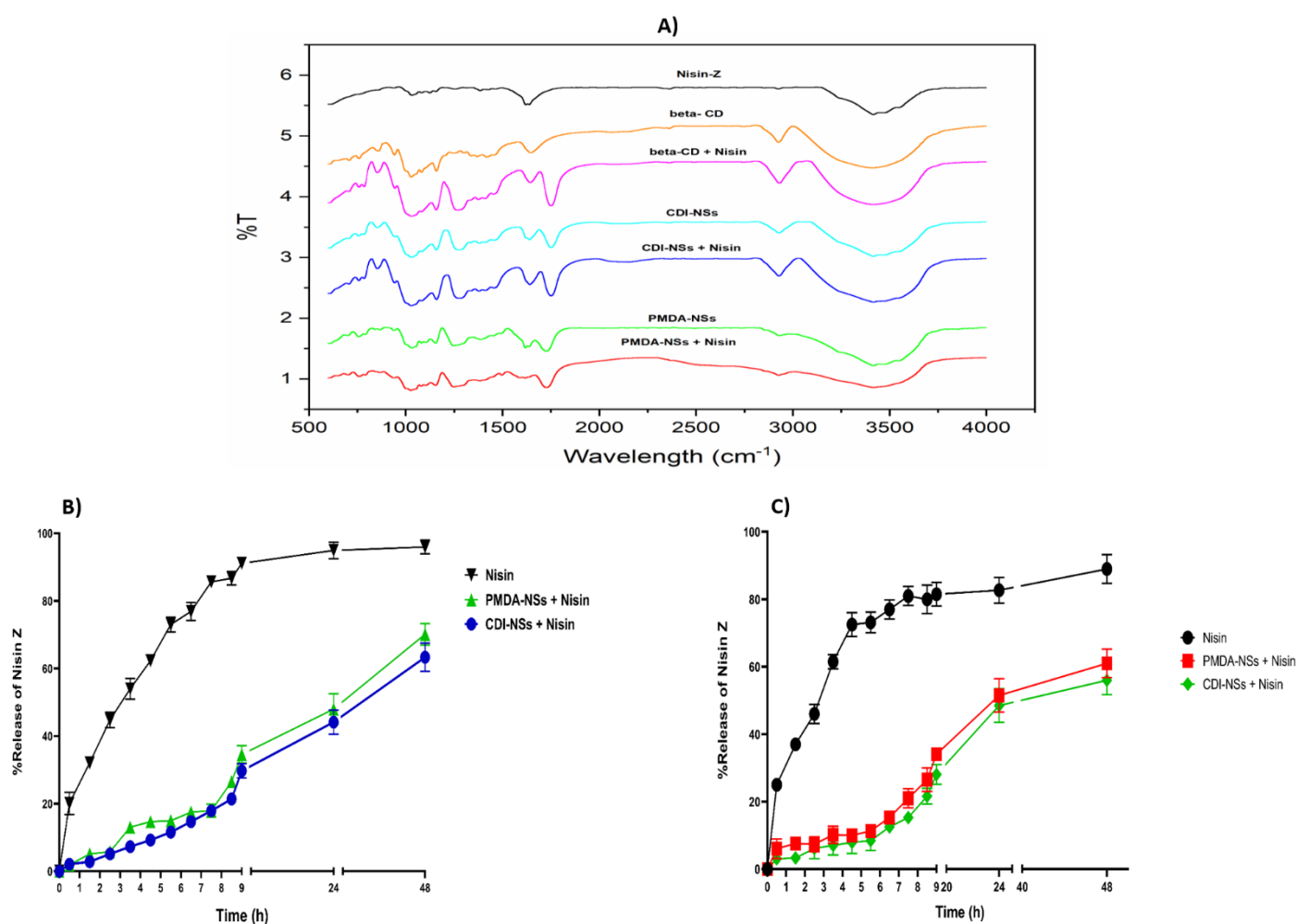


Figure 13. (A) FTIR spectra of samples; (B) release profiles of nisin and nisin-loaded PMDA/CDI-NSs (dissolution medium: phosphate-buffered solution, pH 4, temperature: 37 ± 0.5 °C, rotation speed: 150 rpm); (C) release profiles of nisin and nisin-loaded PMDA/CDI-NSs (dissolution medium: phosphate-buffered solution, pH 7.4, temperature: 37 ± 0.5 °C, rotation speed: 150 rpm; obtained from [33] with permission from Polymers, MDPI, 2022).

Table 3. Particle size and Zeta potential of drug and drug-loaded nanosponges (error reported as SMD).

Carriers	Z-Average (nm)	Zeta Potential (mV)	PDI	EE (%)	DL (%)
CDI-NSs	164.3 ± 1.2	-16.5 ± 0.3	0.22 ± 0.04	-	-
CDI-NSs + Nisin	187.8 ± 2.4	-13.8 ± 0.3	0.41 ± 0.07	91	22.74
PMDA-NSs	308 ± 0.9	-20 ± 0.5	0.41 ± 0.02	-	-
PMDA-NSs + Nisin	369 ± 3.6	-14.0 ± 0.6	0.54 ± 0.09	92	23.3

3.1.1.3.2. Inhibition Zone Diameter

Inhibition zones of nisin-free and nisin complexed with PMDA, CDI, and β -CD, and drug-free formulations (Figure 15) against *S. aureus* and *E. coli* were investigated. As shown in Figure 14, the nisin-free sample exhibited a better inhibition zone against *S. aureus* as a Gram-positive bacterium than against *E. coli* as a Gram-negative bacterium, which showed negligible inhibition zone even at the highest concentration, as reported previously [135, 136]. As a promising approach in food science in recent years, various studies have revealed the effect of different nanoparticles to improve the inhibition activity of nisin against bacteria, especially Gram-positive bacteria, when compared with nisin-free forms[137-139]. This fact is in agreement with the results of the present study, which showed that the complex form in PMDA and CDI increased the inhibition zone approximately 10 times more than the nisin-free form at 5000 $\mu\text{g/mL}$ for both strains ($p < 0.001$). However, the complex of nisin with β -CD indicated a less inhibitory effect. Perhaps due to the affinity of β -CD with some membrane components [140]because β -CD and the polymers presented some antibacterial

capacity, which was higher in the polymeric material. A possible explanation would be the higher number of CD units in the polymer, which would increase the affinity with these molecules, justifying the observed differences. Moreover, the higher entrapment of water into the NS structure might cause a prolonged release of antimicrobial agents such as nisin, leading to a better antibacterial action of nisin in forms complexed with NSs rather than those with β -CD [87].

The results of the present study showed that nisin complexed with CDI-NSs can inhibit the growth of *E. coli* significantly better than the nisin-free form, even at the lowest concentration, which can be attributed to (i) a better formulation that increases the activity of nisin or (ii) the antibacterial combinatorial effect of nisin and the nanocarrier, because drug-free CDI-NSs illustrated a notable bacterial inactivation depending on the concentration (Figure 14). These results match with the results of Desai et al., indicating that CDI-NSs with drug-free formulations considerably inhibited the growth of *E. coli* at 5 mg/mL, by about 78.9% [130, 141]. This antibacterial effect of CDI-NSs against *E. coli* seems to be associated with the interaction among the outer membrane, lipopolysaccharide, or other biomolecules of the bacterial cell membranes because it is presumably the most sensitive site to be attacked by cyclodextrins and its derivatives [141].

These potential antibacterial effects of nisin entrapped in both NSs and β -CD against *S. aureus* and *E. coli* have been demonstrated with other antimicrobials. For example, allyl isothiocyanate (AIT), a food preservative agent, complexed with β -CD, showed a considerably better antimicrobial effect than unentrapped AIT [142]. Another study showed that the antimicrobial activity of chlorhexidine against Gram-negative bacteria was remarkably enhanced by cyclodextrin encapsulation; indeed, the results of TEM and SEM showed cell membrane structural changes and ultrastructural membrane swelling [143]. Recent data manifested new antibacterial aspects of CDs, specifically α -cyclodextrin, which showed a strong ability to inhibit bacterial activities by changing their communications through blocking the quorum sensing [144].

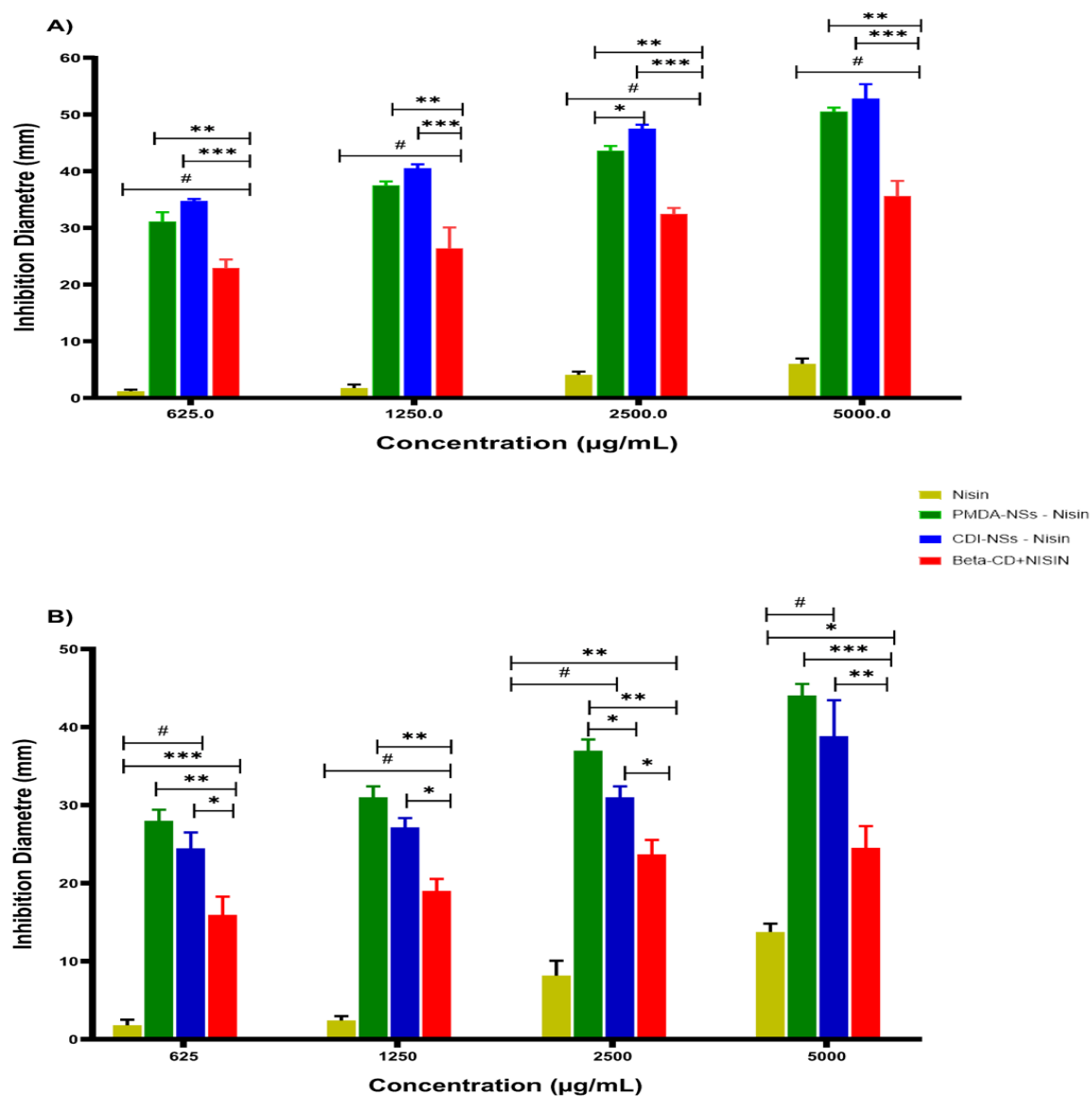


Figure 14. The inhibitory activity (appearance) of nisin Z, PMDA-NSs + nisin Z, CDI-NSs + nisin Z, β -CD + nisin Z. It was evaluated by measuring the growth inhibition zones at different concentrations against *E. coli* (A) and *S. aureus* (B) in well agar diffusion assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.0001$; ns, not significant. The cited concentrations refer to final nisin concentration; see Section 2.3.1.

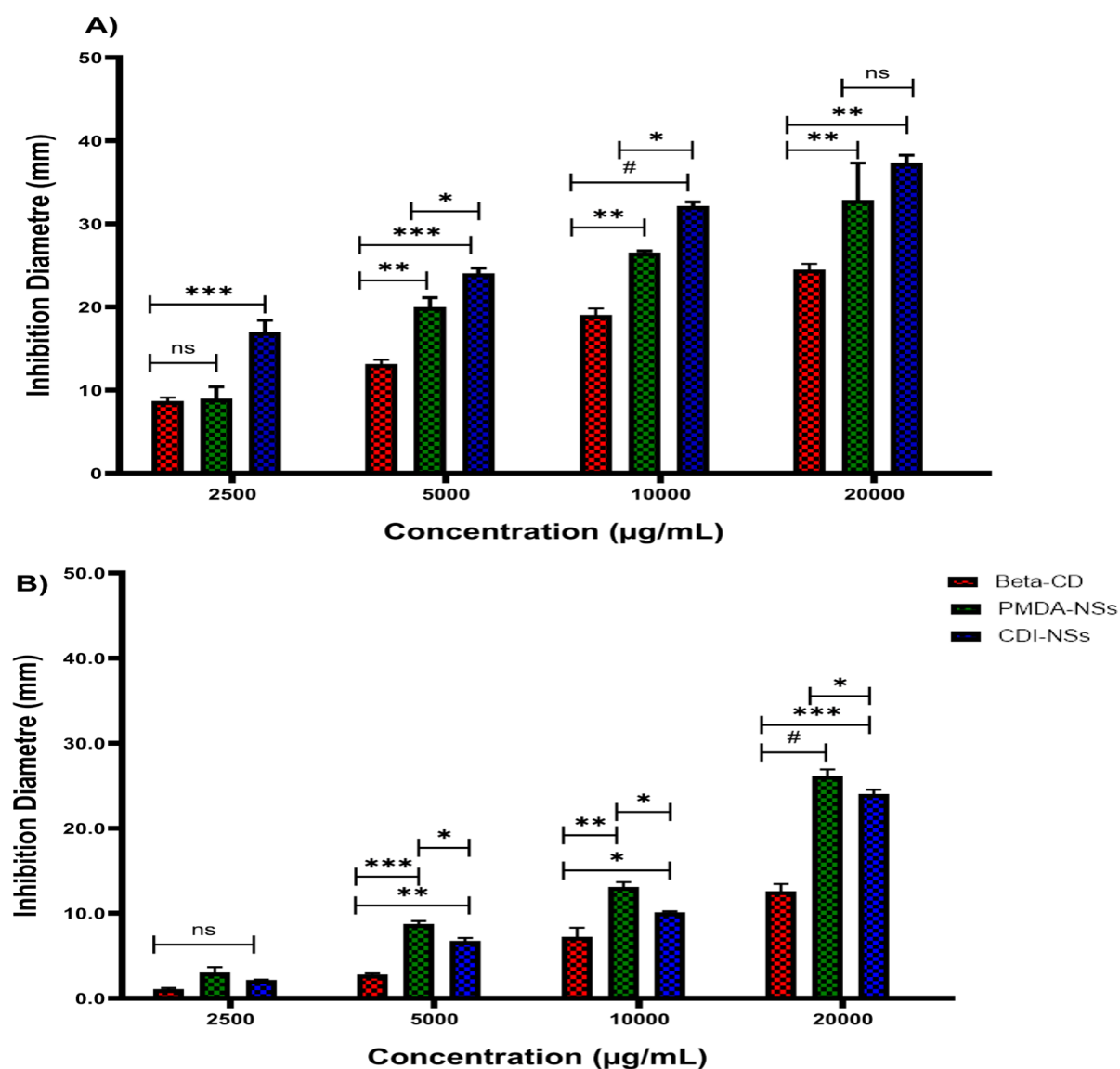


Figure 15. The inhibitory activity (appearance) of CDI-NSs, PMDA-NSs and β -CD free of drugs. It was evaluated by measuring the growth inhibition zones at different concentration against *E. coli* (A) and *S. aureus* (B) in well agar-diffusion assay. *P<0.05, **P<0.01, ***P<0.001, #P<0.0001 and ns (Not Significant).

3.1.1.3.3. Inhibition of Bacteria Growth Rate in BHI

BHI broth was used to evaluate bacterial growth at determined time intervals. According to the growth curves, the nisin-free sample could not effectively delay the bacterial growth rate of *E. coli*, even at the highest concentration. However, *S. aureus* strain growth showed a decline with the nisin-free sample, only at 2500 and 5000 µg/mL, whereas the results illustrated a remarkable decrease in bacterial growth, for both strains to the lowest extent at the determined time intervals, when they were exposed to nisin encapsulated with the PMDA and CDI, while the nisin-free sample did not show effective antibacterial activities at mentioned concentrations (Figures 16 and 17). In addition, a considerable bacterial inactivation at concentrations 2500 and 5000 µg/mL of nisin loaded with β-CD against both strains was observed. The main mechanism effect of nisin against bacteria occurs through direct contact of nisin with the bacteria [145, 146]. It has been reported that CDI-NSs as stable nanosponges formulations are promising carriers for antibacterial protein and prevent depletion of calcium in antibiotic-associated hypocalcemia conditions [147].

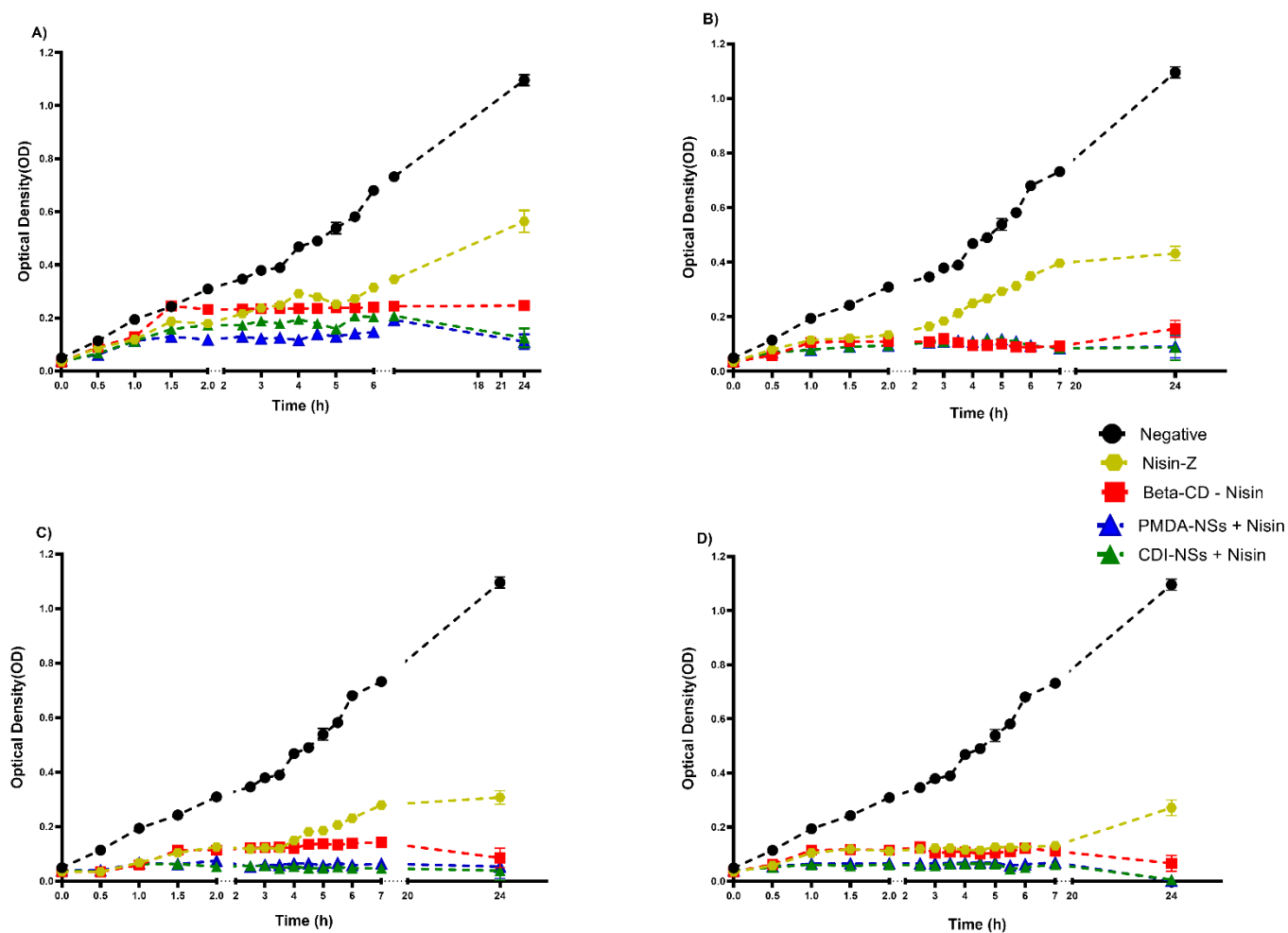


Figure 16. Growth curve of antibacterial activity of nisin-free and complexed in formulation samples against *E. coli*, compared with control and measured by optical density (600 nm) as a function of time: (A) 625, (B) 1250, (C) 2500, and (D) 5000 µg/mL. All data are expressed as mean $\pm$ standard deviation ($n = 3$). The cited concentrations refer to final nisin concentration; see Section 2.3.1.

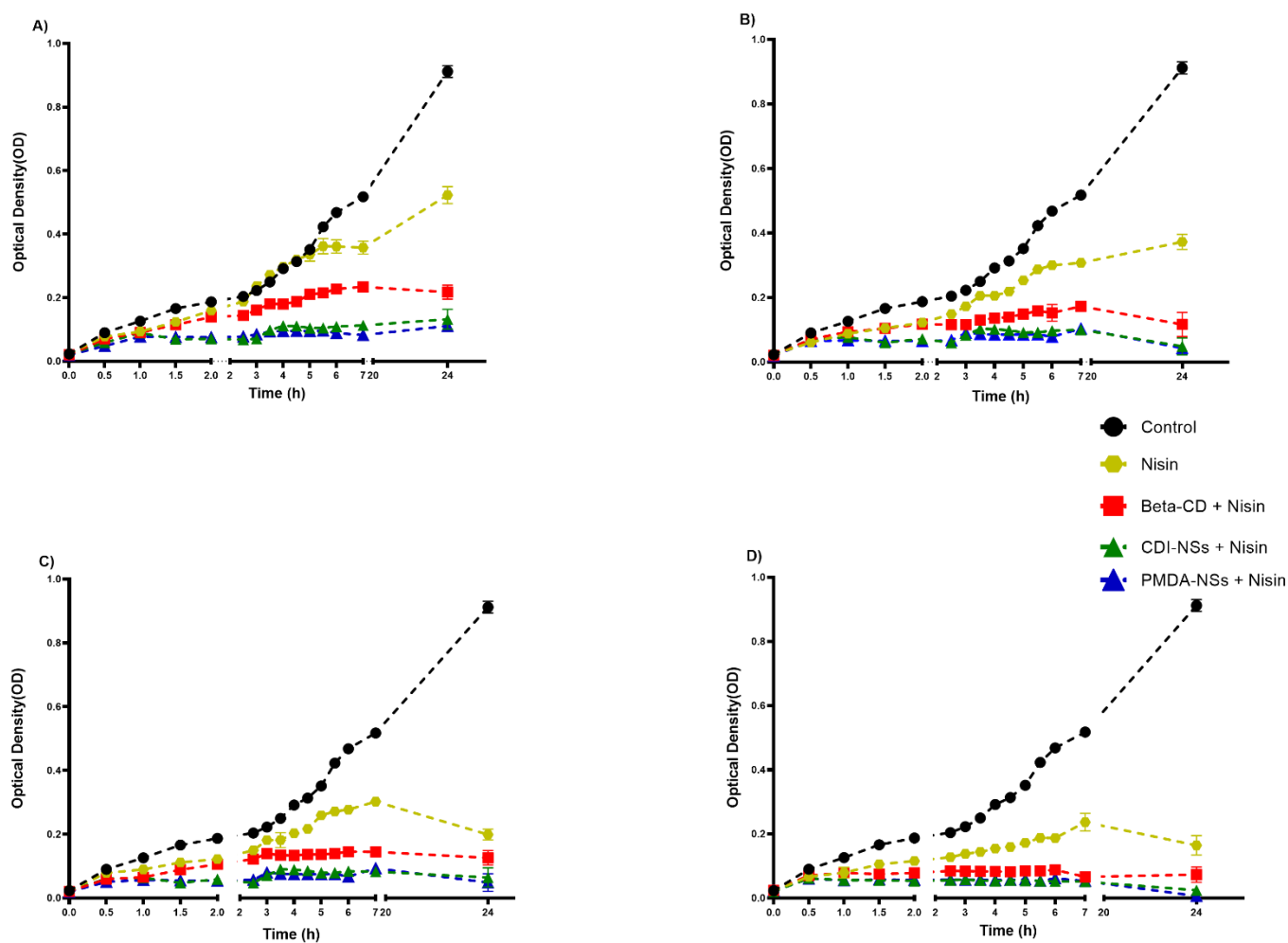


Figure 17. Growth curve of antibacterial activity of nisin-free sample and samples complexed in formulations against *S. aureus*, compared with control and measured by optical density (600 nm) as a function of time: (A) 625, (B) 1250, (C) 2500, and (D) 5000 $\mu\text{g/mL}$. All data are expressed as mean $\pm$ standard deviation ($n = 3$). The cited concentrations refer to final nisin concentration; see Section 2.3.1.

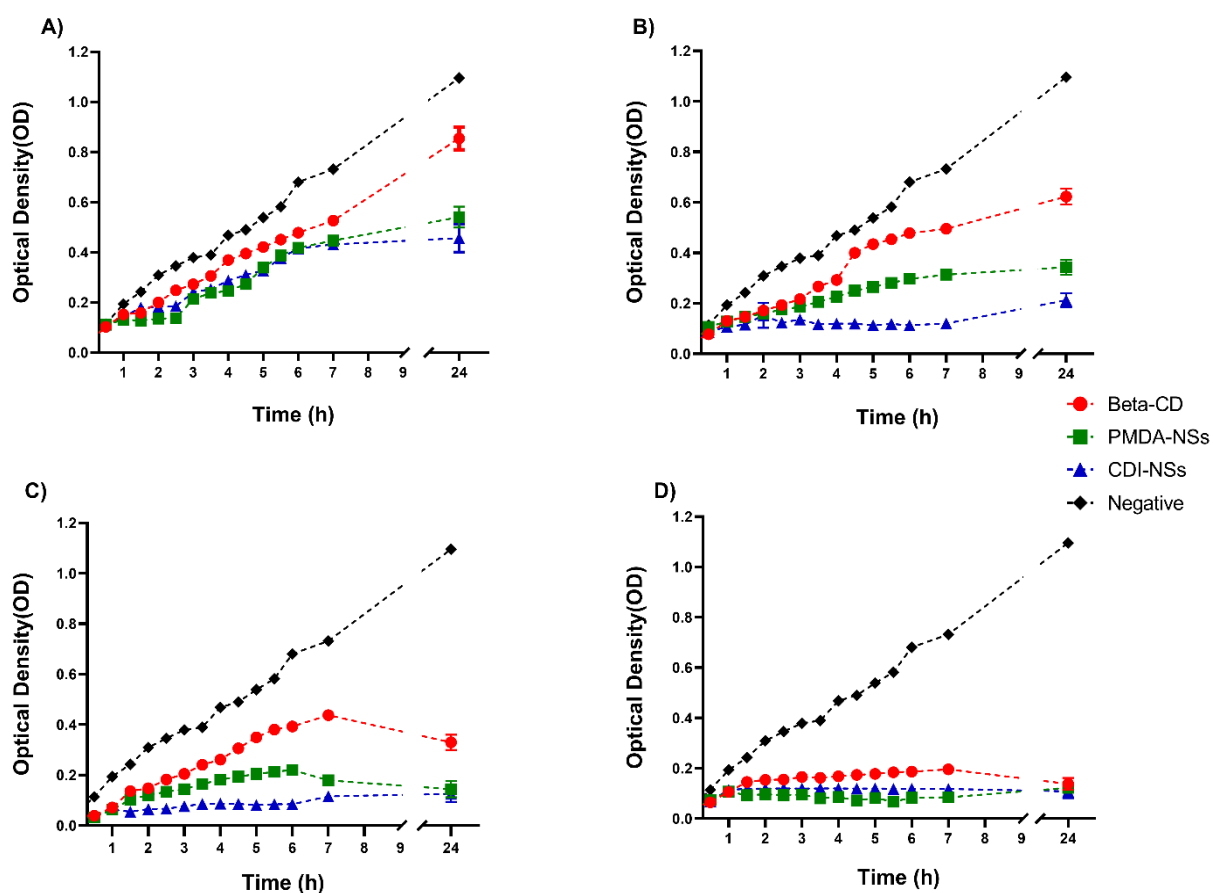


Figure 18. Growth curve of antibacterial activity of drug free formulations against *E. coli* compared to control measured by optical density (600 nm) as a function of time. A: 2500, B: 5000, C: 10000 and D: 20000 $\mu\text{g/mL}$. All data are expressed as mean $\pm$ standard deviation ($n = 3$).

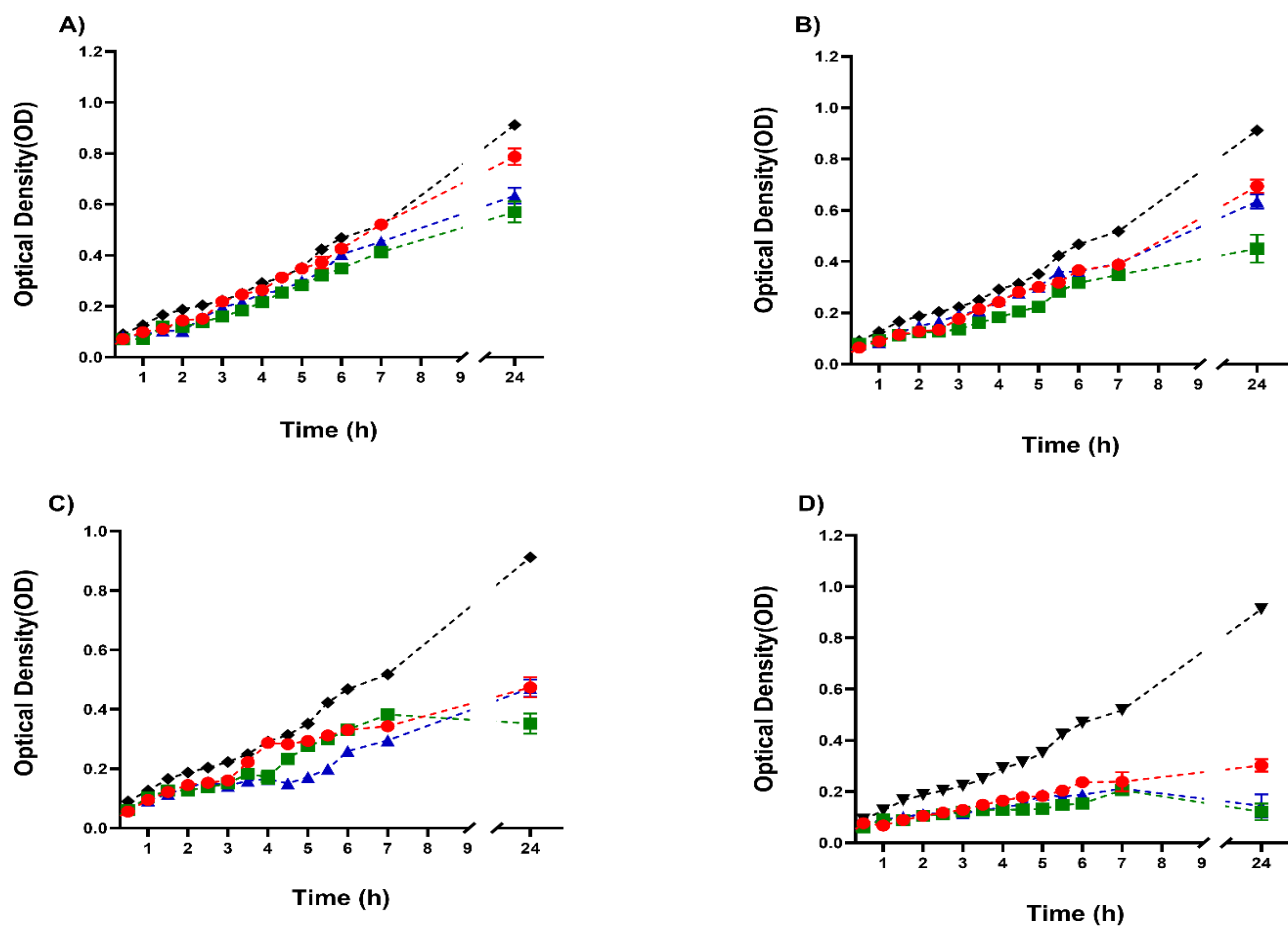


Figure 19. Growth curve of antibacterial activity of drug free formulations against *E. coli* compared to control measured by optical density (600 nm) as a function of time. A: 2500, B: 5000, C: 10000 and D: 20000 µg/mL. All data are expressed as mean $\pm$ standard deviation ($n = 3$).

Interestingly, drug-free CDI-NSs indicated strong growth inhibition against *E. coli* strains after 24 h incubation at 10,000 and 20,000 µg/mL (Figure 18), whereas PMDA

and CDI-NSs showed this inactivation against *S. aureus* (Figure 19) at the last concentration. In contrast, the β -CD sample alone illustrated an inhibitory effect only at 20,000 $\mu\text{g/mL}$ against *E. coli* and *S. aureus*, and it did not show a considerable inhibitory effect for other concentrations, in agreement with the results of agar well diffusion (Figure 15). Furthermore, results of formula size illustrated that the CDI-NS sample had a size of 164.3 nm, which was smaller than other sizes. A strong inhibitory effect of CDI-NS formulation against *E. coli* may be related to its smaller size, as it has been reported that smaller nanoparticles have better bacterial inhibition since they have the highest level of contact with bacteria, so they can easily reach the cytoplasmic content of bacteria [148]; this fact may suggest a higher encapsulation of essential component of bacteria, which would increase the efficacy of the material. The results of this study exhibited antibacterial activities corresponding to rises in both concentration and time.

3.1.1.3.4. MIC and MBC

Table 4 shows the actual representative MIC values for *S. aureus* and *E. coli* exposed to nisin Z and to combined forms with PMDA, CDI, and β -CD. The results emphasized that the antibacterial activity of nisin, when combined with the selected formulations, was strongly increased such that the nisin-free sample did not show an inhibitory effect against *E. coli*, even at the highest concentration, but in complex forms with

both PMDA and CDI, the formulations decreased to 625 µg/mL. It is worthy to note that, as previously explained, the nisin-free form showed a better inhibitory effect against Gram-positive strains. For this reason, nisin had the MIC value against *S. aureus* at the highest concentration, even though this parameter was strikingly decreased to the lowest concentration for nisin after being combined with NSs. The cell wall of Gram-positive strains is wider than that of Gram-negative; therefore, the antibacterial difference results of our formulations in complex with nisin and nisin-free forms against both bacteria can be elucidated based on this fact. This hypothesis agrees with the cases presented by Kim et al. [149]. Interestingly, NSs alone showed promising MIC values, especially for CDI-NSs against *E. coli*, while β-CD only showed at 20,000 against *E. coli* but not for *S. aureus* (Table 5). Although this kind of potentially bacterial inhibitory effect of poly-cross-likened β-CDs free of antimicrobial agents has been reported to kill both *E. coli* and *S. aureus* in wastewater treatment [132] and prevent the *Mycobacterium tuberculosis* infection [131], the main difference between these studies and the present report is the different types of cross-linkers used.

To determine the minimum bactericidal concentrations (MBCs), 50 µL of each incubated broth aliquots containing a nisin-free sample and sampled complexed with formulations, as well as drug-free formulations that had shown the MIC values against

both pathogens, were taken and plated on MHA plates and incubated for 24 h. The results indicated that the bactericidal effect for nisin was not recognized in both strains, while fortified bactericidal activity was revealed for nisin complexed with CDI in the way that the MBC value for both strains was determined at 625 $\mu\text{g/mL}$. Nisin encapsulated with PMDA also exhibited a valuable bactericidal effect for both strains, compared with nisin loaded on β -CD (Table 5). In addition, drug-free formulations other than CDI and PMDA at the last concentration and only for *E. coli* strains did not show MBC value, while the β -CD-free sample showed no effect on either strain (Table 5).

Table 4. MIC and MBC values for nisin-free sample and samples loaded on selected formulations. ND: not determined.

	MIC($\mu\text{g/mL}$)				MBC($\mu\text{g/mL}$)			
	<i>Nisin</i>	<i>PMDA + Nisin</i>	<i>CDI + Nisin</i>	β -CD + <i>Nisin</i>	<i>Nisin</i>	<i>PMDA + Nisin</i>	<i>CDI + Nisin</i>	β -CD + <i>Nisin</i>
<i>E. coli</i>	ND	625	625	1250	ND	1250	625	2500
<i>S. aureus</i>	5000	625	625	1250	ND	1250	625	2500

Table 5. MIC and MBC values for drug-free formulations. ND: not determined.

	MIC($\mu\text{g/mL}$)			MBC($\mu\text{g/mL}$)		
	<i>PMDA-NSs</i>	<i>CDI-NSs</i>	β -CD	<i>PMDA-NSs</i>	<i>CDI-NSs</i>	β -CD
<i>E. coli</i>	10,000	5000	20,000	20,000	20,000	ND
<i>S. aureus</i>	20,000	ND	ND	ND	ND	ND

3.11.3.5. Capacity of Bacteria to Use β -CD and Nanosponges as Carbon Sources

Bacteria have different enzymes; relevant to the present study is amylase [119], which may lead to the breakdown of cyclodextrins as sources of carbon to reduce their antimicrobial activity. Therefore, we used a minimal agar medium free of carbon to evaluate this hypothesis. Interestingly, the results showed that both bacteria grew on plates containing β -CD even at the highest concentrations, which may confirm the hypothesis that β -CD could be used as a carbon source for bacterial growth, while NSs at different concentrations were difficult to digest by the preformed biofilm of *E. coli*

or *S. aureus* (Figure 20A, B), in comparison with β -CD alone. Therefore, it can be concluded the NSs are not so suitable as β -CDs to use as carbon sources, likely due to their different 3D structure, which would make degradation by amylases difficult [150].

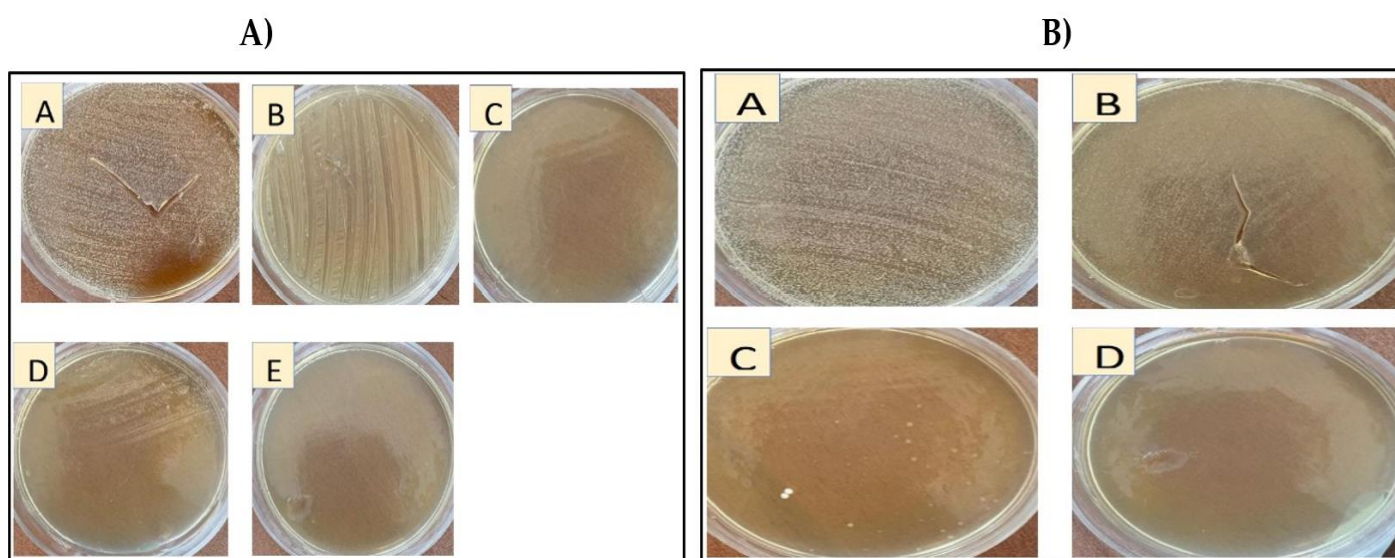


Figure 20. (A) Growth of *E. coli* on the surface of minimal agar medium mixed with β -CD and nanosponges as carbon sources: A, β -CD 20,000 $\mu\text{g/mL}$; B and C, PMDA-NS 10,000, 20,000 $\mu\text{g/mL}$, respectively; D and E, CDI-NSs 5000, 10,000 $\mu\text{g/mL}$, respectively; (B) growth of *S. aureus* on the surface of minimal agar medium mixed with β -CD and nanosponges as carbon sources: A, β -CD 5000 $\mu\text{g/mL}$; B, CDI-NSs 20,000 $\mu\text{g/mL}$; C and D, PMDA-NSs 10,000, 20,000 $\mu\text{g/mL}$, respectively

As a consequence, they were more effective to carry different antimicrobials. Taken together, these results show that PMDA-NSs and CDI-NSs presented interesting antimicrobial activities against Gram-positive and Gram-negative bacteria. The influence of comrade interaction among β -CD monomer and NSs formulations with microorganisms was seen to delay bacterial growth, and in some cases, they could induce the complete inhibition to both strains [151], illustrating a possible mechanism for this inhibition in combination with the delivery of some antimicrobial agents.

3.1.1.3.6. Total Viable Counts (TVCs)

One of the most common tests for studies regarding meat quality and shelf-life assessment to investigate meat freshness is total viable counts (TVCs) [119, 152]. The antibacterial effects of the nisin-free sample and samples complexed with CDI, PMDA-NSs, and β -CD were defined by the changes in TVCs during storage. Moreover, as a digestive enzyme, pepsin was used to contact with mentioned formulations and nisin-free form to define the protective effect of NSs, changing the antibacterial activities of the nisin-free sample and samples using selected formulations in cooked chicken meat. Table 6 shows the TVC results for cooked chicken meat samples when treated by nisin Z and by nisin encapsulated in various formulations. The initial TVC of the control group was $3.6 \log \text{CFU/mL}$ on day 0, while for nisin-free, it was 3.08 CFU/mL, whereas nisin in formulations and in contact with pepsin showed less inhibitory behavior on

bacterial growth on day 0. However, over time, at each time point of detection, the effect of nisin and complexes decreased, i.e., the presence of the materials was very insignificant during the first 3, 7, and 14 days, especially in nisin-Z-loaded CDI-NSs. After 30 days, statistical differences in TVCs started to decrease, perhaps due to the degradation of the materials by biotic or abiotic conditions. Statistical differences were observed between the group treated with nisin-free and samples treated with nisin complexed with CDI-NSs ($p < 0.001$) and PMDA-NSs ($p < 0.01$), whereas the nisin sample loaded on β -CD ($p < 0.05$) showed less effect, compared with other formulations.

Table 6. Total viable counts in cooked chicken meat during storage at 30 °C: (C), distilled water; (N), 0.05% nisin; (N-P), 0.05% nisin and 0.15% pepsin; (N- β -CD), 0.05% nisin and 0.1% β -CD; (N- β -CD-P), 0.05% nisin, 0.1% β -CD, and 0.15% pepsin; (PMDA-N), 0.05% nisin and 0.1% PMDA-NSs; (PMDA-N-P), 0.05% nisin, 0.1% PMDA-NS, and 0.15% pepsin; (CDI-N), 0.05% nisin and 0.1% CDI-NSs; (CDI-N-P), 0.05% nisin, 0.1% CDI-NS, and 0.15% pepsin. Mean values in the same column with the different lowercase letter are significantly different (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ for nisin vs. CD-N, CDI-N and PMDA-N. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, ns, not significant for N-P vs. CD-N-P, CDI-N-P, and PMDA-N-P). Data are presented as Mean (log CFU/mL) $\pm$ SD ($n = 3$).

TVC (log CFU/mL)					
Treatments	Storage Time (d)				
	0	3	7	14	30
C	3.6 $\pm$ 0.04	4.8 $\pm$ 0.09	5.86 $\pm$ 0.05	7.05 $\pm$ 0.06	8.8 $\pm$ 0.03
N	3.08 $\pm$ 0.02	4.43 $\pm$ 0.04	5.28 $\pm$ 0.05	6.63 $\pm$ 0.04	8.5 $\pm$ 0.06
N-P	3.45 $\pm$ 0.05	4.72 $\pm$ 0.01	5.35 $\pm$ 0.04	6.85 $\pm$ 0.07	8.71 $\pm$ 0.01

β -CD-N	3.38 ± 0.03	$4.30 \pm 0.02^*$	$5.17 \pm 0.06^*$	6.57 ± 0.02^{ns}	$8.2 \pm 0.07^*$
β -CD-N-P	3.55 ± 0.02	$4.64 \pm 0.03^\#$	$5.20 \pm 0.02^\#$	$6.70 \pm 0.05^\#$	$8.38 \pm 0.06^\#$
CDI-N	3.20 ± 0.06	$3.9 \pm 0.04^{**}$	$4.81 \pm 0.04^{***}$	$5.54 \pm 0.05^{****}$	$7.88 \pm 0.04^{***}$
CDI-N-P	3.34 ± 0.03	$4.18 \pm 0.04^{###}$	$4.94 \pm 0.06^{##}$	$5.98 \pm 0.03^{###}$	$8.07 \pm 0.02^{###}$
PMDA-N	3.24 ± 0.03	$4.24 \pm 0.05^*$	$5.04 \pm 0.05^{**}$	$6.33 \pm 0.04^{**}$	$8.08 \pm 0.05^{**}$
PMDA-N-P	3.52 ± 0.05	$4.45 \pm 0.04^{##}$	$5.15 \pm 0.03^\#$	$6.54 \pm 0.06^{##}$	$8.25 \pm 0.05^{##}$

This significant inhibitory effect of nisin Z in formulations might be attributed to the increase in properties such as solubility and stability, as well as the prolonged release of nisin from formulations that remained in samples, showing statistically significant differences during the 30 days of storage time. In comparison, samples treated with nisin in the presence of pepsin showed higher microbial counts (Figure 21), which is similar to the findings of other studies [153, 154]. Interestingly, the microbial counts of the nisin-free sample and samples complexed with formulations after contact with pepsin were increased, even though this increase was considerably more for nisin-free than that for complexed samples in NSs and β -CD ($p < 0.05$), especially CDI-NSs ($p < 0.001$). Altogether, the marked reduction in TVCs of chicken meat treated with nisin complexed with the NSs and β -CD, especially NSs, as well as the better antibacterial effect of nisin when encapsulated in formulations in presence of pepsin, might be attributed to the protective effects of NSs and β -CD on nisin. In their study, Li et al. showed that nisin loaded on β -CD could delay the breakdown of nisin and increase its stability in the presence of trypsin[133]. The results of the present study indicated

that the combination of nisin with NSs and β -CD could act as an effective sanitizer for microbial decontamination in cooked chicken meat. Another study illustrated the inclusion of β -CD complex inhibited the growth of aerobic microflora and reduced survival of *L. monocytogenes* in packaged freshly cut onions [142]. The conclusions drawn in previous studies indicate that nisin activity as an antimicrobial agent in active food packaging can be increased by loading on cellulose nanocrystals [138], chitosan microcapsules [155], chitosan nanoparticles [156], and chitosan–carrageenan nanocapsule [157]. Indeed, the results revealed a strong antibacterial activity of nisin complexed with NSs against both strains, whereas nisin loaded on β -CD did not show any effect, compared with NS formulations, which may result from further entrapment of any essential biomolecule in the structure of NSs.

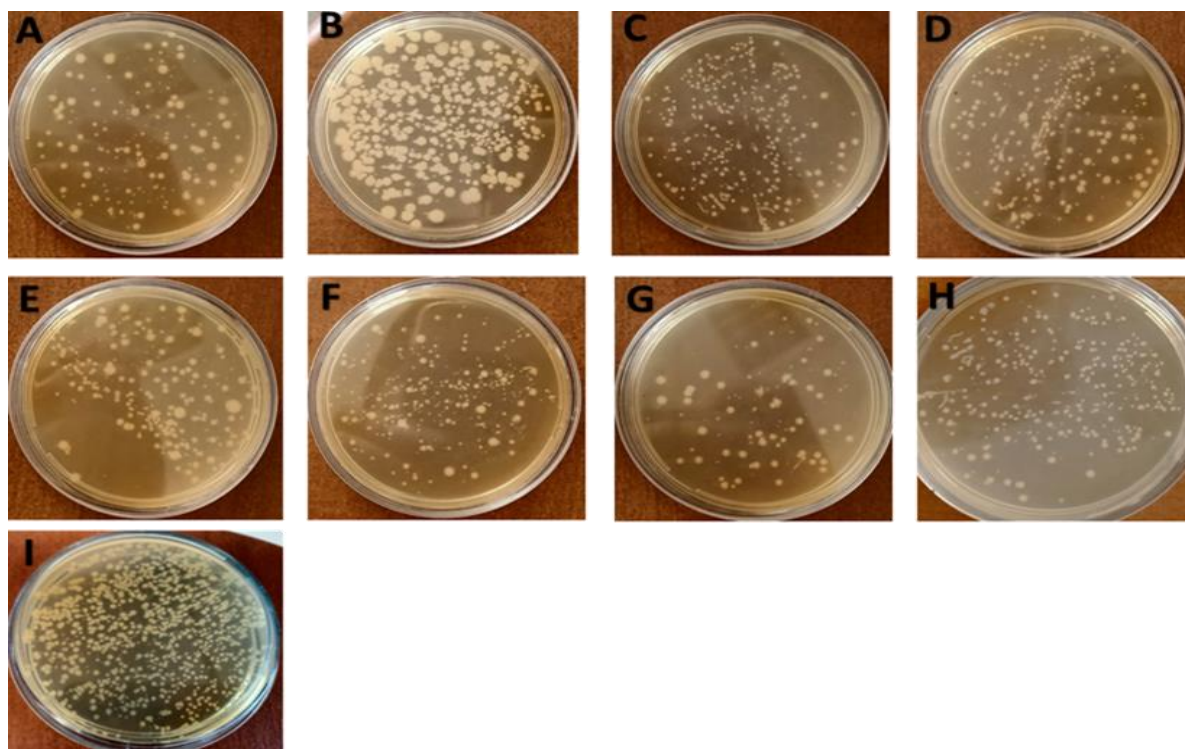


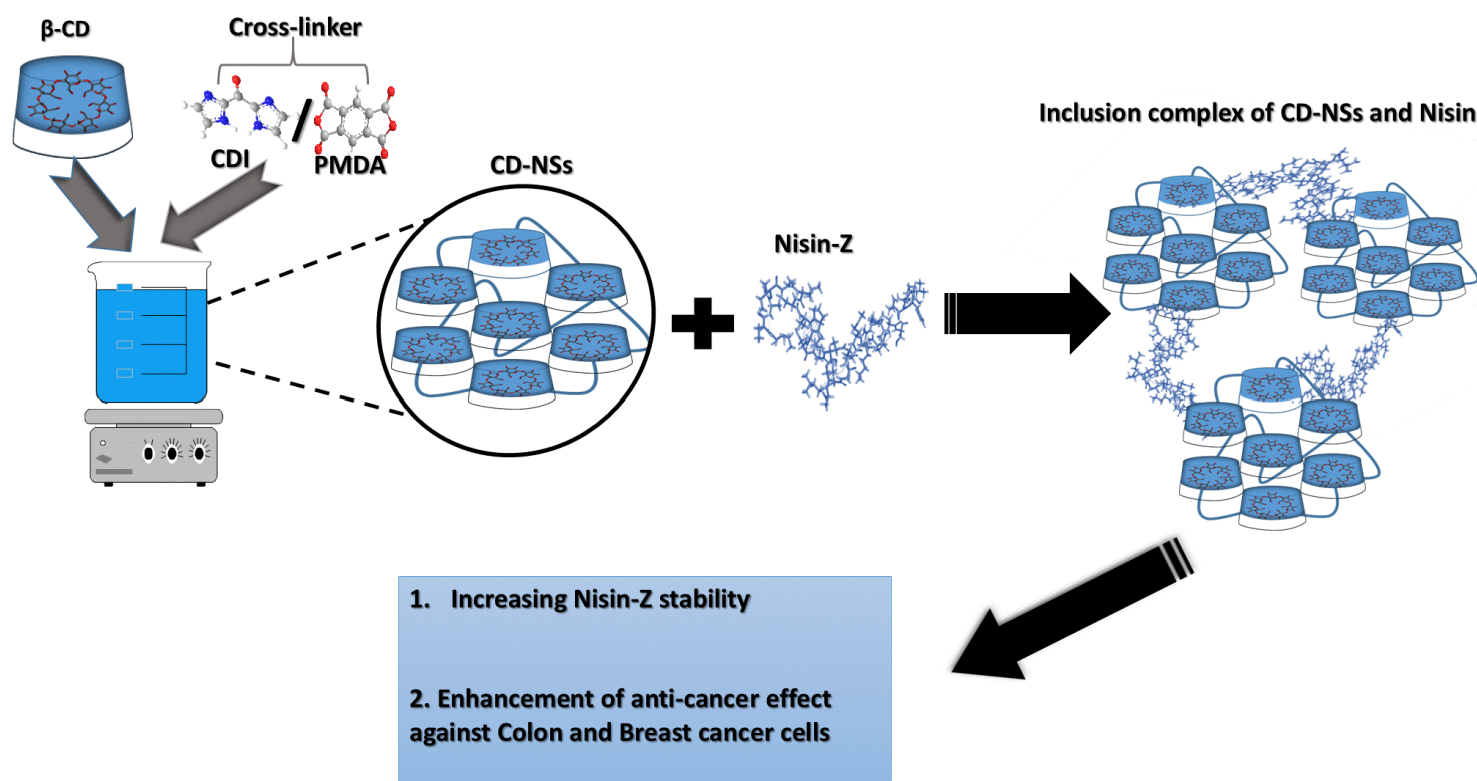
Figure 21. Total viable counts in cooked chicken meat during storage at 30 °C on agar medium after 24 h incubation: (A) 0.05% nisin; (B) 0.05% nisin and 0.15% pepsin; (C) 0.05% nisin and 0.1% β -CD; (D) 0.05% nisin, 0.1% β -CD, and 0.15% pepsin; (E) 0.05% nisin and 0.1% PMDA-NSs; (F) 0.05% nisin, 0.1% PMDA-NS, and 0.15% pepsin; (G) 0.05% nisin and 0.1% CDI-NSs; (H) 0.05% nisin, 0.1%, CDI-NS, and 0.15% pepsin (I) distilled water (control).

It should be noted that various experiments in the present study showed that nisin loaded on PMDA-NSs and the sample free of drug carrier was more effective against Gram-positive bacteria, while nisin complexed with CDI-NSs and its carrier had a higher inhibitory effect against Gram-negative growth; these results might be justified by the different compositions of membranes and the nature of Gram-positive and

negative bacteria [141], as well as the different sizes and zeta-potential values of NSs, as previously reported [130, 148].

To sum up, the results of this study led us to conclude that PMDA and CDI-NSs have potential antimicrobial activities themselves, as well as the ability to increase nisin stability and antimicrobial activity, which is one of the most common attributes of this food preservative agent and suggests its potential application in the food industry. These results support the idea of a novel antimicrobial formulation based on CD-NS alone or in complexes with some antimicrobials.

Second Project: Stabilization and Anticancer Enhancing Activity of the Peptide Nisin by Cyclodextrin-Based Nanosponges against Colon and Breast Cancer Cells



Graphical Abstract

Aim of study:

The great variability of cancer types demands novel drugs with broad spectrum, this is the case of Nisin, a polycyclic antibacterial peptide that recently has been considered for prevention of cancer cells growth. As an accepted food additive, this drug would be very useful for intestinal cancers, but the peptide nature would make easier its degradation by digestion procedures. For that reason, the aim of present study to investigate the protective effect of two different β -cyclodextrin-based nanosponges (carbonyl diimidazole and pyro-mellitic dianhydride) and their anti-cancer enhancement effect of Nisin-Z encapsulated with against colon cancer cells (HT-29). To extend its possible use, a comparison with breast (MCF-7) cancer cell was carried out. The physicochemical properties, loading efficiency, and release kinetics of Nisin complex with nanosponges were studied. Then, tricin-SDS-PAGE electrophoresis was used to understand the effect of NSs on stability of Nisin-Z in the presence of gastric peptidase pepsin. In addition, the cytotoxicity and cell membrane damage of Nisin Z were evaluated by using the MTT and LDH assay, which was complemented via Annexin-V/ Propidium Iodide (PI) by using flowcytometry.

1.1.2.1. Introduction

Currently, cancer is one of the most important diseases to get a universal therapy due to the diversity of cancer lines [158]. In particular, colon cancer caused nearly 881,000 deaths in 2018 [159]. To overcome this challenge, the research of novel broad-spectrum drugs is one of the most promising alternatives. Recently, there has been reported the anti-cancer effect of food preservative and bacteriocin agent, Nisin [160, 161], a polycyclic antibacterial peptide containing 34-amino acid with rarely amino acids, such as dehydroalanine (Dha), dehydrobutirine (Dhb), lanthionine (Lan), and methyl-lanthionine (Melan) [159]. Thermal stability and antibacterial activity of Nisin are related to these unusual amino acids [159, 162]. Different variants of Nisin have been discovered from a range of taxonomically distinct organisms isolated from a broad range of environments[162]. The differences among Nisin-Z and Nisin A, the first discovered natural Nisin, is attributed to the present histidine and asparagine amino acids in the residue position 27, respectively, which led to enhance the diffusion of Nisin Z [163]. Increased solubility and stability of Nisin in acidic medium as well as its stability in autoclave, at 121°C for 15 min and pH 2–3 without denaturation, has been reported [164]. While Nisin mainly has been used in the food industry as a food protective against bacterial growth through disrupting the integrity and formation of

the short-lived pore inside the bacterial cells, leading to a change in membrane potential [165], it has only recently been considered for prevention of cancer cells growth: in the last few years, extensive research has confirmed that the cytotoxicity and antitumor effects of Nisin are mediated through the activation of preferential apoptosis, cell cycle arrest, and reduced cell proliferation [160, 166, 167]. The cationic amino acids moieties of the Nisin interact with negatively charged phospholipid heads from the cell membrane of the molecule, while the hydrophobic portion of Nisin interacts with the nucleus of the membrane core [168]. Protein and peptide-based drugs have shown great biological activities against cancer cells because of their high biocompatibility, high specificity, and low toxicity as new therapeutically agents with a broad-spectrum of anti-cancer activities to inhibit tumor growth [169, 170]. Whereas, the main limitation in regard to these agents is low stability in-vivo due to enzymatic cleavage by peptidases, therefore, some delivery approaches were implied to improve plasma half-life of the peptide drugs [170, 171]. On the other hand, the delivery of effective and safe therapeutic agents to human cancer cells has been studied by various methods [172-174]. Among others, polymer science is used to design such drug delivery systems against cancer cells. As a potential polymer system, cyclodextrin-based (CD-NS) or cross-linked cyclodextrins nanosponges have often been used in drug delivery systems to deliver various anticancer drugs due to their low cost and high biocompatibility [70].

Nisin-Z as anti-tumor agent can be decomposed by proteases in the digestive system, while it is unstable at physiological pH. Consequently, these issues are the main concerns to use this peptide in clinical practice[175, 176]. Therefore, we aimed to investigate the enhancement of stability and anti-cancer effect of Nisin Z encapsulated with two different CDNSs (PMDA-NSs and CDI-NSs) against colon and breast cancer cells in lines.

3.1.2.3. Results and Discussion

3.1.2.3.1. Tricine-SDS-PAGE of the Nisin-Z Free and Loaded with β CDNSs

The Nisin activity is affected by protein degradation enzymes in the environments such as the digestive system. The stability and protective effects of Nisin by combination with PMDA/CDI-NSs were evaluated in the presence of pepsin, the principal acid protease of the stomach, through the Tricine-SDS-PAGE gel electrophoresis, the results of which are shown in Figure 22A. Nisin has a relative molecular mass of 3.5 kDa [177], which is located in lane 1. Meanwhile, PMDA-NSs (lane 3) and CDI-NSs (lane 4) showed no characteristic bands. The results exhibited the band of Nisin exposed to pepsin was disappeared, while when Nisin was encapsulated inside the CD-NSs in the presence of pepsin showed the bands, which might be attributed to the protective effect of PMDA/CDI-NDSs, which prevent degradation. Even a second band appeared, maybe caused by any conformational change or the complexation generated a running delay in a proportion of Nisin, creating two bands [178, 179]. This result was in accordance with the outcomes of other studies that revealed the high encapsulation efficiency of resveratrol, oxyresveratrol, bovine serum albumin (BSA), and insulin complexed by different nanosponges [83, 87, 180]. The release profile and pepsin degradation indicate that CD-NSs are able to protect the peptide of specific degradation and improve its release of the peptide. These aspects are important in digestion processes to achieve the tumor with the peptide in functional

conditions [33]. It has been suggested that for insulin, as a peptide case, stability can be ameliorated in the presence of hydroxypropyl- β -cyclodextrin (HP- β -CD) and cationic cyclodextrin polymers (CP β CDs) in different environments such as in simulated intestinal fluid, which was mainly because of retaining in the core of the nanoparticles and well protected against degradation in simulated gastric fluid [181]. As a case of CDNSs to protect peptides, there has been shown insulin complexed by crosslinking β -cyclodextrins with pyromellitic dianhydride increases the intestinal permeation when loaded inside the nanosponge and in-vivo studies confirmed the presence of insulin in rat plasma and a marked hypoglycemic effect in diabetic mice after oral administrations [87].

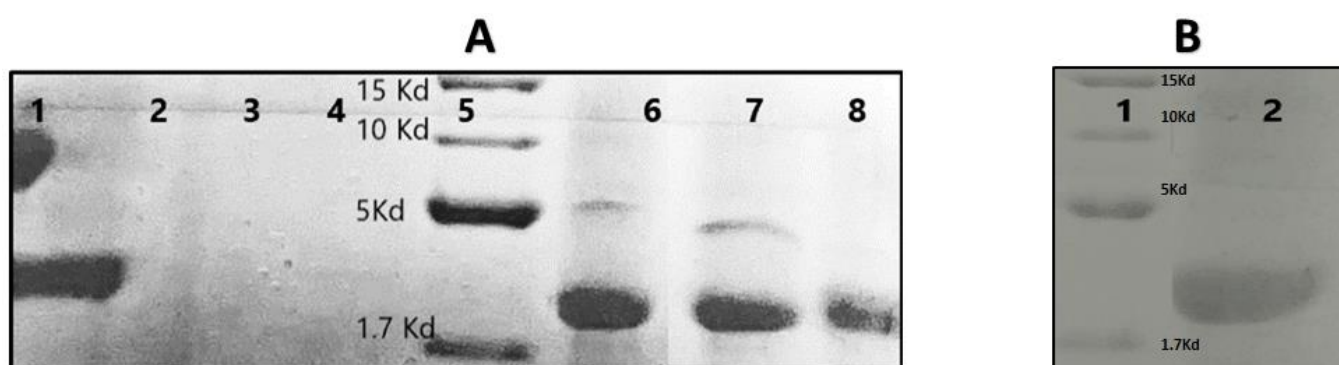


Figure 22. Tricine-SDS-PAGE analysis of Nisin with different treatments. Lane 1: Nisin (4 mg/mL); Lane 2: Nisin (4 mg/mL) and pepsin (7.5 mg/mL); Lane 3: PMDA-NSs (15 mg/mL); Lane 4: CDI-NSs (15 mg/mL); Lane 5: Marker; Lane 6: Nisin and PMDA-NS; Lane 7: Nisin and CDI-NSs; Lane 8: Nisin, CDI-NSs and pepsin (7.5 mg/mL). B) Lane 1: Marker; Lane 2: Nisin, PMDA-NSs and pepsin (7.5 mg/mL). The same protein quantity was loaded.

3.1.2.3.2. Intracellular Uptake of Free Coumarin-6 (C-6) in Comparison Coumarin-6-Loaded PMDA-NSs and CDI-NSs

To evaluate the possible intracellular uptake of Nisin when complexed by PMDA-NSs and CDI-NSs, coumarin-6 (0.1 mg/mL), a fluorescence biomolecule, was used as template to simulate the uptake of Nisin. Coumarin-6 was loaded into the fixed concentration of nanosponges (10 mg/mL) for 4 h. Then, their qualitatively and quantitatively uptakes were analyzed by Cytation-5 Cell Imaging Multi-Mode Reader and Fluorescence-activated Cell Sorting (FACS), respectively, in two different cancer cells (HT-29 and MCF-7). Fluorescence images approved that coumarin-6-loaded PMDA-NSs and CDI-NSs was taken up in both HT-29 and MCF-7 cells (Figure 23A), while the HT-29 showed higher up-take (Figure 24A). However, coumarin-6-loaded PMDA-NSs was highly taken up in both cells compared with CDI-NSs. Perhaps, CDI-NSs present higher complexation strength for Coumarin-6 than PMDA. To support these results, we used the quantitative method (FACS).

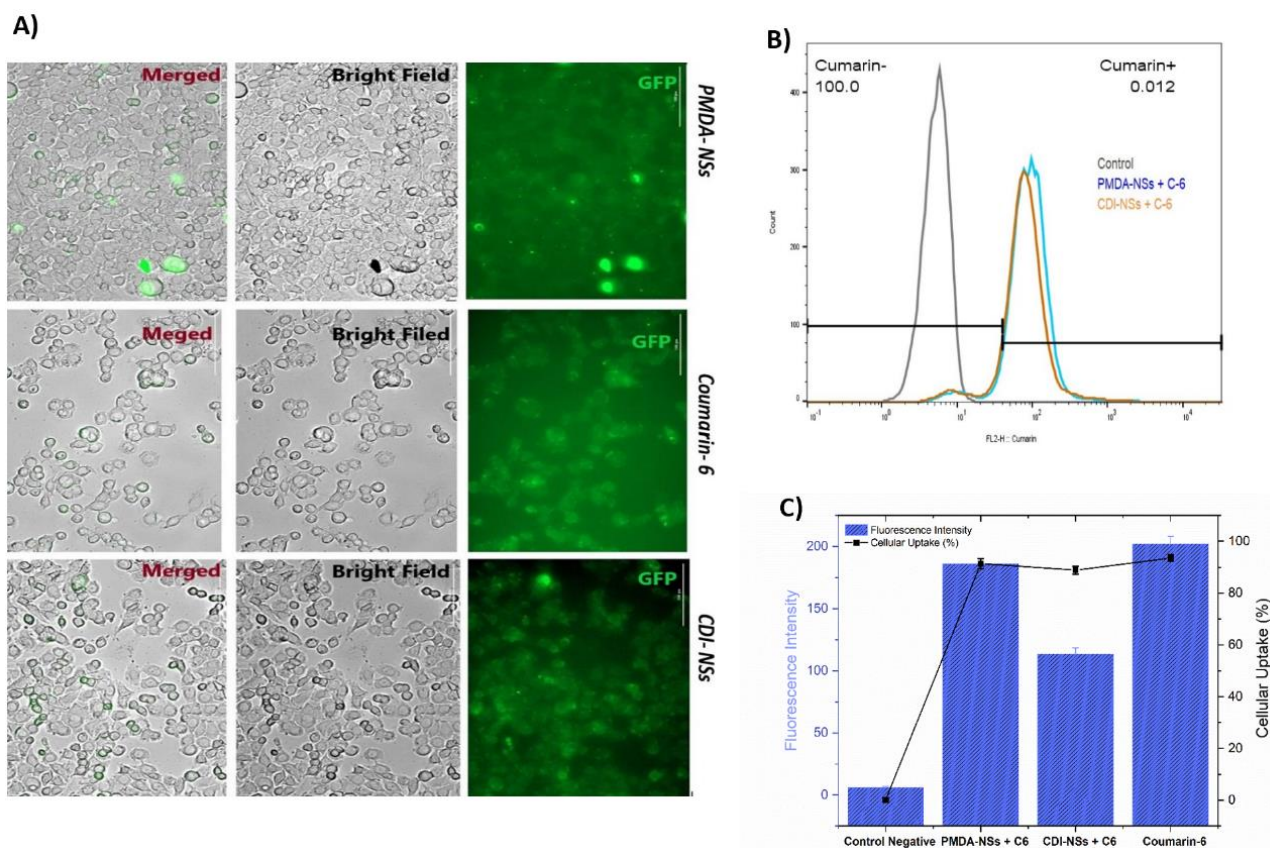


Figure 23. Uptake of coumarin-6 labeled CDNs on HT-29 cells at 37°C for 4 h was observed and investigated by fluorescent microscopy and flowcytometry. (A) was observed by fluorescence microscope. (B): Flowcytometry assay, (C): Mean of Fluorescence Intensity of HT-29 cells by flowcytometry assay. Data are expressed as mean $\pm$ SD ($n = 3$). One-way ANOVA analyses was used.

As shown in Figures 23C and 24C, the cellular uptake percentage of C-6 complexed in PMDA-NSs was notably greater than CDI- NSs in HT-29 cell line ($p < 0.0001$), whereas in the MCF-7 cell, there was not seen differences, furthermore, the mean of fluorescence intensity (MFI) of PMDA-NSs compared to CDI-NSs ($p < 0.0001$) in both

cell lines was considerably higher. Surprisingly, a comparative analysis indicated the MFI and cellular uptake percentage of both CDNSs in HT-29 cells were significantly higher than MCF-7 ($p < 0.0001$), which was in agreement with microscopic fluorescence results (Figure 50). These results suggest a better uptake of selected biomolecules inside the cancer cells to release targeted drugs when they are complexed [32]. Previous studies indicated that the nanosponges as drug carriers can effectively deliver the biological agents inside the cancer cells [134, 175].

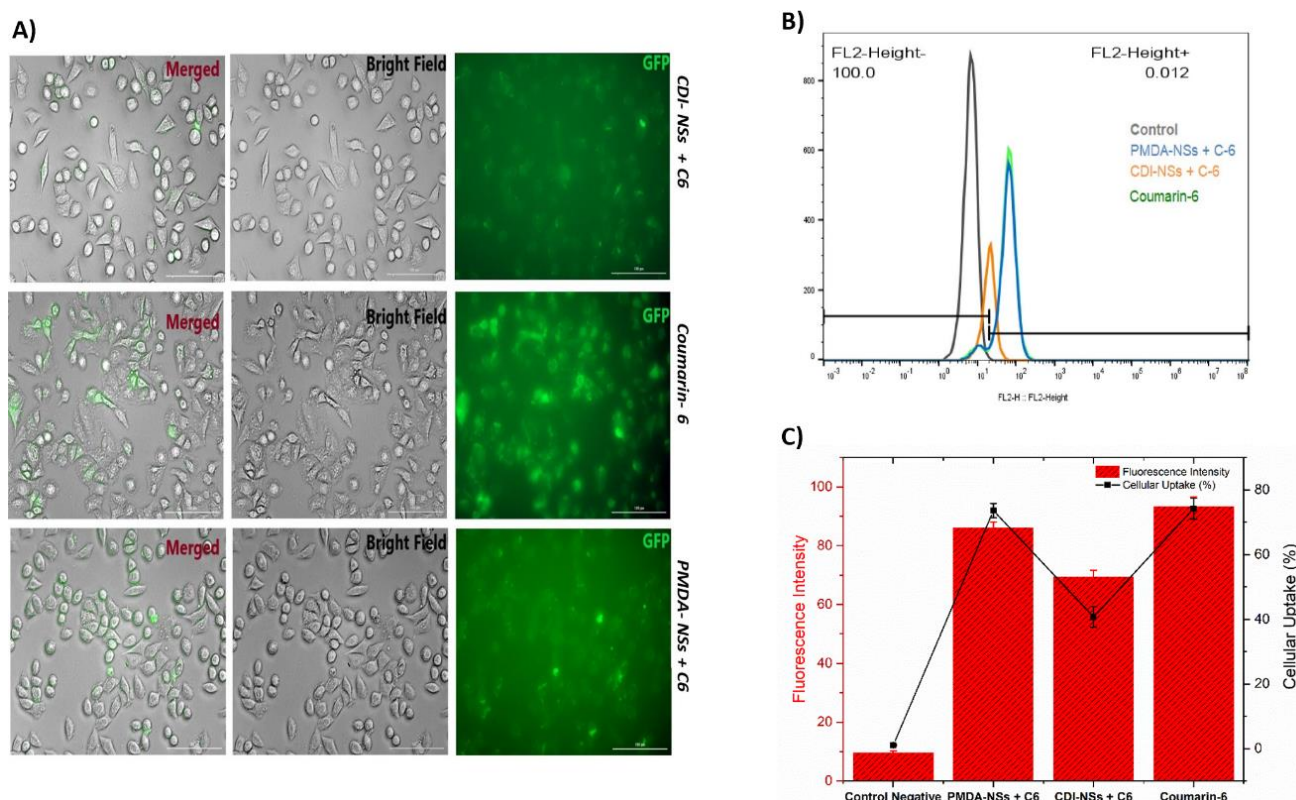


Figure 24. Uptake of coumarin-6 labeled CDNSs on MCF-7 cells at 37 °C for 4 h was observed and investigated by fluorescent microscopy and flowcytometry. (A) was observed by fluorescence microscope; (B,C) were analysis of flowcytometry data; (B): Flowcytometry assay, (C): Mean of Fluorescence Intensity of MCF-7 cells by flowcytometry assay. Data are expressed as mean $\pm$ SD (n = 3). One-way ANOVA analyses was used.

3.1.2.3.3. MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl Tetrazolium Bromide) Assay

The cell viability was evaluated by the MTT assay. Firstly, the cytotoxic effect of blank CDI-NSs and PMDA-NSs was checked in the two cell lines (Figure 25C, D). Nanoparticles did not show cytotoxicity even at a high amount of CDNSs (250 μ g/mL) toward the cells at 24 h, indicating that selected nanoparticles are nontoxic to tumor cells at selected concentrations. Nisin Z free and loaded on both nanoparticles showed a dose-dependent cytotoxicity to both of the tumor cells. As shown in Figure 6B, the cytotoxicity of free-Nisin was not significant against MCF-7 cells, but interestingly, Nisin loaded on both NSs showed the viability of cells was considerably lower in all concentrations compared to Nisin ($p < 0.0001$), while there was not seen the differences cytotoxicity effect between Nisin encapsulated with PMDA-NS and CDI-NSs. On the other hand, free Nisin indicated a good cytotoxicity effect against colon cancer cell in comparison with breast cancer, which might be associated to differences of the Nisin anti-cancer mechanism effect in these cells (Figure 25A, B). Furthermore, Nisin loaded on PMDA-NSs illustrated greater cellular toxicity than free Nisin and

encapsulated with CDI-NSs even at 250 $\mu\text{g/mL}$ Nisin loaded on PMDA-NSs displayed cytotoxicity about two folds higher than that of free-Nisin after 24 ($p < 0.0001$). However, it seems due to the lack of minimum dosage that is required to obtain the significant toxicity, both Nisin and Nisin loaded on CDI-NSs exhibited similar toxicity to the MCF-7 cells at the low concentration. This result suggested that the enhanced cytotoxicity of Nisin loaded on PMDA-NSs in both cells might be due to the higher bioavailability of PMDA-NSs which increased the intracellular concentration of Nisin in both cells, in particular HT-29 [32].

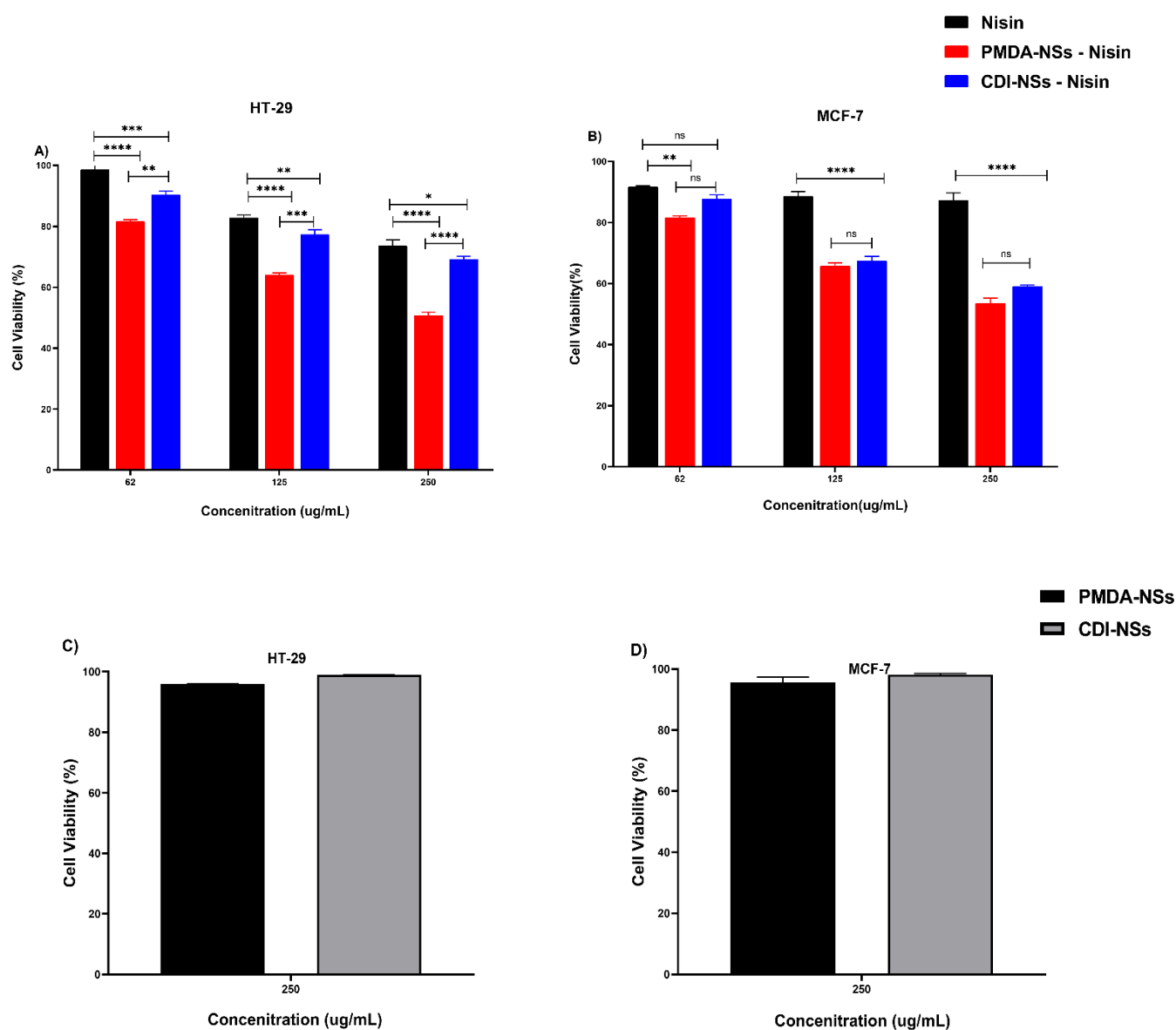


Figure 25. Cell viability results for colon cancer (HT-29) and breast cancer (MCF-7) cells exposed to Nisin-Z and loaded on nanosponges (A, B) and plain nanosponges (C, D) for 24 h. Results are expressed relative to the

untreated control, which was set as 100% viable. Data are expressed as mean $\pm$ SD ($n = 3$). Two-way ANOVA analyses was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns (Not Significant).

3.1.2.3.4. LDH Release

Lactate dehydrogenase (LDH) was used to determine whether the observed cytotoxicity of free Nisin Z and loaded on nanosponges against both cancer cells was related to cell membrane damage. The LDH method, which measures the release of cytosolic enzyme lactate dehydrogenase (LDH) in culture media after cell plasma membrane damage, is theoretically directly comparable to the number of lysed cells. Firstly, the release of LDH in two cancer cells, which treated with the blank CDI-NSs and PMDA-NSs, was evaluated (Figure 26C, D). Nanoparticles did not show notable amount of LDH release even at a high concentration of CDNSs (1000 $\mu\text{g/mL}$) against cancer cells at 24 h, suggested that our nanoparticles are not able to damage the cell membrane of tumor cells. Interestingly, Nisin free and loaded on both nanoparticles was dose-dependent to stimulate tumor cells to release the LDH. This assay illustrated that the colon cancer cells treated by free Nisin resulted in a considerably larger increase in LDH release than MCF-7 cells, indicating membrane damage, while Nisin encapsulated by CDI-NSs except in last concentration did not show differences effect on LDH release compared to Nisin free in both cells (Figure 26A, B). On the other side, colon cancer cells exposed to Nisin loaded on PMDA-NSs resulted in a dramatic

increase in LDH release about two times more at 250 $\mu\text{g/mL}$ compared to free Nisin ($p < 0.0001$), also this formulation showed more increment in LDH release in MCF-7 cells at the last concentration than free Nisin. The enhanced cytotoxicity effect of Nisin loaded on both CDNS β against the colon and breast cancer cells was concordant with other studies outcomes that showed a significant reduction of cell viability in colon [182], melanoma [160], and MCF-7 [167] cancer cells when they were exposed to Nisin-Z, whereas in this study the Nisin Z cytotoxicity effect was remarkably enhanced in complexed with NSs against both cancer cells, which might be related to better entrance of nanosponges into the cells to release Nisin inside the cancer cells. In addition, it has been suggested that the net positive charge of Nisin Z with anionic cell membrane of the cancer cells preferentially interact with phosphatidylcholine, leading to a better transmembrane potential than normal cell membranes because of more cholesterol content in normal cells [165, 168, 183].

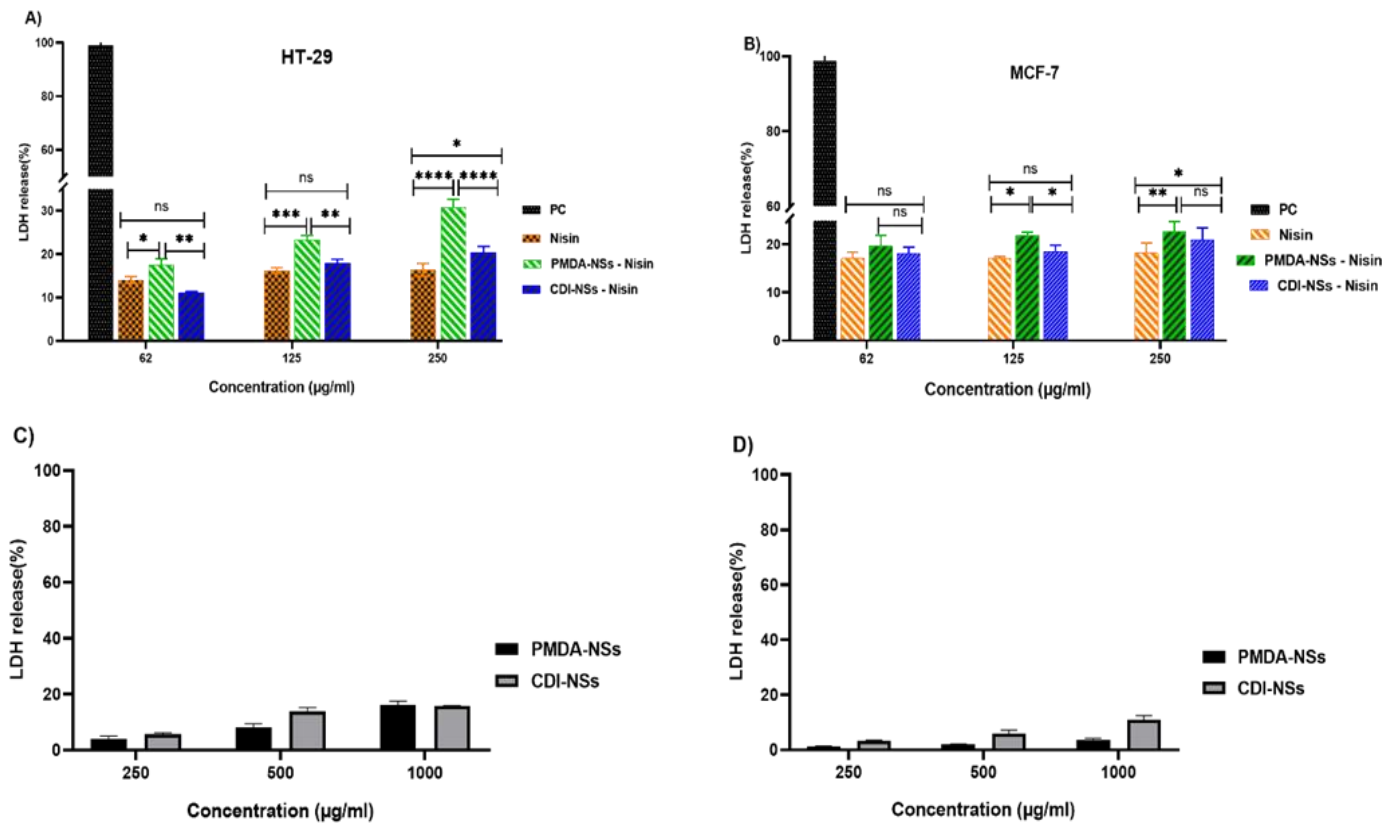


Figure 26. LDH release from HT-29 and MCF-7 cells following treatment with free Nisin Z and loaded on nanosponges (A, B) and plain nanosponges (C,D) for 24 h. Results are expressed relative to the untreated control and the maximum release sample (Positive Control (PC)), which was set as having 0 and 100% LDH release, respectively. Data are expressed as mean $\pm$ SD ($n = 3$). Two-way ANOVA analyses was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ and ns (Not Significant).

3.1.2.3.5. Detection of Apoptosis and Late Apoptosis/Necrosis with Flow Cytometry

Cytotoxicity and LDH release assays showed a concentration-dependent decrease in cell viability and an increase in LDH release among cancer cells treated with free Nisin Z and encapsulated by nanosponges. The assays also suggested that free and loaded Nisin Z on PMDA-NSs appeared to be more selectively toxic to colon cancer cell line, while Nisin loaded to CDI-NS showed less toxic effect on the breast cancer cells. Therefore, we implied the flow cytometry to understand that if the observed lower cell viability in cancer cells was of necrotic or apoptotic origin. Cultured colon and breast cancer cells were exposed to the same concentration of free Nisin Z (250 $\mu\text{g/mL}$) and CDNSs suspension containing the same amount of entrapped Nisin Z based on the results of the cytotoxicity assays and LDH release, the FITC AnnexinV flow cytometry assay was used for analysis. The results of MCF-7 cells treated with free Nisin Z indicated that with approximately 30% of the cells undergoing late apoptosis/necrosis while the Nisin/PMDA-NSs complex showed notable increase about two times more than free Nisin ($p < 0.0001$). Interestingly, breast cancer cells exposed to free Nisin and Nisin/CDI-NSs presenting early apoptotic markers of about 20%, which was two times higher to Nisin/PMDA-NSs. In addition, the % of cell viable in Nisin entrapped by both NSs was markedly less than Nisin ($p < 0.0001$) (Figure 27). On the other hand, the percentage of live cells in HT-29 cancer cells was dramatically reduced to 20.35 and 26.1% for Nisin/PMDA-NSs and Nisin/CDI-NSs, respectively, which was two times more than free Nisin ($p < 0.0001$). These results suggest that death of HT-29

cancer cells exposed to Nisin loaded on both NS is likely due to activation of an apoptotic pathway (late apoptosis/necrosis), while MCF-7 cancer cells underwent early apoptotic pathway (Figure 28). The Nisin-Z /PMDA-NSs induced far better apoptosis in both cancer cells compared to Nisin free and Nisin/CDI-NSs. These outcomes substantiate the cytotoxicity data of MTT assay and LDH release that showed free Nisin Z and loaded on PMDA/CDI-NSs are more selectively toxic towards HT-29 cells than MCF-7 cancer cells. One of the main mechanisms of cancer cells is to strongly suppress the apoptosis (programmed cell death), which may lead to cancer chemotherapy resistance and uncontrolled condition [184], as previous studies revealed Nisin plays its role in apoptosis by increasing mitochondrial intrinsic pathway of apoptosis to either up regulate pro-apoptotic molecules or down regulate anti-apoptotic molecules [184-187]. In addition, Hosseini et al.'s study showed Nisin could suppress proliferation of SW48, HT29, and Caco2 cells by regulation of the metastatic genes in colorectal cancer cells [182] as well as Ahamdi et al.'s study showed that the bax / bcl-2 ratio in the colon adenocarcinoma cell line raised during treatment with Nisin due to activation of the apoptotic pathway [188]. So, implying Nisin as an inducer of apoptosis agent maybe could control the uncontrolled condition in cancer cells, in agreement with these studies, the results of apoptosis assay in present assay showed the percentage of live HT-29 and MCF-7 cancer cells when exposed to free Nisin Z was considerably higher compared to loaded on selected NSs in the way that Nisin

encapsulated with NSs moved the colon cancer cells mainly to the late apoptosis/necrosis stage. Interestingly, breast cancer cells exposed to free Nisin Z and Nisin/CDI-NSs present early apoptotic markers. The results of Preet et al.'s study emphasized the therapeutic efficacy of gold nanoparticle-assisted co-delivery of Nisin, doxorubicin, and Nisin-doxorubicin combination against murine skin cancer, probably through ROS mediated apoptotic effects and immunomodulation either alone or synergistically [161]. In another study, Goudarzi et al. evaluated the cytotoxicity effects of Nisin and Nisin-loaded PLA-PEG-PLA nanoparticles on gastrointestinal (AGS and KYSE-30), hepatic (HepG2), and blood (K562) cancer cells, the results of which showed that nanoparticles loaded with Nisin increased the percentage of apoptotic cells and the effect of cytotoxicity on the mentioned cancer cell lines more than free Nisin [189].

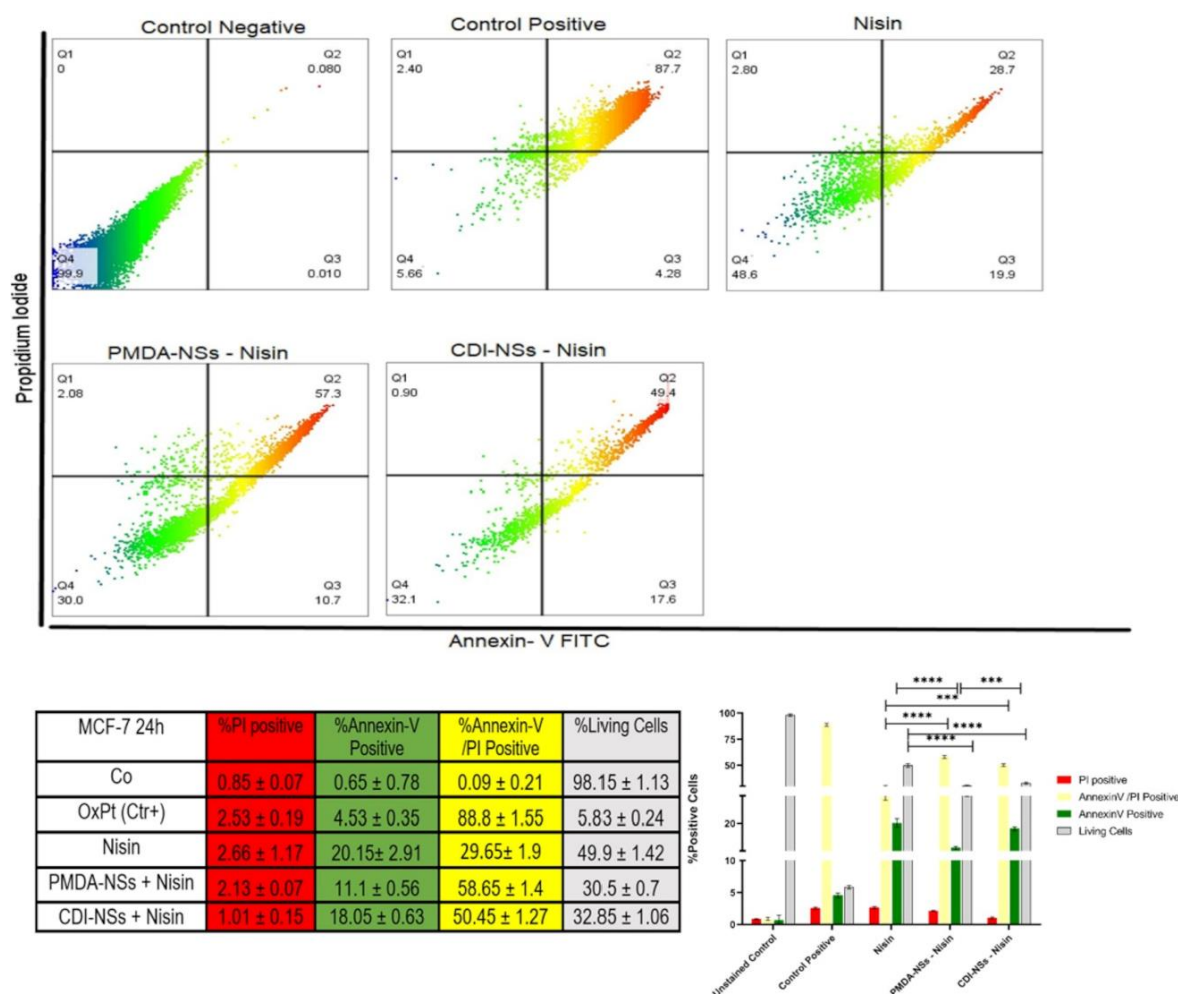


Figure 27. Representative flow cytometric dot-plots indicating the population sizes of apoptotic and necrotic in MCF-7 cells after exposure to 250 µg/mL of Nisin Z and loaded on PMDA/CDI-NSs for 24 h. Analyses were performed with annexin-V-FITC (FITC) and propidium iodide (PI). The untreated control was not exposed to Nisin Z and the complexes, and positive control was treated with oxaliplatin (OxPt). Experiments were done in triplicate and independently repeated. Bar graphs illustrate the percentage of apoptotic, necrotic, and late-stage apoptotic MCF-7 cells after exposure to free Nisin Z and encapsulated by the NSs. *** $p < 0.001$, **** $p < 0.0001$ apoptosis relative to the free Nisin Z. Data are expressed as mean ± SD ($n = 3$). Two-way ANOVA analyses was used.

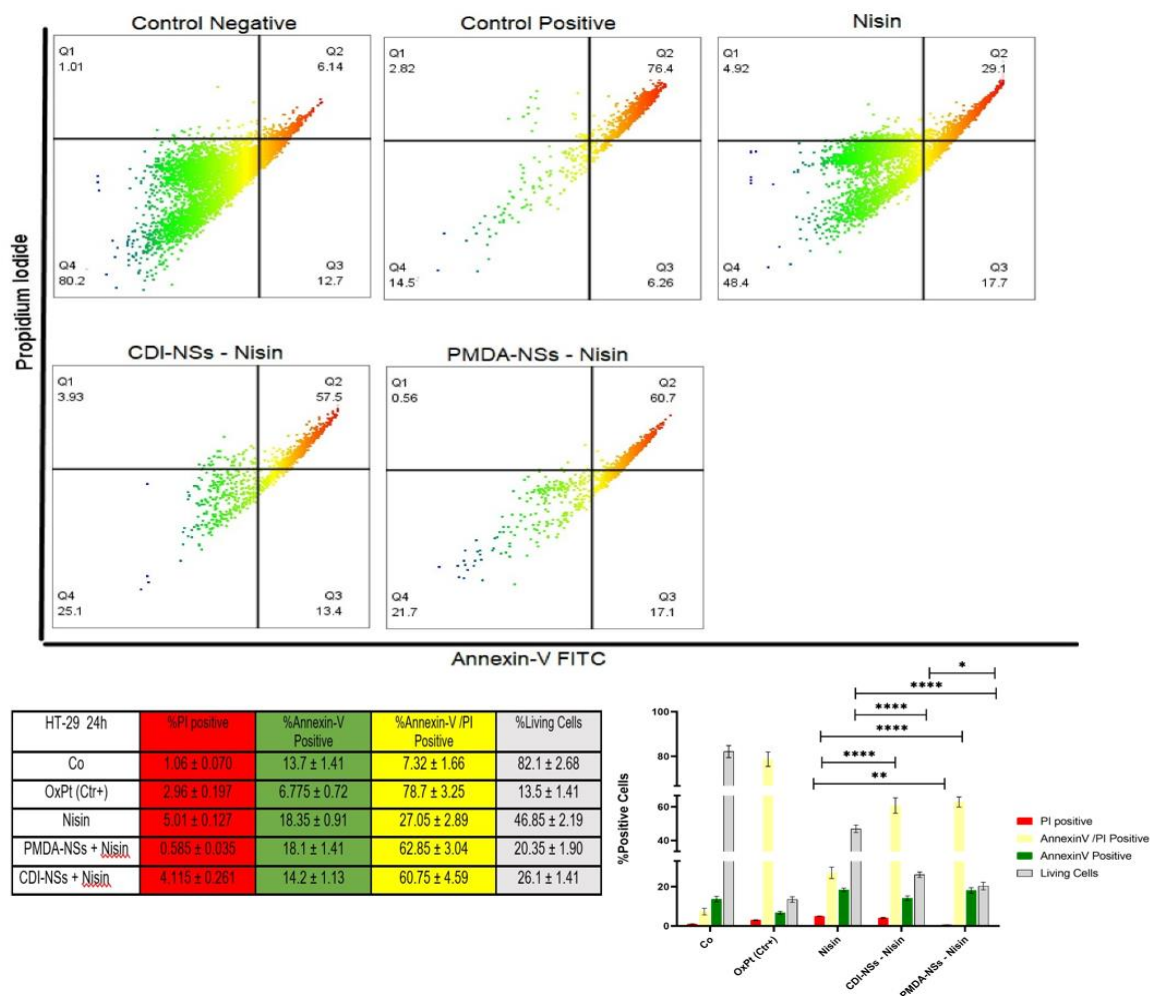
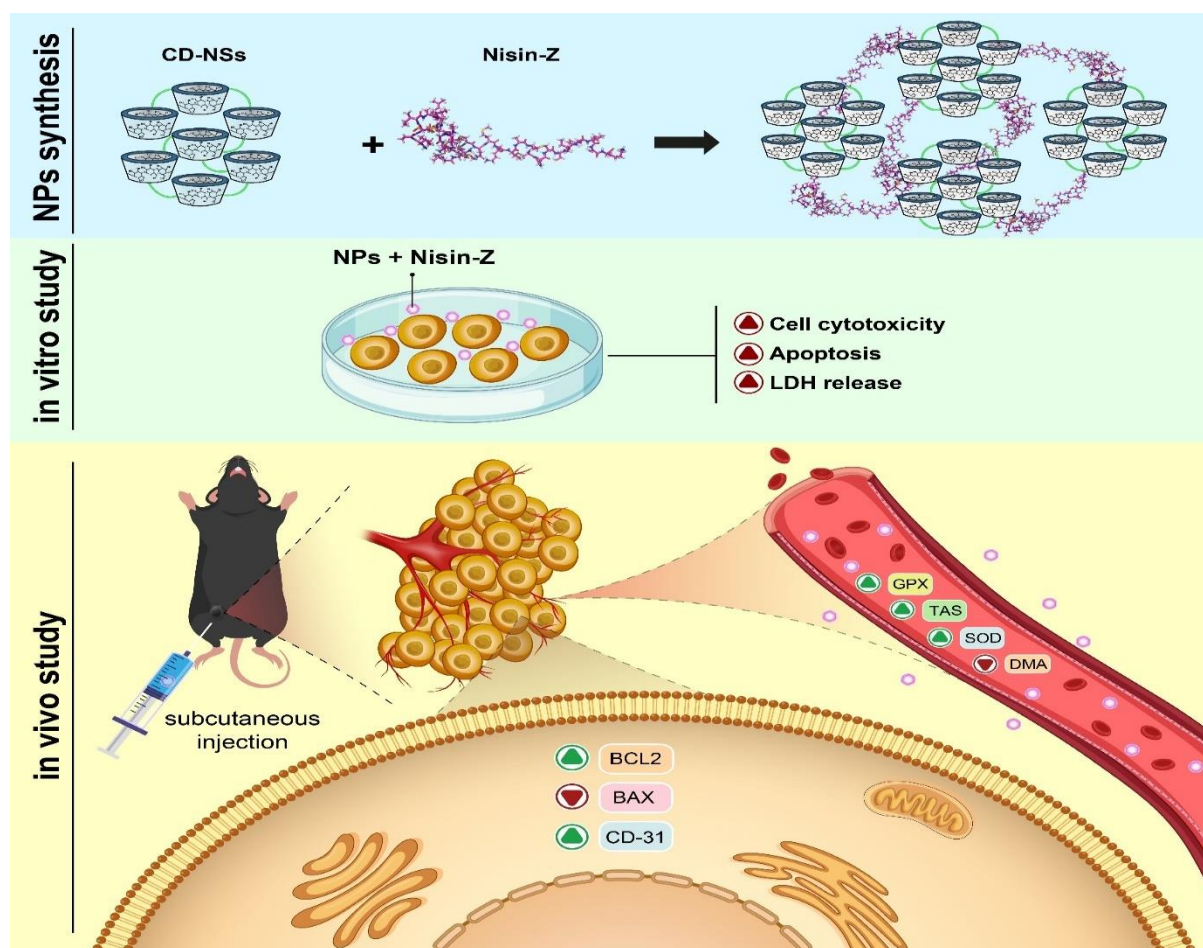


Figure 28. Representative flow cytometric dot-plots indicating the population sizes of apoptotic and necrotic in HT-29 cells after exposure to 250 µg/mL of Nisin Z and loaded on PMDA/CDI-NSs for 24 h. Analyses were performed with annexin-V-FITC (FITC) and propidium iodide (PI). The untreated control was not exposed to Nisin Z and the complexes, positive control was treated with oxaliplatin (OxPt). Experiments were done in triplicate and independently repeated. Bar graphs illustrate the percentage of apoptotic, necrotic, and late-stage apoptotic HT-29 cells after exposure to free Nisin Z and encapsulated by the NSs. * p < 0.05, ** p < 0.001, , **** p < 0.0001. Data are expressed as mean ± SD (n = 3). Two-way ANOVA analyses was used.

To sum up, based on the results of this study, we can conclude that PMDA and CDI-NSs polymeric particles are able to carry Nisin Z, a peptide drug. This non-toxic delivery system showed notable uptake into MCF-7 and HT-29 cell lines, and the higher toxicity and cell membrane damage were shown by Nisin-Z encapsulated with NSs as compared to that of free Nisin Z against both cancer cells in MTT and LDH assays. Furthermore, Nisin Z free and loaded on PMDA/CDI-NSs are more effective towards HT-29 cells than MCF-7 cancer cells and indicates that the cell death observed in these cells is most likely due to the activation of an apoptotic pathway. Finally, the pepsin degradation study and release profile showed that the stability of Nisin-Z in presence of CD-NSs complexes was increased, which suggests the complexes would be interesting to deliver protein drugs, which have low stability in the gastrointestinal tract, suggesting a promising future application.

Third Project: Nisin Delivery by Nanosponges Increases Its Anticancer Activity Against *In-vivo* Melanoma Model



Graphical Abstract

Aim of study:

Nisin, a small antimicrobial peptide, has been recently suggested as a novel potential therapeutic approach to treat cancer with no toxicity in humans. The current study used cross-linked-cyclodextrin nanosponges (CDNS) to load Nisin-Z in order to study the capacity of the complexes against melanoma cancer in vitro and in vivo.

3.1.3.1. Introduction

High morbidity and mortality from human cancer have remained a major challenge throughout the world. In particular, melanoma is a skin cancer with a high rate of incidence [158, 160, 190]. Currently, the most common treatment for cancer is chemotherapy, which involves sending a cytotoxic chemical to cancer cells. However, typical cancer chemotherapies have failed due to anti-cancer drug resistance and side effects [191-193]. Therefore, new advances in cancer treatment will be a major step forward with the development of a novel class of anticancer drugs with lower toxicity and chemical resistance than chemotherapeutic agents[193].

In our previous works, the complex between Nisin-Z with PMDA and CDI-NS was investigated for antimicrobial and in-vitro anticancer enhancing properties [194, 195]. Our results confirmed the possibility of Nisin complex with CDI and PMDA-NSs by different characterization methods including SEM, FTIR, and DSC [194, 195]. In addition, both of them have shown a good encapsulation efficiency of about 90% under the optimized conditions [194]. Furthermore, Nisin's in vitro release in complexes with NSs demonstrated a sustained and prolonged release profile at both physiological and acidic pH, implying that Nisin is entrapped inside the nanosponges' and its availability is increased. However, we still need to determine the capacities in an *in vivo* model for eventual therapies. Therefore, we designed this study to evaluate

the anticancer effect of Nisin-Z against animal melanoma cancer. The first step was to understand the possible enhancement effect of Nisin in complex with PMDA-NSs and CDI-NSs in an *in vitro* against melanoma cancer cells (B16-F10). Then, based on the *in-vitro* experiment results that showed a potent antitumor effect of Nisin in complex with PMDA-NSs and to extend these observations, we have examined the antitumor growth and antioxidant effects of Nisin loaded on PMDA-NSs in induced mice with melanoma cancer.

3.1.3.2. Results and Discussion

3.1.3.2.1. MTT assay against the B16-F10 cancer cell lines

MTT analysis was used to determine the cytotoxicity effect of Nisin free, and Nisin in complexes with β CDNSs against melanoma cancer cells (B16-F10) after 24h exposure. Firstly, the cytotoxic effect of blank CDI-NSs and PMDA-NSs in both melanoma cancer cells (Figure 29) and fibroblast (Figure 30), as a normal cell, were evaluated. The results showed that free-CDNSs even at a high concentration did not induce cytotoxicity in both cells. The cytotoxicity of both Nisin Z, and Nisin complexed with β CDNSs, was dose-dependent against melanoma cancer cells. In addition, Nisin free did not show significant cell death effect even at the last concentration which was less than 20 percent, while the viability of cancer cells exposed to Nisin loaded on both NSs was considerably decreased in all concentrations in compared to Nisin free. It is noticeable that the Nisin complexes with PMDA-NSs exhibited higher cytotoxic effects than free Nisin or Nisin encapsulated with CDI-NSs, in specific at the concentration of 500 μ g/mL, its cytotoxicity effect was about three folds higher than that of free-Nisin ($p < 0.0001$) (figure 31). This result was consistent with earlier research findings showing a significant reduction in viable cells in colon cancer cells[182], melanoma [190, 196] and MCF-7[189] cancer cells when they were exposed to Nisin-Z, notably

the anti-cancer effect of Nisin Z loaded on PMDA-NSs against melanoma cancer cells, was significantly boosted in this work.

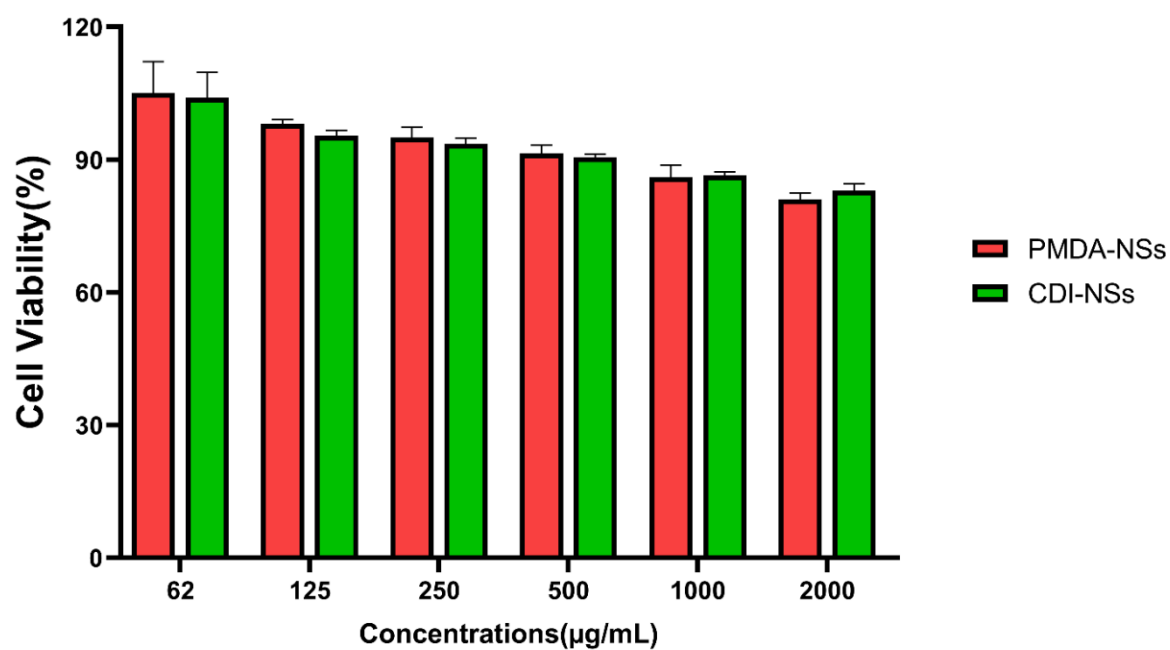


Figure 29. Effect of empty β CDNSs cytotoxicity effect on B16-F10 cells viability was evaluated after a 24 h incubation with different concentrations

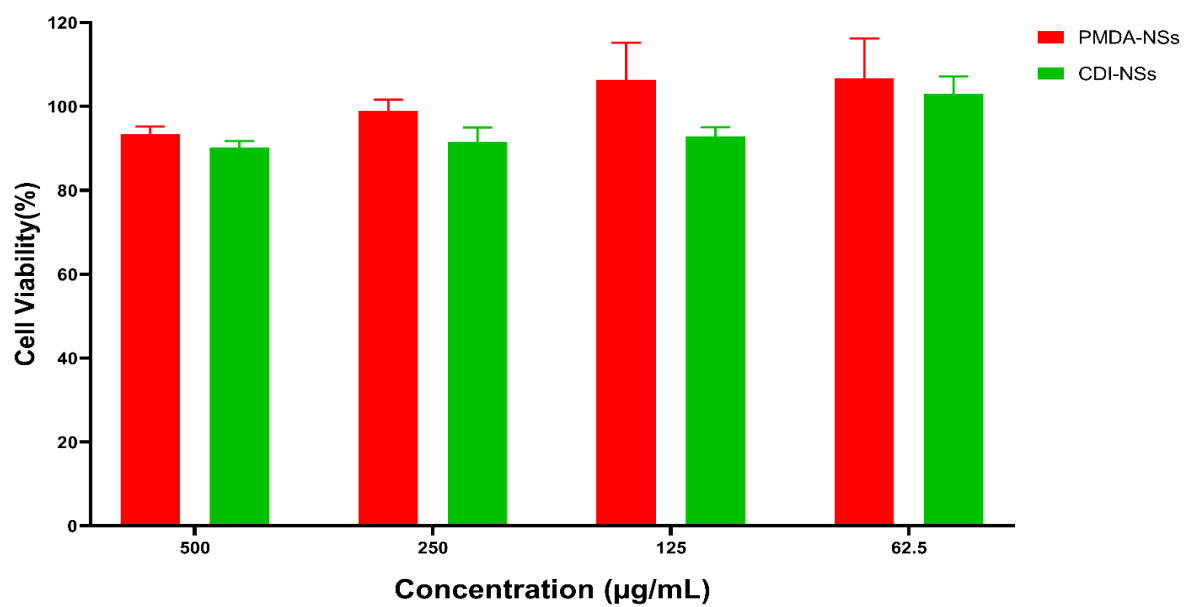


Figure 30. Effect of empty β CDNSs cytotoxicity effect on fibroblast cells viability was evaluated after a 24 h incubation with different concentrations.

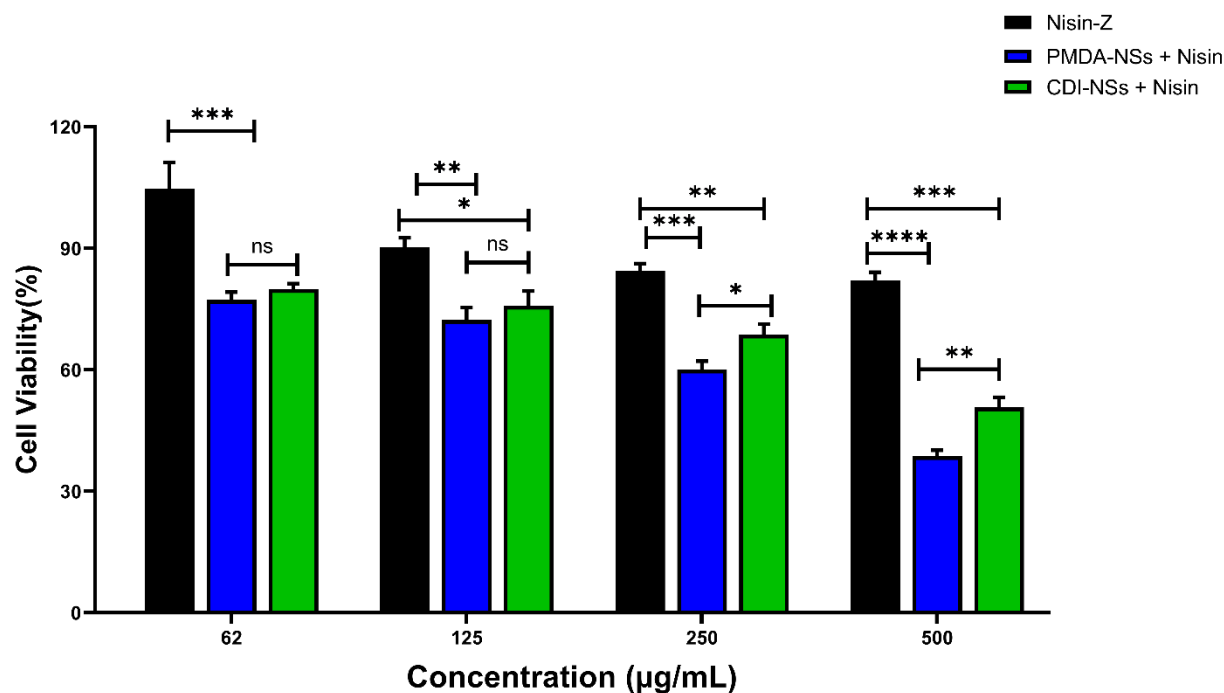


Figure 31. The effect of free Nisin and loaded with β CDNSs on viability of B16-F10 cells was evaluated after a 24 h incubation with different concentrations. (n = 5); *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.1.3.2.2. LDH release

The amount of LDH released from melanoma cancer cells was evaluated to understand whether the cell cytotoxicity effect of free Nisin Z and loaded on both nanosponges was associated with cell membrane damage. As shown in Figure 33, both free CD-NSs did not show considerable LDH release even at 500 µg/ml against melanoma cancer cells, this suggests that our nanoparticles are not capable of damaging tumor cell membranes. Also, the data showed a dose-dependent trend for the release of LDH

from cancer cells in contact with Nisin free and loaded on both β CDNSs (Figure 32). Nisin encapsulated with both β CDNSs led to an increase in the amount of LDH leakage at all concentrations compared to free-Nisin, while the LDH release of cancer cells treated with nisin in complex with PMDA-NSs was substantially higher than free nisin at 62 and 500 $\mu\text{g/ml}$, about four and two times ($P < 0.0001$), respectively.

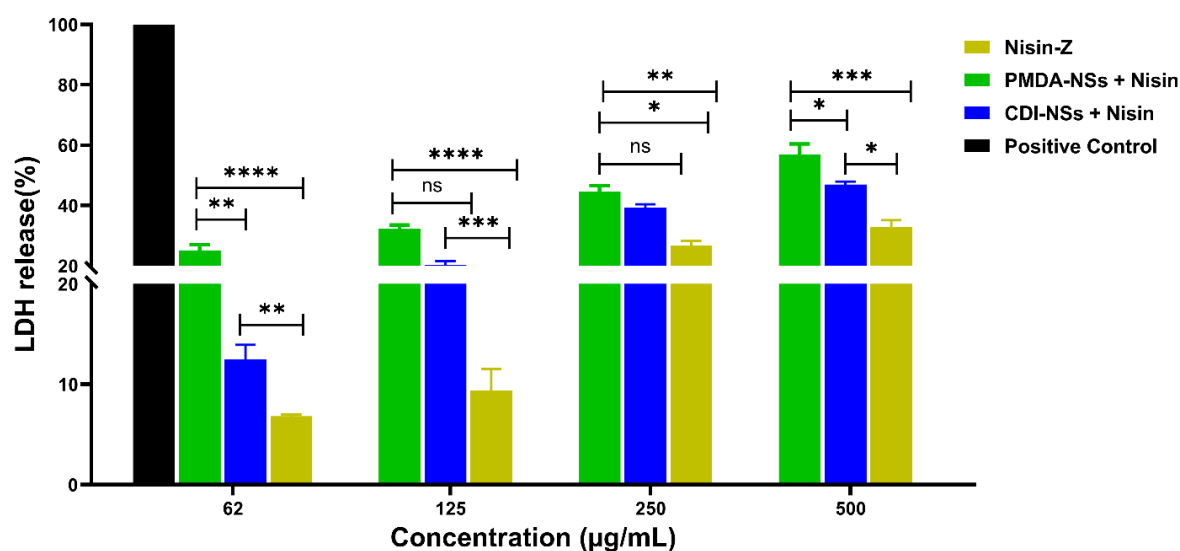


Figure 32. LDH release from B16-F10 cells following treatment with free nisin Z and loaded on β CDNSs for 24 h. The positive and negative controls were considered as having 0% and 100% LDH release, respectively. Data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ and ns (Not Significant).

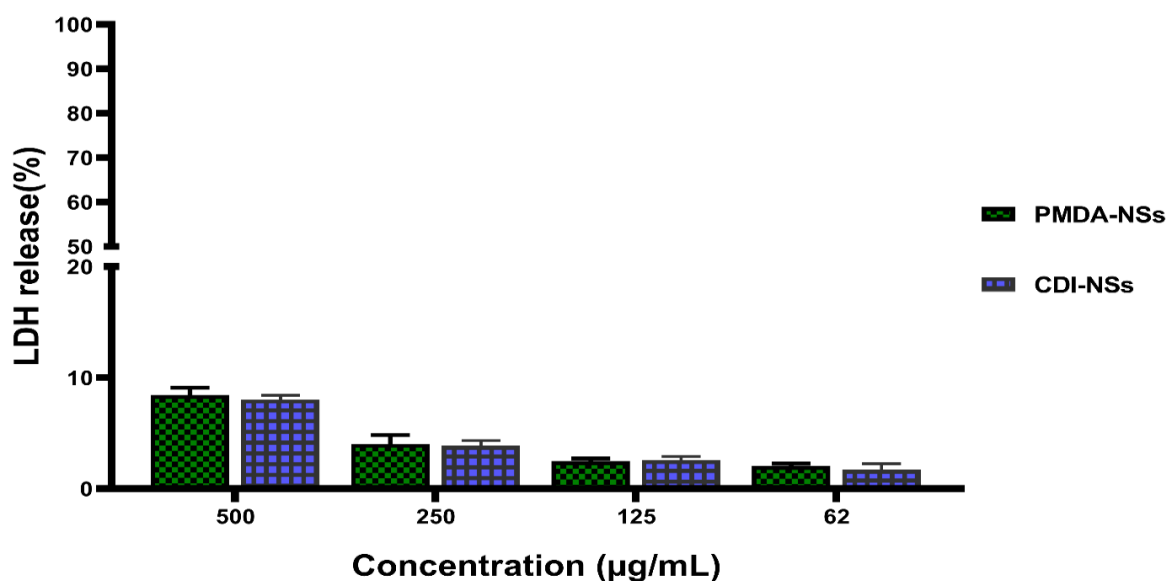


Figure 33. LDH release from B16-F10 cells following treatment with plain nanosponges for 24 h. Data are expressed as mean $\pm$ SD.

3.1.3.2.3. Free coumarin-6 (C-6) internalized uptake in compared to coumarin-6-loaded PMDA-NSs and CDI-NSs

To test cellular uptake, PMDA-NSs and CDI-NSs were loaded with coumarin-6 (0.1 mg/ml), a fluorescent biomolecule, to imitate Nisin uptake. Melanoma cancer cells were then treated for 4 hours with coumarin-6 put into a predetermined concentration of nanosponges (10 mg/mL), and the cellular fluorescence qualitative and quantitative were determined by fluorescent microscope and flowcytometry, respectively. Fluorescence images illustrated a strong uptake of coumarin-6 loaded on both CDI-NSs, while, coumarin-6-complexed with PMDA-NSs showed higher cellular uptake

compared to CDI-NSs (Figure 34A). Flow cytometry findings showed that the cellular uptake level of C-6 complex in PMDA-NSs is higher than CDI-NSs, while there is no significant difference between free C-6 and loaded in CDI-NSs (Fig 34B). In addition, the mean fluorescence intensity (MFI) for C-6 complexed with PMDA-NSs was higher than CDI-NSs, and these data were consistent with microscopic fluorescence outcomes. The C-6 encapsulation efficiency results within PMDA-NSs and CDI-NSs were about 80% and 88%, respectively, suggesting that PMDA complex C-6 with less strength than PMDA (data not shown). As the complex is a dynamic equilibrium, the lower the strength, the faster the release. Furthermore, this issue might be due to the higher bioavailability of Nisin in PMDA-NSs that led to increase its stability [194], which would increase the intracellular concentration of Nisin in melanoma cancer cells [32, 194], as evidenced by the higher coumarin-6 absorption. This effective cellular uptake was in agreement with the results of previous studies, which showed the biological agents loaded nanosponges could be fruitfully delivered inside cancer cells [93, 134]. Classically, CD derivatives are uptake by endocytosis processes, releasing the drug carried inside the cell in direct treatments [197]. Taking into account this point, better uptake of C-6 in complex with PMDA-NSs may led to conclude that the targeted drugs encapsulated by this NS could be taken up and released inside the cancer cells fastly [32, 87].

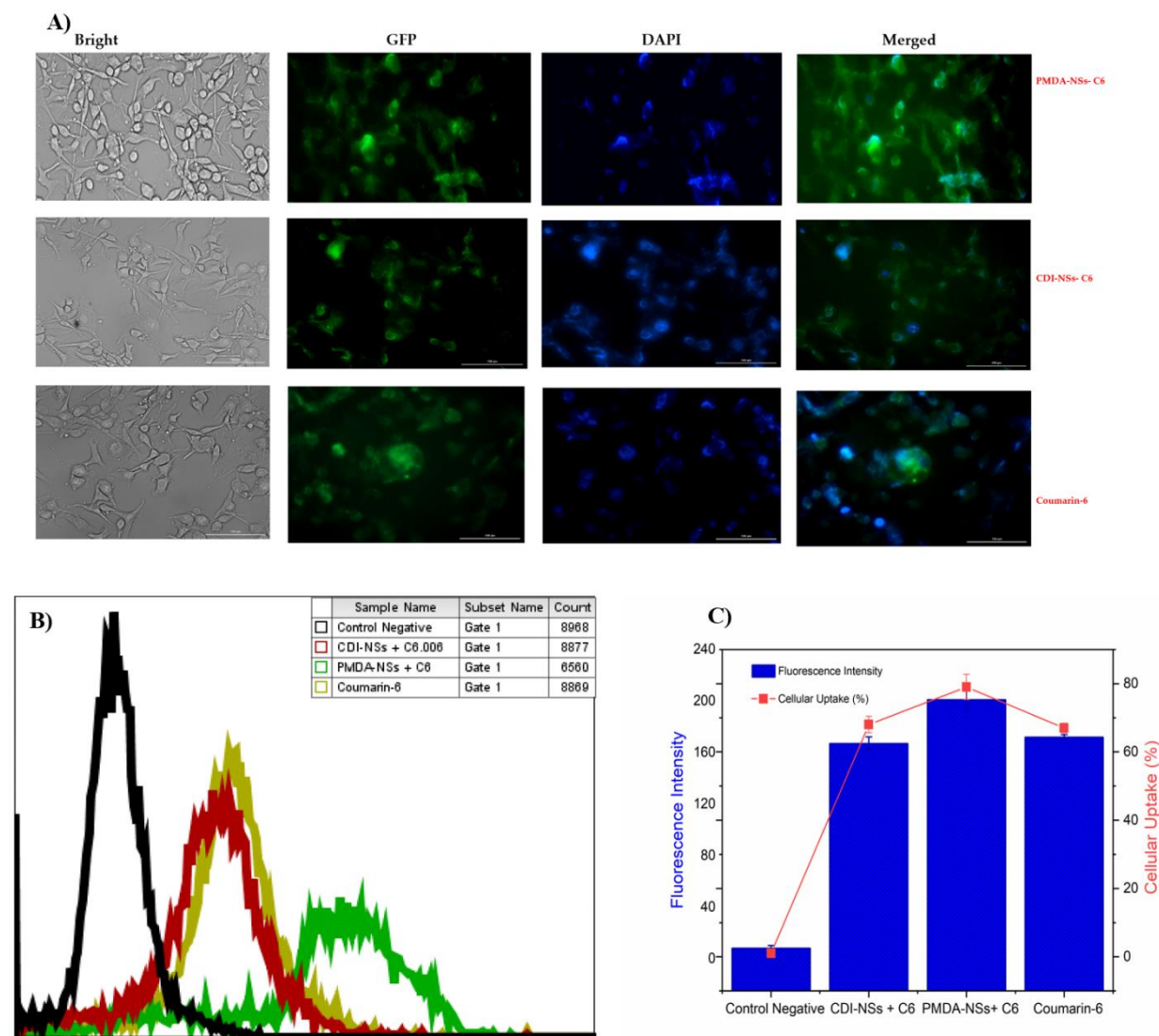


Figure 34. The cellular uptake of coumarin-6 loaded CDNSs on B16-F12 cells.. A; Fluorescent microscope (37°C, 4h), B; Flow cytometry data (37°C, 4h), and C; was the mean fluorescence intensity of B16-F10 cells measured using a FACS assay. The data is presented as a mean standard deviation (n=3). Differences in statics between PMDA-NS, CDI-NSs, and control (**p 0.01).

3.1.3.2.4. Detection of apoptosis and late apoptosis/necrosis with flow cytometry

The apoptosis status of cell lines was evaluated using flow cytometry analysis to understand if the death of cancerous cells due to cytotoxicity and LDH release was related to either necrosis or apoptosis. Previous reports have indicated that the annexin-V and PI can be used for detecting a primary apoptotic and late-stage apoptotic cells (called secondary necrosis cells)[198]. According to the results of cytotoxicity assays, the melanoma cancer cells were treated with free nisin Z at the concentration of 500 µg/ml and the same concentration in complexes with βCDNSs. As shown in Figure 35, the cancer cells were mainly in the early apoptotic stage in the way that the cells treated with free Nisin showed a 30% annexin-V positive rate, while this percentage was outstandingly increased to approximately 50 and 75% when nisin was entrapped with CDI and PMDA-NSs, respectively. Interestingly, the results concluded that the percentage of viable cells declined about three times when the cells were exposed to Nisin loaded on PMDA-NSs in compared to free Nisin($P < 0.0001$).

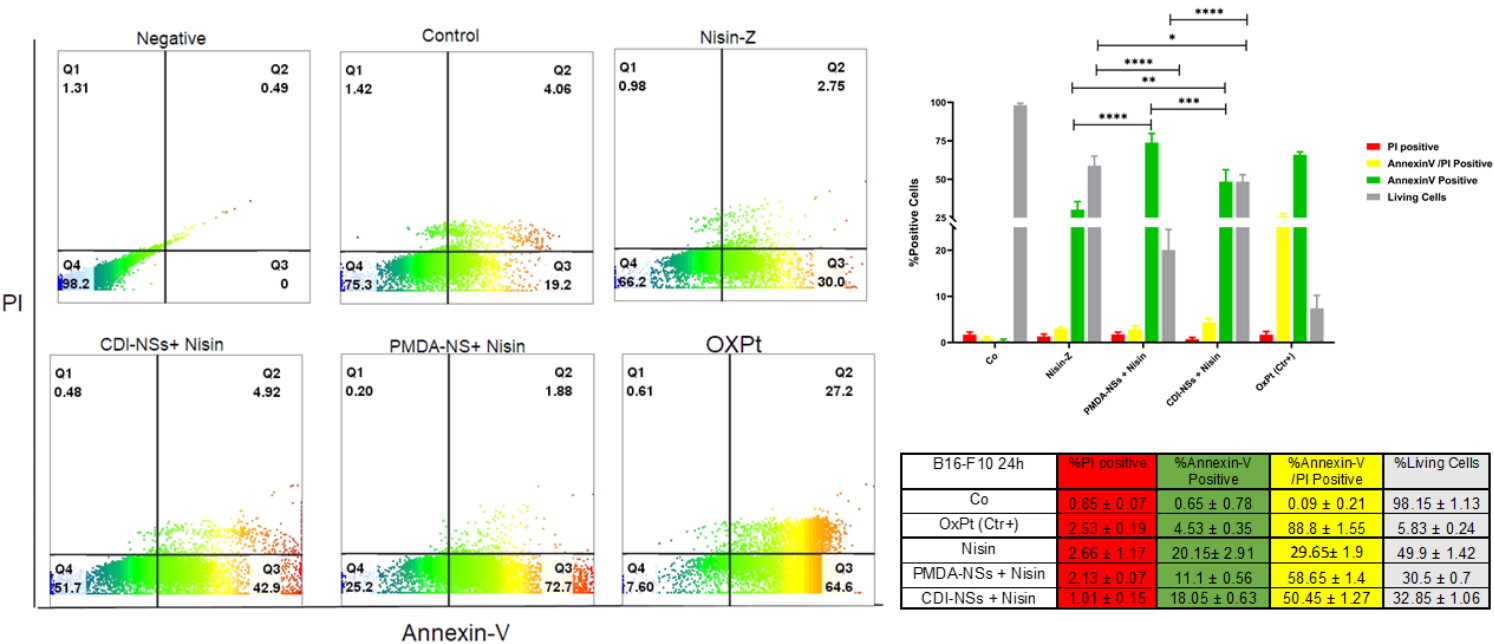


Figure 35. Flow cytometric dot-plots of the population distribution of apoptotic and necrotic in B16-F10 cells after exposition to 500 µg/ml nisin Z and loading on both PMDA and CDI-NSs for 24 hours. In the untreated control, the complexes were not exposed to nisin Z, and the positive control was given oxaliplatin (OxPt). In comparison to free nisin Z, apoptosis was found to be *p 0.05, **p 0.01, ***p 0.001, and ****p 0.0001. The data is presented as a mean standard deviation (n=3).

3.1.3.2.5. Inhibition effects of Nisin-Z alone and complexed with PMDA-NSs on the in vivo tumor growth

C57BL6/J mice with palpable melanoma tumors were treated subcutaneously with either free Nisin, Nisin in complexes with CDNS, empty CDNS, or the same volume of saline every 2 days for a 10-day treatment duration, and tumor growth was evaluated every 2 days to determine the anti-cancer therapeutic efficacy of Nisin and loaded on

PMDA-NSs. As shown in Figure 36, Nisin free illustrated a low effect in reducing tumor growth and weight, while the Nisin in complexes with PMDA-NSs led to a significant reduction in both tumor volume and weight compared to the free Nisin (P 0.001) and untreated group (P 0.0001), whereas the free CDNS did not show an anti-tumor effect. These data was in the same line of other studies results that showed nisin combination with 5-florouracil loaded on oligomeric chitosan coated silver nanoparticles showed both in mean tumor volume (68.34 %) and mean tumor burden (82.39 %); restored skin histoarchitecture and improved oxidant/antioxidant status in murine skin cancer model[199]. In addition, the tumor growth reduction because of nisin treatment alone and in combination were reported [123, 161, 166, 199, 200], while our data illustrated enhanced tumor suppression perhaps for nisin due to the effects of nanoformulation [32].

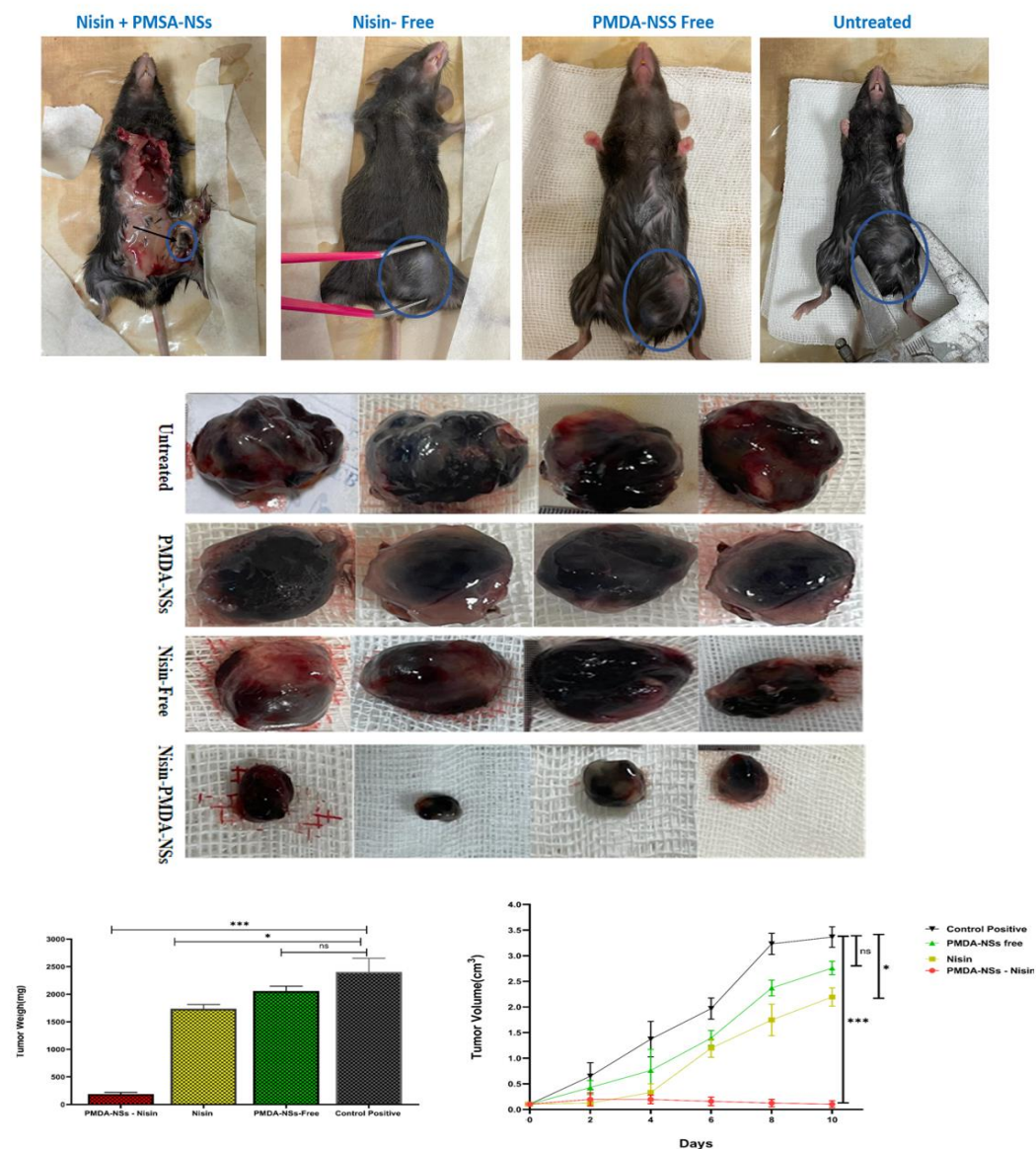


Figure 36. Effect of Nisin Z and loaded CDNS; and free CDNS on the growth of B16-F10 tumors in C57BL6/J mice.

Every two days the tumor volume (cm³) was evaluated after the first treatment (T0, when the tumor was palpable)

and tumors were weighted at the last day when the mice were sacrificed after 10 days. * $P \leq .05$, *** $P \leq .001$, **** $P \leq .0001$ and ns (Not Significant).

3.1.3.2.6. Effects of Nisin-Z alone and complexed with PMDA-NSs on histopathology of solid melanoma cancer mice

Histopathological examination of tumors in the untreated group revealed a large necrotic area in the center of the tumor surrounded by a peripheral area of living cells (Figure 37A). The necrotic core contained tumor cells with pycnotic and karyorrhectic nuclei with low eosinophilic cytoplasm and cell debris. The development of a centrally located necrotic area is attributed to inadequate vascular supply to the area. The nuclei of the cells in the viable peripheral growth zone were large, ranging in shape from ovoid to round to rather irregularly polygonal (Figure 37b). Furthermore, this group indicated a high mitotic index. The microscopic view of the tumor revealed that the tumor was relatively vascular, and many of the blood vessels appeared to be congested. The cells were often arranged in irregular cords or as swirls around a blood vessel. In some areas, the cells with light brown granules, presumably melanin, were abundant. Invasion of the melanoma cells into surrounding skeletal muscle, collagenous tissue, and adipose tissue occurred in areas where the tumor was not encapsulated (Figure 37c). The tumors in the group treated with the free PMDA-NSs showed the same characteristic vascular arrangements and were histologically comparable to those in the untreated group. The parenchyma of the melanoma

consisted chiefly of degenerated tumor cells arranged in circular or oval groups around the blood vessels. Also, the nucleus of the cells displayed characteristics of degeneration, including swelling, vacuolization, rupture, and fragmentation. The cytoplasm of degenerated melanoma cells was stained slightly basophilic and contained varying amounts of fine, light brown melanin pigment (Figure 37d, e, f). The tumors in the treated groups with free Nisin-Z showed a proportionate increase in vascular degeneration and necrotic areas with a remarkably small blood supply (Figure 37 g, h), while they did not show the morphological differences compared to the untreated groups. In the group treated with Nisin-Z loaded on PMDA-NSs, the tumor showed extensive vacuolation of the tumor cell because the tumor tissue was relatively degenerated, while it was noticeable that the mitotic index was significantly depressed compared to the untreated group, as the malignant cell population was lower in this group compared to other groups. The degenerated tumor cells were clearly pigment-free, and the malignant cell population was still less than the other groups, and the cell aggregations were relatively diffuse (Figure 37 I, j).

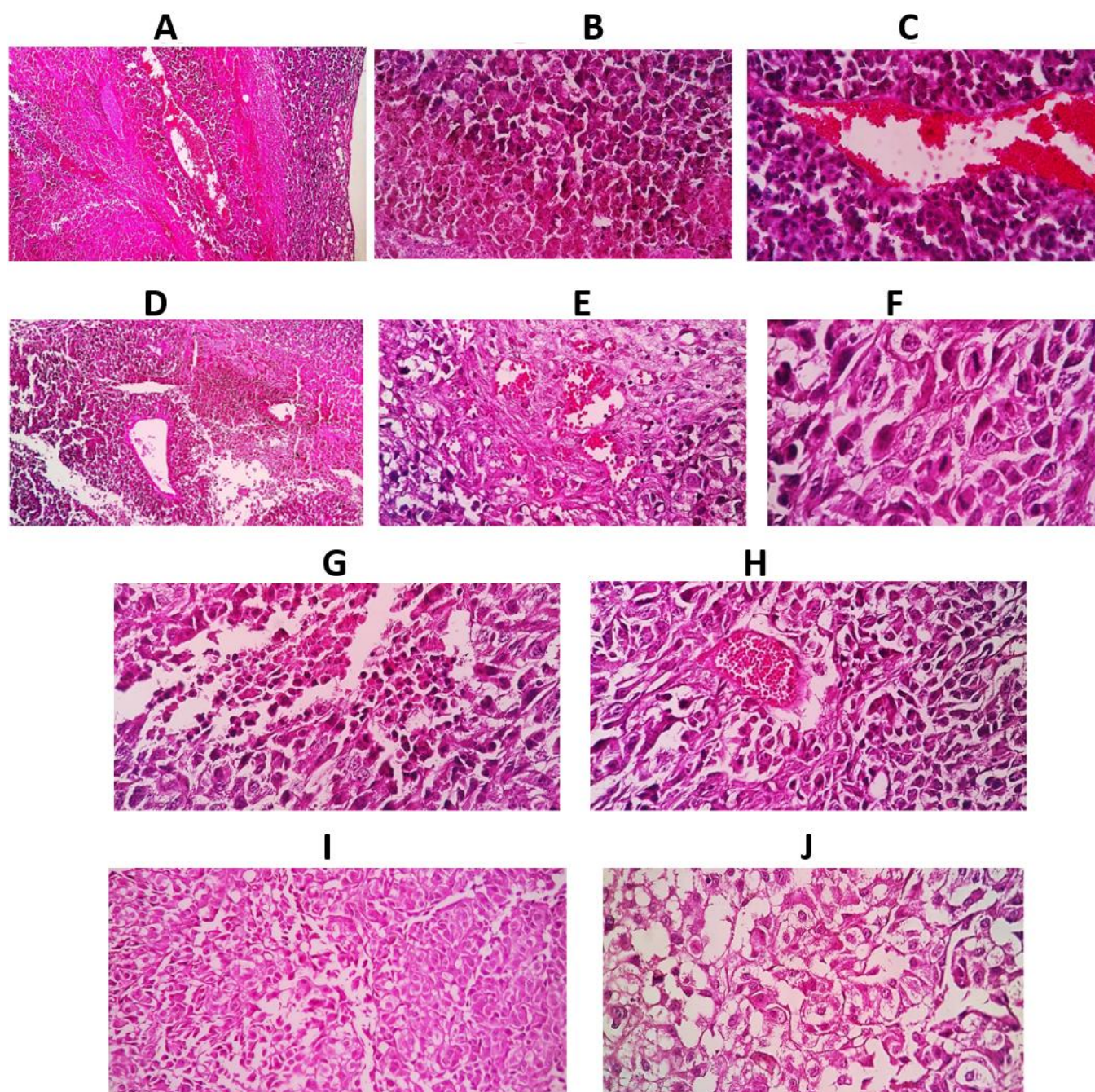


Figure 37. Histopathology experiment. A; Representative section of tumor mass from untreated group (H&E, 120 $\times$). B; Microscopic appearance of tumor mass from untreated group (H&E, 400 $\times$). C; Microscopic view of tumor mass from untreated group (H&E, 400 $\times$). D; Representative section from group treated with free PMDA-NSs (H&E, 202

120×). E; Morphological changes in the tumor mass from group treated with free PMDA-NSs (H&E, 400×). F; Photomicrograph from tumor mass from group treated with free PMDA-NSs (H&E, 800×). G; Photomicrograph from tumor mass of a Nisin-Z free treated group (H&E, 400×). H; Histologic appearance from tumor mass of Nisin-Z free treated group (H&E, 400×). I; Microscopic appearance of tumor mass in treated group with Nisin-Z loaded on PMDA-NSs (H&E, 400×). J; Microscopic view of tumor mass in treated group with Nisin-Z loaded on PMDA-NSs (H&E, 400×).

3.1.3.2.7. Immunohistochemical examination of anti-CD31 activity

The angiogenic marker, CD31[201], was conducted by the immunohistochemistry (IHC) technique to show the tumor angiogenesis status. Furthermore, the CD31-positive area was measured by digital scanning to determine the tumor microvessel density (TMD). As shown in figure 38A, the expression of vascular CD31 level was lower in the group treated with Nisin loaded on PMDA-NSs compared to free Nisin, free PMDA-NSs, and untreated groups. On the other hand, the TMD value in the group treated with Nisin in complexes with PMDA-NSs was significantly less (about four times) than the untreated control and free PMDA-NSs (P 0.0001), whereas treatment with free Nisin showed a negligible decrease compared to untreated and free CDNS (P 0.05) (Figure 38B). Therefore, it can be concluded that the anticancer effect of nisin in complexes with CDNS is increased by suppressing the angiogenesis of cancer cells. This was in line with the results of other studies which evidenced the enhancement effect of nanosponges on various encapsulated agents to decrease angiogenesis as a tumor growth inhibition mechanism [202, 203].

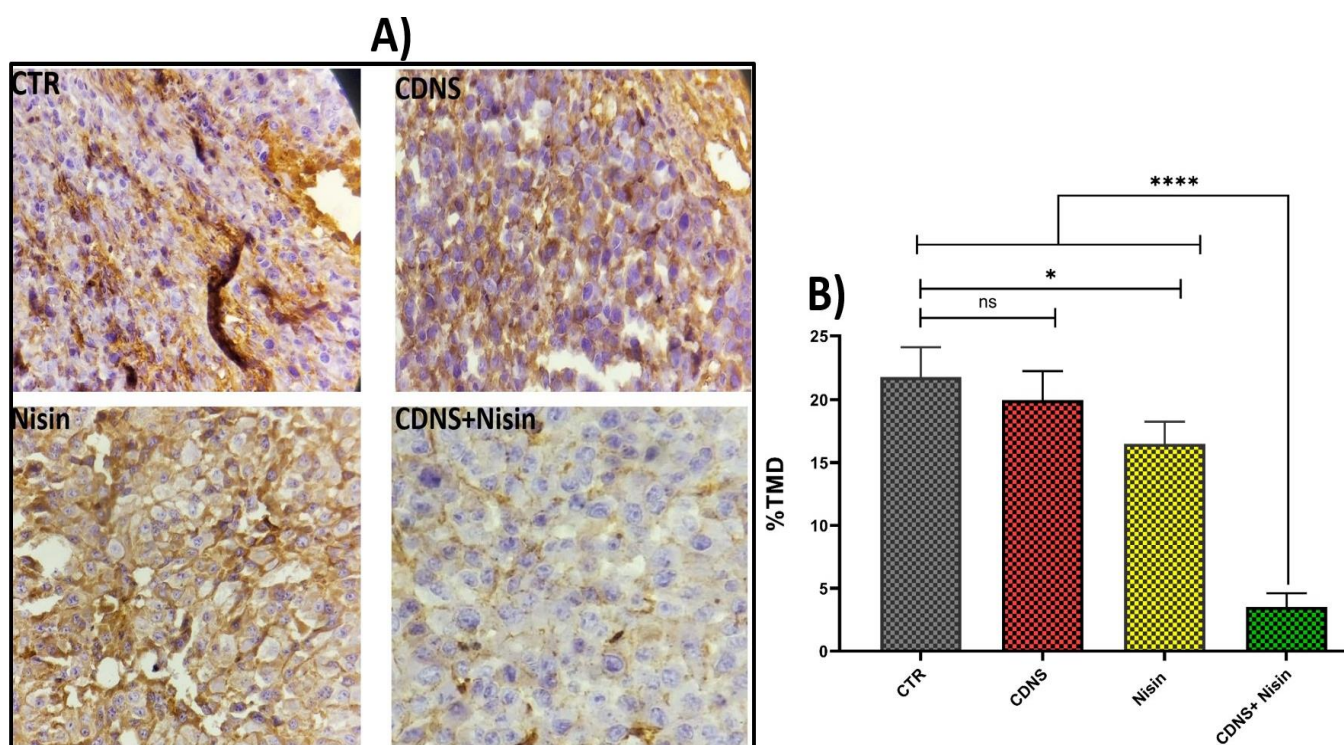


Figure 38. A; Immunohistochemical staining for anti-CD31 of tumors tissue sections from mice treated with saline (CTR), PMDA-NSs alone and Nisin-Z in complexes with PMDA-NSs. Microphotographs of CD31 staining from a representative experiment (brown; magnification 200 $\times$). B; The CD-31 positive area on the tumor sections was assessed to calculate the tumor microvessel density (TMD). Three randomly selected areas from three tumors from each group were analyzed. * $P \leq .05$, **** $P \leq .0001$ and ns (Not Significant).

3.1.3.2.8. Immunohistochemical examination of BAX

The expression of BCL2 associated X protein (Bax), proapoptotic agent, [105] was assessed in different induced melanoma cancer groups. As shown in Figure 39, untreated mice showed low positive (+ve) immunostaining for BAX, and its value in tumor sections of mice treated with free PMDA-NS and free-Nisin indicated low

positive(+ve) and moderate positive(++ve) immunostaining, respectively, while the tumor sections from mice treated with Nisin in complexes with PMDA-NSs has revealed a strong positive (++++ve) of immunostaining for BAX. In addition, the quantification results of bax positive area showed that the expression of proapoptotic protein was about three times increased when Nisin was complexes with PMDA-NSs than free nisin, while there was not seen a differences between free PMDA-NS and untreated group (Figure 39Right).

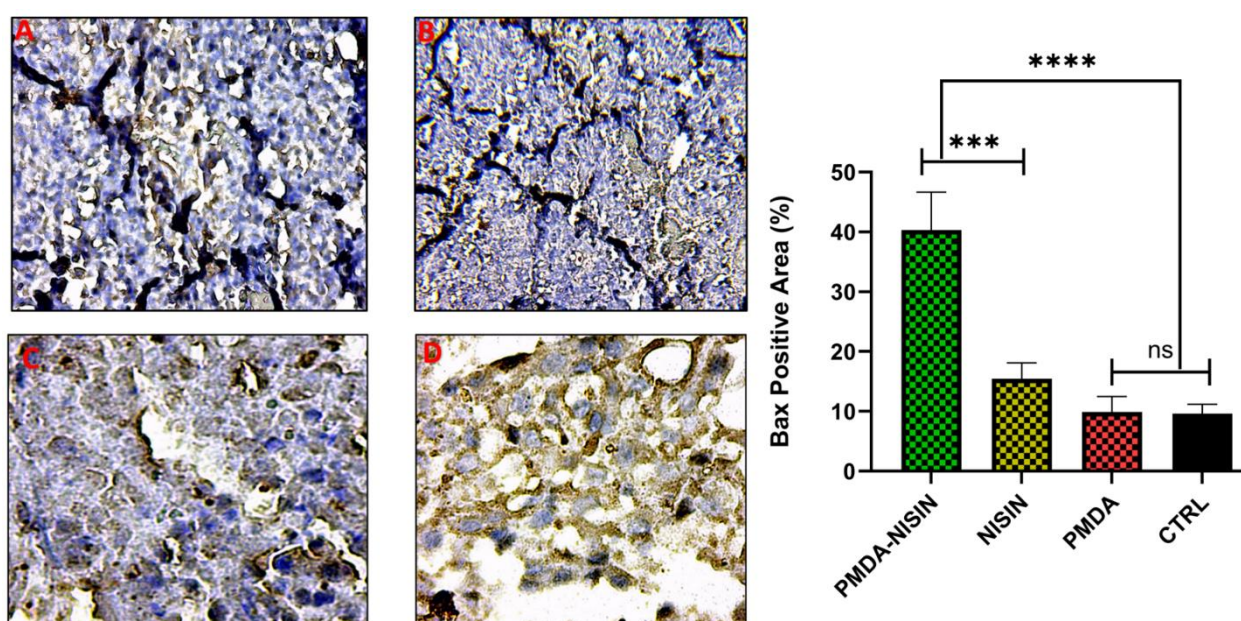


Figure 39. Left: Photomicrographs of sections from different treatments for BAX, A: Untreated group, B: PMDA-NS, C: Free-Nisin, D: Nisin/PMDA-NS ($\times 200$ X). Right: Quantitative analyses of tumor sections stained for Bax. The nuclei were stained blue and the target protein was stained brown. *** $P \leq .001$, **** $P \leq .0001$ and ns (Not Significant).

3.1.3.2.9. Immunohistochemical examination of BCL-2

The expression of B-cell lymphoma 2 (BCL2), an anti-apoptotic agent, [105] was evaluated in different induced melanoma cancer groups by immunohistochemical staining. The results illustrated that the immunostaining for Bcl-2 in tumor sections from untreated mice was graded as strongly positive (++++ve), the similarity status was reported for the treated group with free β CDNS (strongly positive (++++ve)), while the Bcl-2 expression in sections of mice treated with free Nisin and Nisin in complexes with PMDA-NSs was indicated as moderate positive (+++ve) and low positive (+ve) immunostaining, respectively (Figure 40). Furthermore, the quantification results of the bcl-2 positive area indicated that the expression amount of this anti-apoptotic protein was decreased significantly in tumor tissue of mice treated with nisin in complexes with PMDA-NSs compared to nisin-free ($p < 0.01$) and other groups ($p < 0.0001$) (Figure 40 right). These results were in the same line with our previous study that showed Nisin-Z in complexes with PMDA-NSs induced far better apoptosis in both colon and breast cancer cells compared to Nisin free and Nisin/CDI-NSs[194]. On the other hand, the expression of Bax, a proapoptotic agent[105], and Bcl-2, anti-apoptotic agent [105], was outstandingly raised (about 3 times) and depressed (about 2 times) of tumor tissues in mice treated with Nisin in complexes with PMDA-NSs, respectively, compared to treated group with free Nisin. This apoptosis-inducing effect of nisin in combination with PMDA-NSs may be related to the increased cellular uptake of Nisin by cancer cells in complexes with CDNS. The apoptogenic effect of nisin in this

investigation was consistent with other studies where they claim that nisin is a performing bacteriocin that has the ability to alter ionic slopes within cells, resulting in a net calcium influx[200] which this calcium increase may have an important role in inducing apoptosis in cancer cells. In addition, Hosseini et al. study results indicated that Nisin could suppress the proliferation of SW48, HT29, and Caco2 cells by regulating the metastatic genes in colorectal cancer cells[188]. Moreover, according to Ahmadi et al.'s study, the bax/bcl-2 ratio in the colon adenocarcinoma cell line increased during Nisin treatment due to apoptotic pathway activation[204], Nisin-Z also caused apoptosis in human umbilical vein endothelial cells (HUVEC), which resulted in fewer vascular sprouts and a lower density of intertumoral micro vessels [205]. A recent study on the anticancer effect of nisin showed that it causes cell death by manipulating apoptosis and arresting the cell cycle (G0/G1 phase) and inhibits Non-Small Cell Lung Cancer (NSCLC) progression via non-membranolytic pathways by mitochondrial membrane depolarization and elevation in reactive oxygen species (ROS) generation in both 2D and 3D cell culture[206].

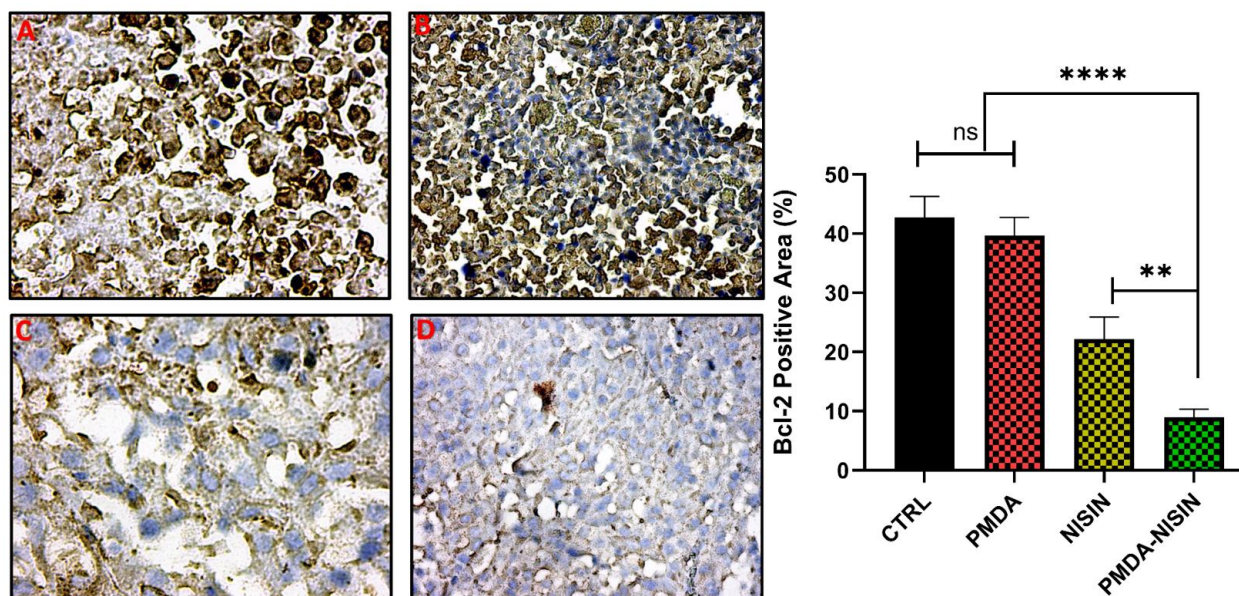


Figure 40. Left: Photomicrographs of sections from different treatments for BCL-2, E: Untreated group, F: PMDA-NS, G: Free-Nisin, H: Nisin/PMDA-NS ($\times 200$ X). Right: Quantitative analyses of tumor sections stained for Bcl-2. The nuclei were stained blue and the target protein was stained brown. $**P \leq .01$, $****P \leq .0001$ and ns (Not Significant).

3.1.3.2.10. The effect of formulations on anti-oxidant agent's level in melanoma cancer mice

Cancer cells produce more reactive oxygen species (ROS) in compared to the normal cells, which is partially related to flawed mitochondrial oxidative metabolism, and this increase in ROS levels is linked to the onset of cancer, tumorigenesis, and resistance to chemotherapeutic agents. On the other hand, these ROS levels are neutralized by increasing the antioxidant capacity in cancer cells. This common occurrence implies that therapeutic approaches can increase ROS production and/or decrease antioxidant

defenses to push cancer cells beyond the point of destruction and activate different pathways of cell death to limit cancer progression[207]. In fact, a wide range of anticancer agents effectively kill cancer cells and sensitize different cancer cells to chemotherapeutic agents by modulating ROS production [208]. Therefore, we have evaluated the effect of the formulations on the level of total antioxidant status (TAS), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) as important antioxidant enzymes in different treated groups. The results indicated that the values of TAS, GPx, and SOD were slightly higher in the group treated with Nisin free than in the untreated group ($P < 0.05$), but their levels were significantly increased when Nisin was loaded on PMDA-NSs than in other groups ($P < 0.0001$) (Figure 41). Interestingly, the level of malondialdehyde (MDA), is a marker used to evaluate the level of lipid peroxidation because of oxidative stress rising [209], was decreased in group treated with Nisin in complex with PMDA-NSs compared to other formulations. It has been suggested that nisin's anticancer properties may also include ROS-mediated apoptotic actions[210-212]. These data support the hypothesis that Nisin in complexes with PMDA-NSs might lead to an increase in the level of antioxidant agents as an anti-cancer defense mechanism and were in agreement with the statement of previous studies that showed nisin alone or in combination with drugs or nanoparticles could suppress tumour growth by increasing the level of anti-oxidant agents[161, 199, 204, 210, 213, 214]. The results of a prior study that demonstrated

that Nisin can activate human polymorphonuclear leukocytes (PMNs) in vitro can be used to support the significant increase in the effect of Nisin-Z on the level of antioxidant agents when complexed with PMDA-NSs in this study. As a result, Nisin increased neutrophil intracellular superoxide levels, which are necessary for the formation of NET and are well known for neutralizing virulence factors[215].

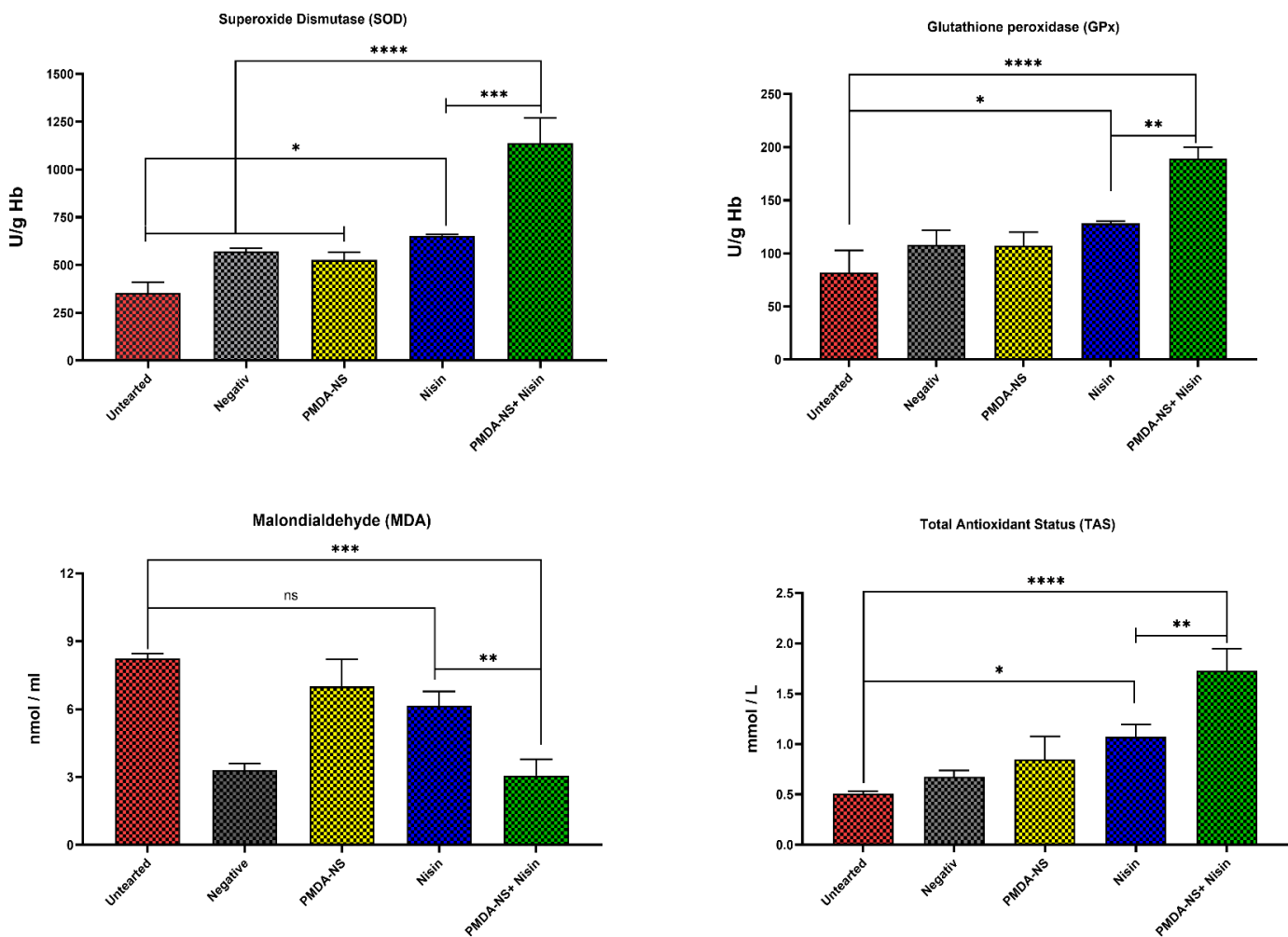


Figure 41. Effect of PMDA-NS alone, nisin-CDNS and Nisin free on antioxidant agents including; SOD activity (U/g Hb), GPx levels (U/g Hb), TAS (nanomoles/L), and MDA (nanomoles/mL) in induced melanoma cancer mice blood stream. Values are represented as Mean $\pm$ SD of four independent values. ** $P \leq .001$, *** $P \leq .001$, **** $P \leq .0001$ and ns (Not Significant).

3.1.3.2.11. The effect of formulations on biochemical parameters level in melanoma cancer mice

In this study, we examined various biochemical parameters for the liver and kidney to understand the effect of tumor-induced toxicity on those organs. The liver enzymes, AST (aspartate aminotransferase) and ALT (alanine aminotransferase), are evaluated as an indicator to illustrate the liver's proper performance, so increased levels of these enzymes in the blood stream can be conducted as a liver damage indicator[216]. Furthermore, the levels of urea and creatinine in the blood are associated with the functionality of the kidney. As represented in Tabl.7, the results showed that the levels of AST and ALT in the treated group with Nisin in complexes with PMDA-NSs were significantly reduced compared to the untreated group, while Nisin free caused an increase in the release of these enzymes compared to the normal group. In addition, drug-free PMDA-NSs led to increase AST but not ALT level. On the other side, the levels of urea and creatinine in all treated groups did not show any considerable differences, except for the untreated group, where the levels of both parameters were increased. These results as a whole suggest a good performance of the Nisin-loaded PMDA-NS to decrease the cancer metastatic effect on those organs.

Table.7. Biochemical parameters assessment in different groups. (***) $P \leq .001$; Nisin-PMDA-NSs vs untreated)

Parameters	Groups				
	Control	Untreated	PMDA-NSs	PMDA-NSs+ Nisin	Nisin-Free
Urea (mg/dl)	18.83 ± 1.25	21.5 ± 2.5	13 ± 1	14.16 ± 2.84	16 ± 3
Creatinine (mg/dl)	0.23 ± 0.03	0.43 ± 0.21	0.20 ± 0.10	0.16 ± 0.05	0.15 ± 0.01
AST (U/L)	108.66 ± 4.16	383 ± 26.87	121 ± 19.07	61 ± 23.43***	153 ± 5
ALT (U/L)	22.43 ± 0.51	59 ± 12.72	19.66 ± 1.52	17.91 ± 5.14***	26.5 ± 4.5
T.P (g/dl)	1.136 ± 0.05	0.7 ± 0.02	1.53 ± 0.92	1.53 ± 0.92	1.05 0.05

3.1.3.2.12. Histopathology analysis of the liver and kidneys

The side effects of induced melanoma tumor on other organs, including liver and kidneys, in different groups were investigated by histopathology examination to understand the inhibitory effect of the formulation. As shown in Figure 42A, the microscopic features indicated normal hepatocytes and a regular liver lobular architecture with a central vein, sinusoidal spaces, and radiating hepatic cords in the healthy control mice. In mice induced with melanoma cancer and without treatment, severe congestion of the central vein was associated with extensive areas of severe hepatocellular degeneration and necrosis randomly distributed throughout the parenchyma, as well as a moderate increase in inflammatory cell infiltration in tumor affected animals, and also their hepatocytes did not show the usual concentric

arrangement and the sinusoids were moderately dilated and congested (Figure 42B). In the group treated with free Nisin-Z, the mice showed mild hepatocellular degeneration and necrosis along with a slight infiltration of inflammatory cells in the centrilobular regions of the livers, as well as a mild congestion in the central veins, the sinusoids were slightly dilated (Figure 42C). Furthermore, in the mice treated with the free drug nanoparticle, the changes were improved compared to untreated mice, which exhibited moderate centrilobular hepatocellular necrosis and patches of mild inflammatory cell infiltration and moderate congestion of the central vein, and also mildly dilated and congested sinusoids (Figure 42D). While the group treated with nisin loaded on PMDA-NSs was able to significantly prevent pathologic changes in liver tissues, with only minor cytoplasmic vacuolation and central vein congestion observed (Figure 42E), these findings were consistent with the reduction of liver enzymes in mice treated with Nisin in complex with PMDA-NSs. The histopathological changes were graded and summarized in Table 8.

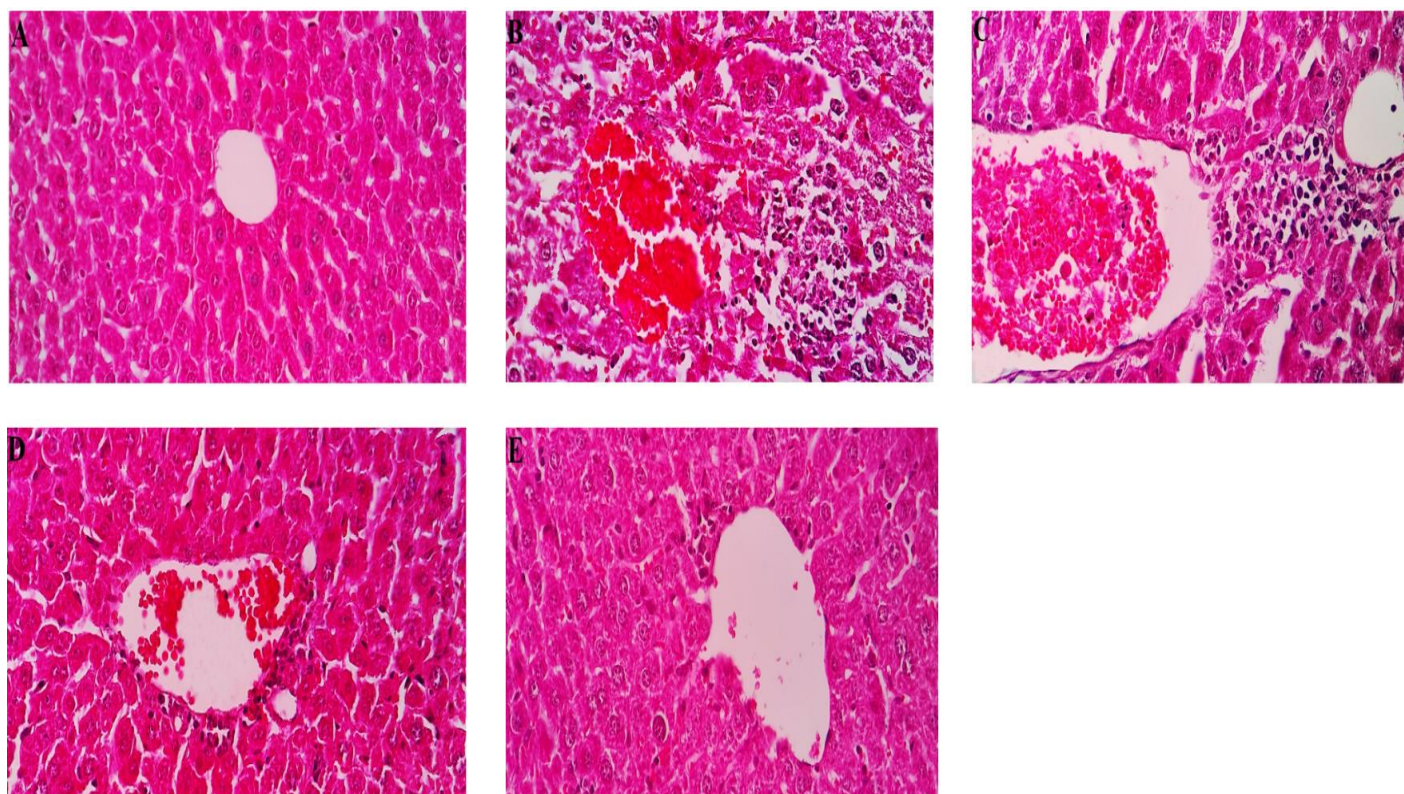


Figure 42. The representative hepatic histopathology. (A) Normal liver from (healthy control) mice. (B) Untreated mice. (C) Mice treated with free PMDA-NSs. (D) Mice treated with Nisin-Z. (E) Mice treated with nisin + PMDA-NSs. Hematoxylin and eosin (H&E) staining (400 $\times$).

The histopathology of kidneys in healthy control mice showed the regular morphology of kidney parenchyma with normal glomeruli and tubuli (Figure 43A). While the renal tissue of mice induced with melanoma cancer which did not receive the treatment showed severe tubular epithelial cell degeneration and necrosis as well as interstitial inflammatory cell infiltration, congestion, edema, and wide spread hemorrhages were obvious, and also a moderate glomerular collapse and shrinkage along with hyperemia

and luminal hyaline cast were seen in these animals (Figure 43B). In the group treated with PMDA-NSs, the severity of necrotic changes and interstitial alterations were alleviated in comparison with untreated mice, as moderate tubule-interstitial changes and a mild glomerular collapse as well as slight hyaline cast formation were seen (Figure 43C). In addition, the mice treated with Nisin free showed some improvement in the damages in comparison with the group treated with nanosponges free of drug, as the mice showed mild tubule-interstitial changes and slight glomerular damage, but there was no evidence of hyaline cast formation in these animals (Figure 43D). Finally, in the group treated with Nisin encapsulated with PMDA-NSs, the complex could considerably prevent the pathologic changes as only slight tubule-interstitial and glomerular alterations were recognized in the renal tissues of these mice (Figure 43E). The histopathological changes were graded and summarized in Table 9.

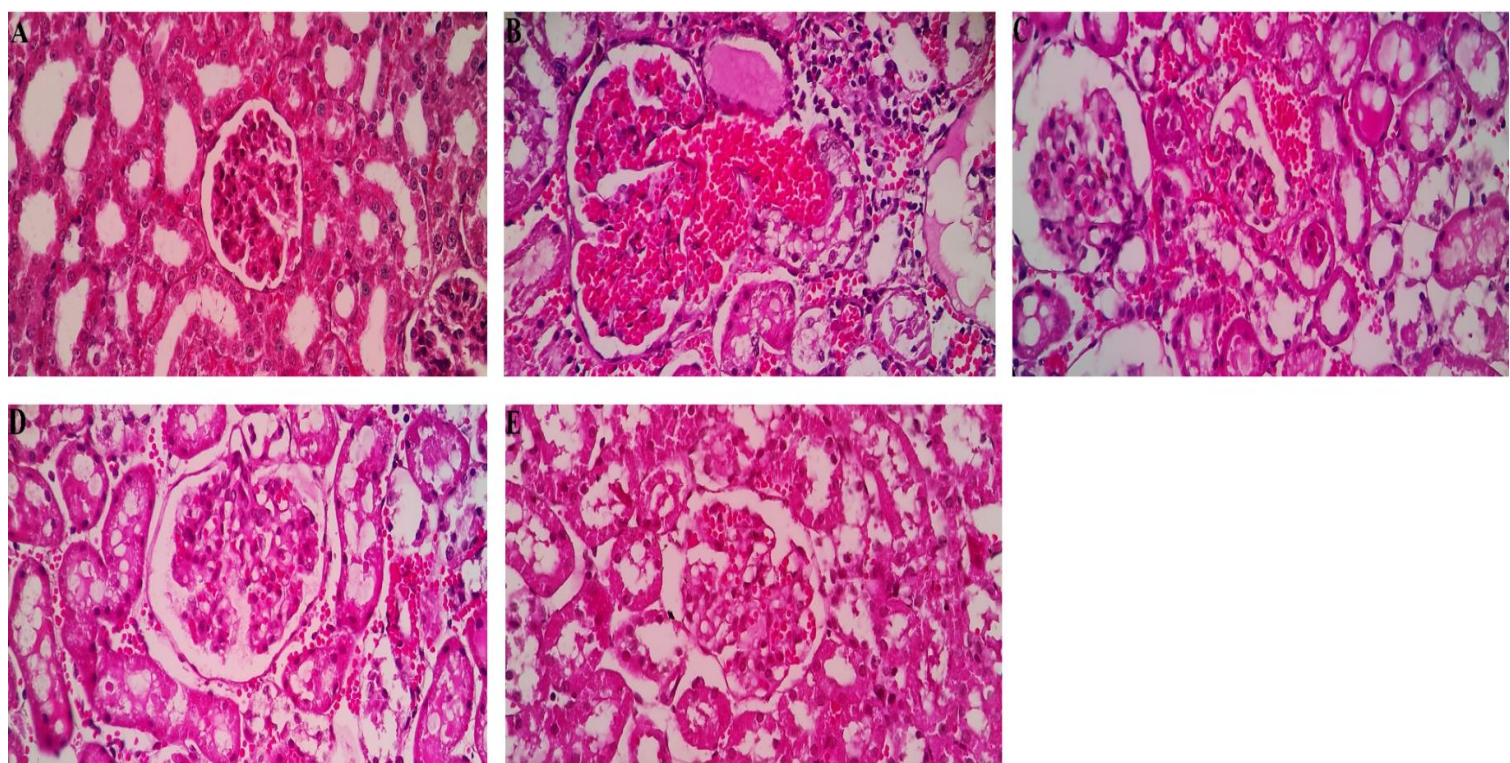


Figure 43. The representative kidney histopathology. (A) Normal Kidney from (healthy control) mice. (B) Untreated mice. (C) Mice treated with free PMDA-NSs. (D) Mice treated with Nisin-Z. (E) Mice treated with Nisin + PMDA-NSs. Hematoxylin and eosin (H&E) staining (400×).

Table 8. Histopathological kidney damages in experimental groups

Groups	Treatment	Microscopic observation			
		Tubular epithelial cells swelling and necrosis	Interstitial inflammatory cells infiltration, congestion, edema and hemorrhage	Glomerular collapse	Luminal hyaline cast
1	Healthy control	0	0	0	0
2	Untreated	++++	++++	+++	+++
3	PMDA-NSs	+++	+++	++	+
4	Nisin-Z	++	++	+	0
5	PMDA-NSs + Nisin	+	+	+	0

The degree of tissue injuries was evaluated semi-quantitatively according to the method reported by Jamshidzadeh et al. Histological damages were scored as follows: 0: absent; +: slight; ++: mild; +++: moderate; and ++++: severe[217].

Table 9. Histopathological liver damages in experimental groups

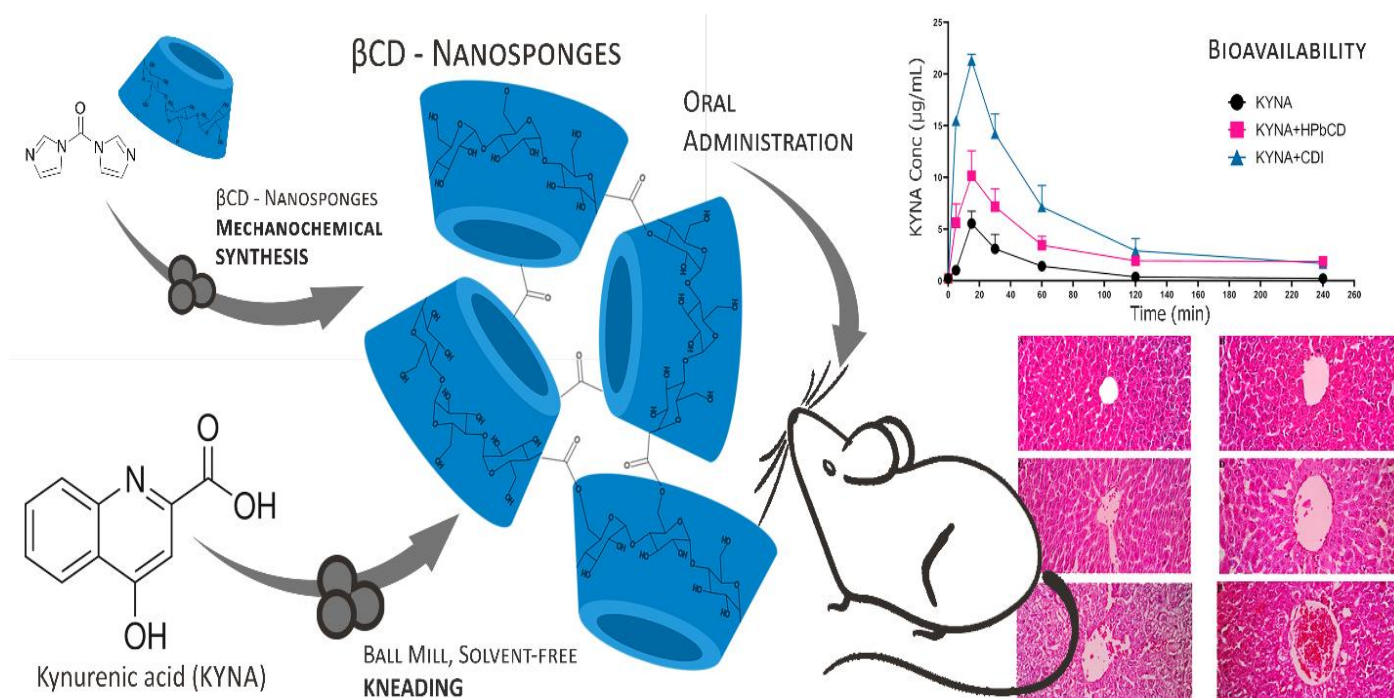
Groups	Treatment	Microscopic observation			
		Necrosis	Central vein congestion	Disorganized sinusoid	Infiltration of lymphocyte
1	Healthy control	0	0	0	0
2	Untreated	+++	+++	++	+++
3	PMDA-NSs	++	++	+	++
4	Nisin-Z	+	+	+	+
5	PMDA-NSs + Nisin	+	+	0	0

The degree of tissue injuries was evaluated semi-quantitatively according to the method reported by Jamshidzadeh et al.. Histological damages were scored as follows: 0: absent; +: slight; ++: mild; +++: moderate; and ++++: severe[217].

To sum up, the in-vitro results showed that Nisin encapsulated in both CDNSs (PMDA and CDI-NSs) behaves as an effective antitumor agent by increasing the cytotoxicity, and apoptosis against melanoma cancer cell lines (B16-F10). Furthermore, Nisin loaded on PMDA-NSs significantly inhibited the in-vivo growth of melanoma cancer in a mouse model compared to free Nisin-Z. Indeed, weight and volume of melanoma tumors were outstandingly decreased in the group treated with Nisin-PMDA-NSs compared to free Nisin. On the other hand, the anti-cancer effects of Nisin related to its apoptosis and anti-oxidant activity were remarkably increased in complex with PMDA-NSs. Moreover, Nisin in nano-formulation led to considerably decrease of CD31,

an angiogenesis factor, expression in tumor tissues. Our findings suggest that incorporating nisin into nanosponges as a safe carrier may allow improving Nisin's anti-tumor effect in melanoma cancer animal models.

Section 2: β NS- CDI in comparison with HP- β CD for nutraceutical delivery



Graphical Abstract

Aim of study:

Many of nutraceuticals presents problems of bioavailability due to their poor water solubility or stability. In this context, the complex between KYNA with HP β -CD and β NS-CDI nanosponges was prepared by complete comparison between kneading in ball milling and classical inclusion complex preparation. Then, the complexes were orally administered to study the eventual effect of these complexes to enhance the bioavailability of KYNA in mice. Consequently, different points such as the antioxidant capacity, general biochemistry and histopathology were studied.

3.2.1.Introduction

Kynurenic acid (KYNA, 4-Hydroxyquinoline-2-carboxylic acid, Figure 44A) is a tryptophan naturally metabolite of our body by the kynurenine pathway. However, it can be found in several foods like spices, potatoes, honey, or broccoli[218, 219]. KYNA also is present in breast milk, and its content increases with lactation indicating the role of neonatal nutrition in general growth with long-term health effects[220]. Furthermore, exogenous KYNA is an ingredient in food including commercial baby formulas and honey[218, 220]. KYNA presents more than an attractive antioxidant capacity, in recent years, its role as ionotropic glutamate and alpha 7-nicotinic receptor antagonist has been investigated, and, indeed, it was proposed as neuroprotective for cerebral ischemia, Parkinson's disease, Huntington's disease, schizophrenia, epilepsy, or depression[221]. Unfortunately, its low aqueous solubility, stability, and fast metabolism limit the therapeutic application of some supplementation[222] although it would be helpful. Even, the uptake is around 25% after oral administration (calculated from[218]). For that reason, different authors have reported many strategies to increase the solubility and the bioavailability of KYNA: for example, in 2016 Varga et al. synthesized KYNA-loaded nanoparticles of bovine serum albumin (BSA)[222] or Lopez et al., earlier, reported the loading of KYNA on silica nanoparticles[223].

A promising strategy to prepare novel supplements is to increase drug and bioactive compounds' solubility, stability, and improves the release profile. Cyclodextrin-based delivery systems provide all these points: the inclusion complex of poorly water-soluble drugs and bioactive compounds with α , β , γ -cyclodextrin is widely reported in foods[4, 23] and several efforts have been made to improve performances and overcome drawbacks associated with native cyclodextrins (CDs). One of this strategies is the polymerization of CDs to obtain both soluble or insoluble[34, 224], which present many advantages against native CDs, for example the possibility to form complexes with a wider variety of molecule[70, 225], being able to exploit both the slightly hydrophobic cavity of the cyclodextrins and the hydrophilic external part.

In the last 20 years, our research group demonstrated remarkable results with specific insoluble β -cyclodextrin polymers, the so-called β CD Nanosponges (CD-NSs), in various applications but especially in the pharmaceutical field as drug delivery systems[226]. However, novel approaches to synthesize the materials are necessary based on green-chemistry principles to "minimize the quantity of final waste and to avoid hazardous or toxic solvents" [227]: CD-NSs themselves, since are synthesized from starch derivatives and biodegradable, are a very promising material but more sustainable syntheses of CDs polymer are of particular interest. Focused on KYNA, we recently reported the synthesis and characterization of complexes of KYNA with several

CDs[110] and with CD-NSs[74], evidencing the formation of inclusion complexes and demonstrating a better antioxidant activity of KYNA loaded on NSs compared to free KYNA. Moreover, the KYNA-loaded NSs were found nontoxic in the cytotoxicity assay.

In this work, several points were studied:

1. A new green approach for both synthesis and drug loading of CD-NSs through mechanochemistry was proposed. A mechanochemical process involves the application of mechanical forces to drive and control chemical reactions by providing the energy necessary to chemical bonds to react. Mechanochemistry in inorganic chemistry is quite established[228, 229] but recently is becoming a more mainstream technique also for organic reactions[230-232] since can promote reaction between solids quickly and quantitatively.
2. The inclusion complexes were prepared directly kneading in ball mill (with different parameters) the KYNA powder with, respectively, Hydroxypropylated β -cyclodextrins (HP- β -CD) and the previously mentioned β CD Nanosponges. The use of kneading permits to overcome all the limitations related to poorly soluble or insoluble compounds, permitting to completely avoid the use of solvents and to speed-up the preparation of the inclusion complex[233, 234].

3. The complexes were orally administered to study the eventual food supplementation. Different points such as the bioavailability, antioxidant capacity, general biochemistry and histopathology were studied

3.2.2. RESULTS AND DISCUSSION

3.2.2.1. Comparative study between ball milling and freeze-drying complexes: Thermal, structural and stability studies

The preparation of inclusion KYNA inclusion complexes, using the “classic” solvent-based method is schematically summarized in Figure 44B, following.

As shown, both the synthesis and the loading are completely carried out without the use of any solvents: the preparation of an inclusion complex in solution/dispersion is widely known, while the use of solvent-free techniques is still a more marginal approach. The same argument can be, even more, valid for mechanochemical polymer syntheses. However, in the last year the use of kneading is reported in many experimental work[234] showing good results.

What we want to highlight in this experimental work is that the solvent-free approach is not only interesting from a green perspective, but it is a method that dramatically speeds up the whole process, both for the synthesis and for the loading of the KYNA. The “classic” solvent approach usually uses 3 days (1 day of mixing, 2 days of lyophilization) but the ball milling only takes 30 minutes of complex preparation, considerably reducing the time to get ready to use complexes.

The materials were fully characterized from a structural and thermal stability point of view before the *in vivo* application. In Figure 44C following the FTIR spectra on the synthesized samples are reported:

A comparison between the free KYNA and the KYNA complexed on NS and HP β -CD, loaded with solvent approach and with ball mill is shown. The FTIR confirm the presence of the carbonate bond peak at 1739 cm⁻¹, characteristic feature of β NS-CDI, confirming the cross-linking reaction in ball-mill [235]. The spectrum of KYNA shows all the strong characteristic peaks at 3095 cm⁻¹ (-N-H stretching), 1660 cm⁻¹ (-C=C stretching), 1362 cm⁻¹ (-OH bending). Interestingly, all the spectra of loaded KYNA, showed the same specific peaks, more intense in the sample prepared by ball-mill (indicating a higher loaded quantity, even starting from the same quantity), but also the disappearance of peaks at 3095 cm⁻¹ (-N-H stretching) signal of a possible interaction (confirmed anyway by other characterizations) of KYNA with NS, and the possible encapsulation within the structure of the drug[74]. The thermal stability, in nitrogen atmosphere, of the starting materials and of the so-described KYNA complexes was investigated (Figure 44D). The KYNA exhibit a degradation that occurs in two main steps: the first step occurs between 100°C and 200°C (Figure 44D, inset a, c and d, pointed by the arrows), the second one that starts at roughly 280°C degrees with a maximum rate weight loss at 300°C. As shown in Figure 44D, inset c

and d (clearly visible in the DTGA) the first step is negligible in the case of complexed KYNA, that is indeed released at 250°C, making possible the assumption of a protective effect of the carrier. Moreover, it possible to hypothesize an efficient “separation” of the KYNA molecules, that can also explain the higher activity that wil

be described later, since the release of KYNA starts at a slightly lower temperature but at a slower rate.

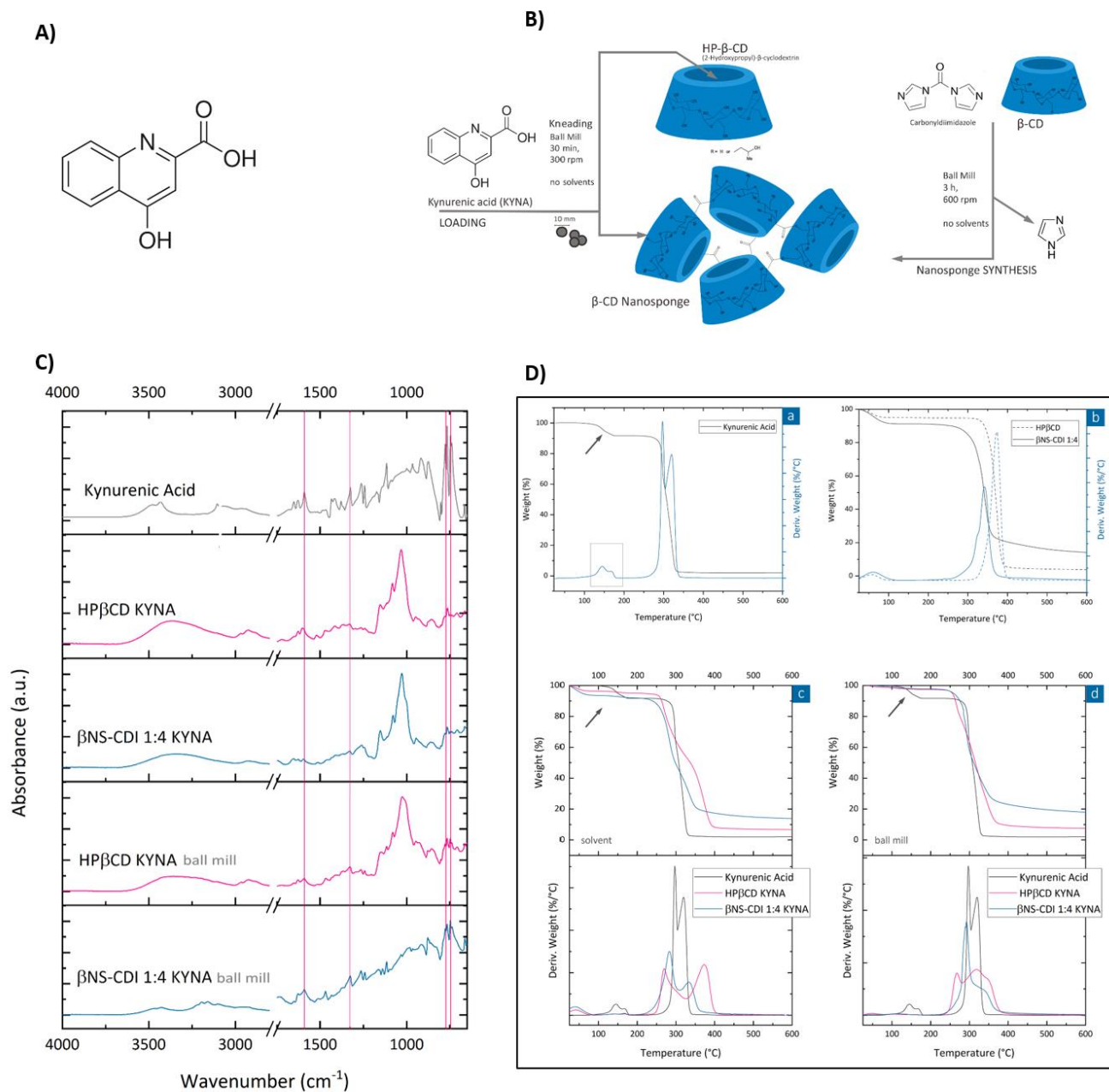


Figure 44. Structural characterization of KYNA complexes. A; Structure of KYNA. B; Schematic β NS-CDI 1:4 ball milling synthesis and kneading complex protocol, C; Comparison: FTIR spectra of KYNA and KYNA-loaded NS and HP β -CD, and D; Comparison of thermo-gravimetric analysis (TGA) and derivative TGA (DTGA) of the different starting material and the inclusion complexes, nitrogen flow from RT to 700 °C, 10 °C/min. Respectively a) Kynurenic Acid b) HP β Cyclodextrins and β NS-CDI 1:4 from ball mill synthesis c) Comparison of inclusion complexes from classic solvent-based method, with free Kynurenic Acid d) Comparison of inclusion complexes from kneading-based method, with free Kynurenic Acid

3.2.2.2. Effect of KYNA complexed in β NS-CDI or HP β -CD orally supplemented on its bioavailability

Although in different studies CD-NS have been proposed as an interesting system to protect and increase the bioavailability of different drugs and proteins[33, 87], until the day there is not a comparative study between CDs monomers and this polymer. For that reason, we selected the interesting bioactive compound KYNA, which pharmacokinetics is considerably fast[236], as a nutraceutical model. A dosage of 50 mg KYNA/kg/day was selected according previous publication and the water consumption per day in mouse[237, 238] administered in free or complexed form.

The results (Figure 45A) demonstrated the fast pharmacokinetics of KYNA obtaining at 15 minutes after administration the maximum. The software PKsolved was used to analyze the curve [239] and the most important parameters are summarized in [table 10](#).

Table 10. Clearance time (CL/F), Cmax, Tmax, Volume of distribution (Vd/F) and bioavailability (F) of measured KYNA

	<i>Control</i>	<i>HPβ-CD</i>	<i>βNS-CDI</i>
<i>CL/F (mg)/(μg/ml)/min</i>	0.000007	0.000001	0.000790
<i>Cmax μg/ml</i>	4.261160	9.560082	21.187298
<i>Tmax min</i>	18.098160	15.658506	12.704157
<i>Vd/F (mg)/(μg/ml)</i>	0.111605	0.052955	0.034259
<i>AUC μg/ml*min</i>	241.901426	751.802754	1352.196742
<i>F (respect control)</i>	1	3.11	5.6

The values of Cmax and Tmax demonstrated the higher and faster uptake of KYNA from the intestinal tissue. HP β -CD is able to reduce the Tmax a 17% and double the Cmax, but the best value was obtained with β NS-CDI, which reduced more the Tmax and increased the Cmax in comparison with HP β -CD. On the other hand, the clearance time (C/F) was increased when the complexed form was administered, possibly to reduce the high concentration in blood. Finally, the AUC showed that the complexed with β NS-CDI was more bioavailable as suggest the relative bioavailabilty (F), giving 5.6 more KYNA in blood than control. It is also important to note that HP β -CD managed to triple the amount of KYNA in the blood, making practically half of the amount supplied bioavailable.

The differences showed between HP β -CD and β NS-CDI could be due to different factors: I) The different release properties: different authors spoke about the better

release properties of CD complexes[4, 240], giving a better bioavailability of the bioactive compound. On the other hand, CD-NS are able to prolong the release of different molecules, even KYNA[74], to obtain a continuous dosage of the molecule due to their intrinsic tissue mucoadhesion capacities[87]. II) The higher stability: Although both carriers have demonstrated a protection during *in vitro* digestion of different drugs[33], CD-NSs presents a higher capacity to protect the compounds against different agents due to the more complex structure and release profile[241].

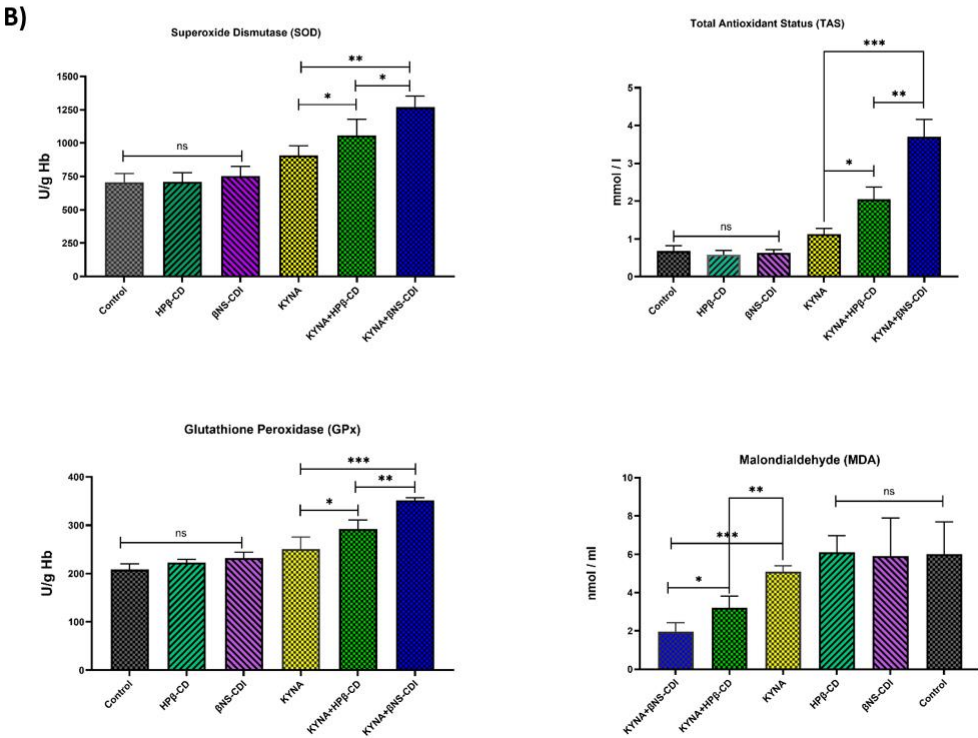
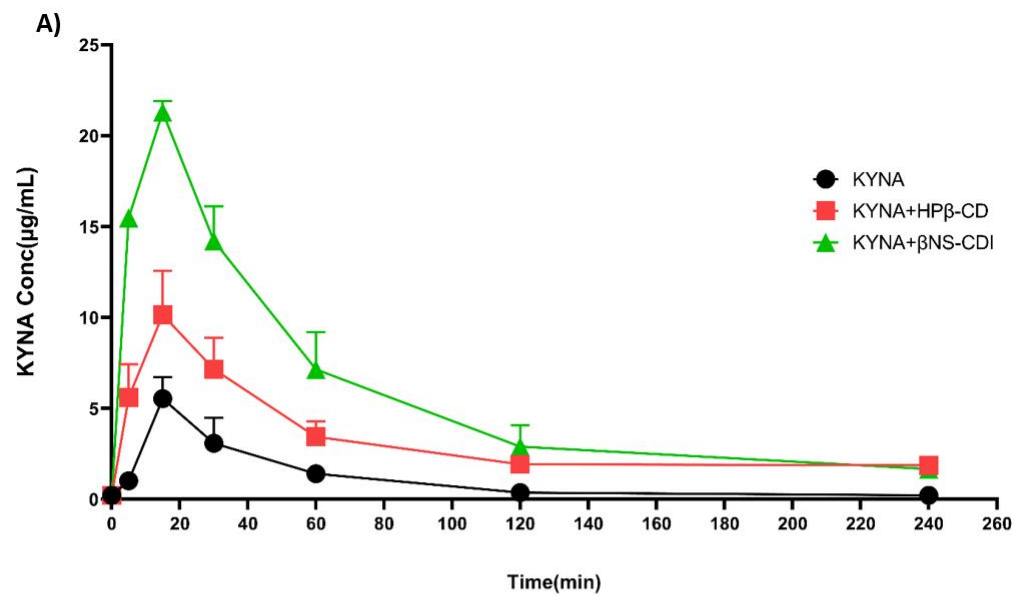


Figure 45. A; Biochemical profile after administration. KYNA plasma concentration at different times after KYNA complex ingestion, B; Effect of β NS-CDI and HP β -CD alone, KYNA in complexes with β NS-CDI and HP β -CD and KYNA free on antioxidant agents including; Superoxide dismutase (SOD) activity (U/g Hb), GPx levels (U/g Hb), TAS (nanomoles/L), and MDA (nanomoles/mL) in mice blood stream. Values are represented as Mean $\pm$ SD of four independent values. **P $\leq$.001, ***P $\leq$.001, and ns (Not Significant).

3.2.2.3. Effect on antioxidant agent levels

Due to the intrinsic antioxidant activity of KYNA, we used this activity to check if KYNA have got any change during liver pass. The effect of KYNA free and in complexed with formulations on the oxidative status of treated animal since the oxidative stress occurs when cells are overproduced of free radicals or deficient in antioxidant defence systems[242]. Therefore, we have evaluated the effect of the formulations on the level of total antioxidant status (TAS), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) as an important antioxidant enzyme in different treated groups (Figure 45B).

The results indicated that the values of TAS, GPx, and SOD were slightly higher in group treated with KYNA encapsulated with HP β -CD than group treated with KYNA free (P<0.05), but their levels were significantly increased when KYNA was complexed with β NS-CDI than free KYNA (P<0.001) (Figure 68B). Interestingly, the level of malondialdehyde (MDA), is a marker used to evaluate the level of lipid peroxidation because of oxidative stress rising[209], was significantly decreased in group treated

with KYNA loaded β NS-CDI compared to other formulations. While there was revealed the better antioxidant effect for KYNA when it was complexed with β NS-CDI than complexes with the HP β -CD in all anti-oxidant agents ($P < 0.01$). These high level of antioxidant status is correlated with several benefits such as antiaging effects [243], more than the own antioxidant status. The higher antioxidant status with KYNA complexes of mice shows that the bioactivities of this bioactive compound reached the body in perfect condition, after complexation by milling and intestinal absorption.

3.2.2.4. Representative biochemical parameters

In this section, we checked a various biochemical parameter for liver and kidney to understand the toxicity effect of complexes. The liver enzymes, AST (aspartate aminotransferase) and ALT (alanine aminotransferase), are evaluated as an indicator to illustrate the liver proper performance, so increased the level of these enzymes in the blood stream can be conducted as a liver damage[216]. Furthermore, the levels of urea and creatinine in blood are associated with the functionality of the kidney. As represented in [table 11](#), the results showed that the level of AST and ALT in treated group with KYNA free were significantly increased in compared to other group (from 64.3 ± 17.16 to 48.2 ± 8.4 U/L with β NS-CDI complex for example), may be this elevation in enzymes level was attributed to some hepatic toxicity of free-KYNA. Interestingly, the toxicity of KYNA was considerably decreased when it was loaded

with formulations, in specially loaded on β NS-CDI. As it is assumed that CDs cannot pass the intestinal barrier[4], but our previous results for antioxidant status demonstrated the increase of this parameter using our formulations but not with free-KYNA, maybe by the increased quantity uptake and better release. This fact could justify the existence of a better behavior to reduce the eventual toxicity of free-KYNA due to eventual damage[244]. On another hand, the levels of urea and creatinine in all treated groups did no show considerable differences except for group treated with KYNA free where showed a slightly increase for urea compared others.

Table 11. Biochemical parameters assessment in different groups. (**P ≤ .01, ***P ≤ .001; KYNA-HPB-CD and KYNA-BNS-CDIs vs KYNA free)

<i>Parameters</i>	<i>Groups</i>						
	Control	HP- β -CD	BNS-CDIs	KYNA	KYNA+ HP-β-CD	KYNA+ BNS-	
<i>Urea (mg/dl)</i>	12 ± 1.1	10.5 ± 0.70	12 ± 2.82	15 ± 1.44	10.5 ± 2.2	12.5 ± 0.71	
<i>Creatinine (mg/dl)</i>	0.13 ± 0.05	0.21 ± 0.03	0.43 ± 0.33	0.23 ± 0.02	0.21 ± 0.2	0.17 ± 0.02	
<i>AST (U/L)</i>	64.3 ± 17.16	67.5 ± 7.7	54 ± 14.12	126 ± 23.43	65.5 ± 12.16***	48.2 ± 8.4****	
<i>ALT (U/L)</i>	27.3 ± 5.65	19 ± 1.71	25.6 ± 7.07	33.51 ± 4.14	19 ± 5.62***	22.5 ± 2.12***	
<i>T.P (g/dl)</i>	0.86 ± 0.05	0.85 ± 0.06	1.1 ± 0.14	0.87 ± 0.042	1.15 ± 0.07	0.95 ± 0.07	

3.2.2.5. Histopathology analysis

3.2.2.5.1. Liver

Histopathological examination of liver sections of the healthy control mice showed distinct hepatocytes arranged in regular cellular architecture with normal concentric cord-like structure around the central vein as well as normal sinusoidal spaces (Figure 46A. a). Microscopic appearance of the livers in mice treated with KYNA complexed by βNS-CDI showed a slight centrilobular degeneration, and the central veins were

slightly congested. Furthermore, there were no evidence of inflammatory cells infiltration in the livers of those mice (Figure 46A. b). The histopathological studies of liver tissues from animals which have been treated with KYNA encapsulated with HP β -CDs illustrated a slightly vacuolation of hepatocytes in centrilobular areas, central vein congestion and sinusoidal dilation (Figure 46A. c).

As shown in figure 46A. d, the β NS-CDI free indicated a slightly hydropic degeneration of the hepatocytes in centrilobular regions, also, a slightly congestion of central veins and sinusoidal dilation were seen in this group. The changes observed in the livers of mice treated by HP β -CD illustrated a mild centrilobular degeneration with slightly central vein congestion and sinusoidal dilation (Figure 46A. e). Finally, in group which was treated by KYNA displayed a variety of pathologic changes in the livers including moderate hydropic degeneration of hepatocytes and congestion of central veins as well as a mild sinusoidal dilation accompanied by slight inflammatory cells infiltration (Figure 46A. f). Quantitative microscopic results of experimental mice are presented in [Table 12A](#).

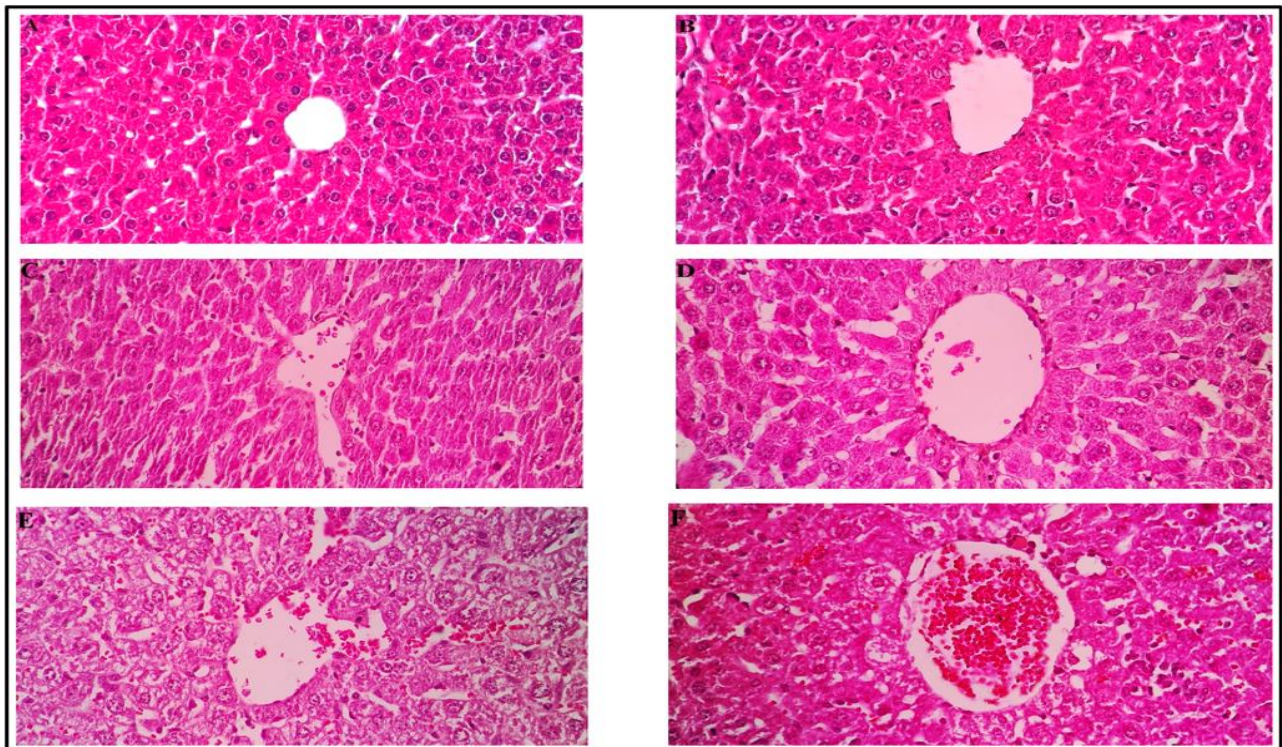
3.2.2.5.2. Kidney

The histopathology of kidney tissue in healthy control mice showed normal morphology of renal parenchyma with distinct glomeruli and tubuli structures (Figure 46B. a). In renal tissue of mice treated with KYNA encapsulated with β NS-CDI

illustrated a slight tubular epithelial cells swelling and interstitial edema and congestion (Figure 46B. b). In group 3, co-administration of KYNA loaded on HP β -CD caused a slight luminal hyaline cast formation along with tubulo-interstitial injury of kidney tissues (Figure 46B. c).

Furthermore, administration of β NS-CDI free to the mice of group 4 caused a slight glomerular and interstitial damages (Figure 46B. d). In group 5 which was treated with HP β -CD, a slightly tubular vacuolization, luminal hyaline cast formation, glomerular changes and tubulo-interstitial injuries was seen (Figure 46B. e). Microscopic observations revealed that in Group 6 treated with KYNA imposed a moderate tubular vacuolization with hydropic degeneration, and also a mild luminal protein cast formation, glomerular hyperemia and interstitial congestion, edema with the hemorrhage (Figure 46B.f). The histopathological changes were graded and summarized in the Table 12B.

A)



B)

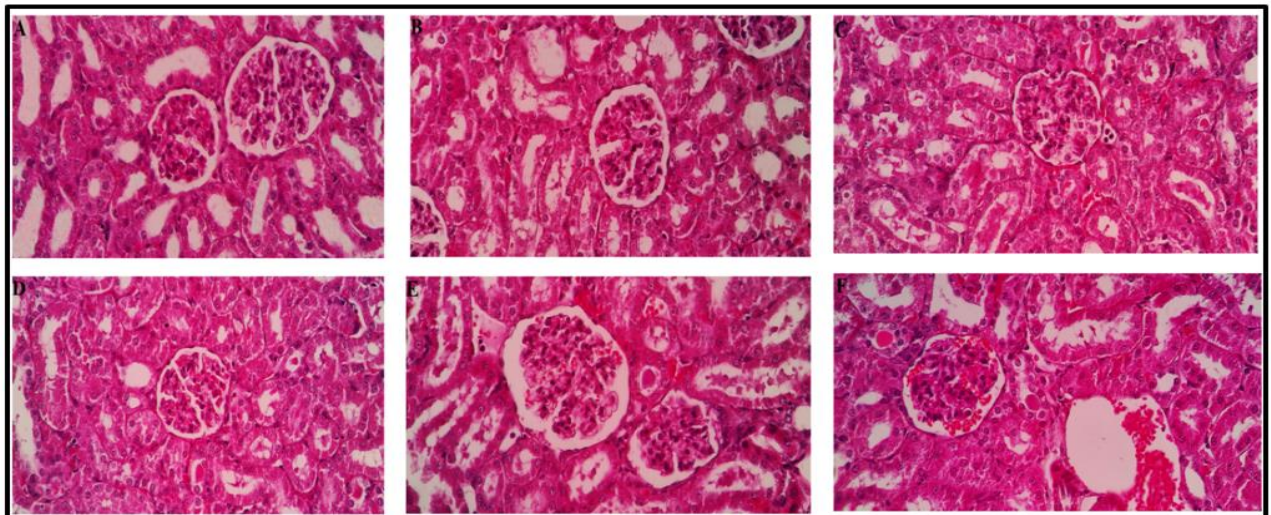


Figure 46: A; Histological analysis. The representative hepatic histopathology. (a) Normal liver from (healthy

control) mice. (b) Mice treated with KYNA+ β NS-CDIs, (c) Mice treated with KYNA+ HP β -CD (d) Mice treated with β NS-CDIs (e) Mice treated with HP β -CD (f) Mice treated with KYNA, B; The representative kidney histopathology. (a) Normal kidney from (healthy control) mice. (b) Mice treated with KYNA+ β NS-CDI, (c) Mice treated with KYNA+ HP β -CD (d) Mice treated with β NS-CDI (e) Mice treated with HP β -CD, (f) Mice treated with KYNA. Hematoxylin and eosin (H&E) staining (400 $\times$).

To having note that the results illustrated that KYNA cytotoxicity in both kidneys and liver organs was considerably decreased when it was complexed with HP β -CD, and specially β NS-CDI, might because of the increase of the antioxidant status of the body and the better bioavailability profile, masking these toxicities. Moreover, these slightly changes showed for carriers can be attributed to different factors like the nature of mice body and other effects on the uptake of nutrients due to the CD or CD-NS complexation[245]. However, the interesting point is the KYNA cytotoxicity was reduced when is complexes with the carriers.

Table 12. Histopathological liver and kidney damages in experimental groups

LIVER

Groups	Treatment	Microscopic observation			
		Vacuolar degeneration	Hyperemia	Disorganized sinusoid	Infiltration of lymphocyte
1	Healthy control	0	0	0	0
2	β NS-CDIs + KYNA	+	+	0	0
3	HP β -CD + KYNA	+	+	+	0
4	β NS-CDI s	+	+	+	0
5	HP β -CD	++	+	+	0
6	KYNA	+++	++	++	+

KIDNEY

Groups	Treatment	Microscopic observation				
		Tubular epithelial swelling	cells	Interstitial congestion, edema and hemorrhage	Luminal hyaline cast formation	Glomerular hyperemia
1	Healthy control	0	0	0	0	0
2	β NS-CDI s + KYNA	+	+	0	0	0
3	HP β -CD + KYNA	+	+	+	0	0
4	β NS-CDI s	0	+	0	+	+
5	HP β -CD	+	+	+	+	+
6	KYNA	++ +	++	++	++	++

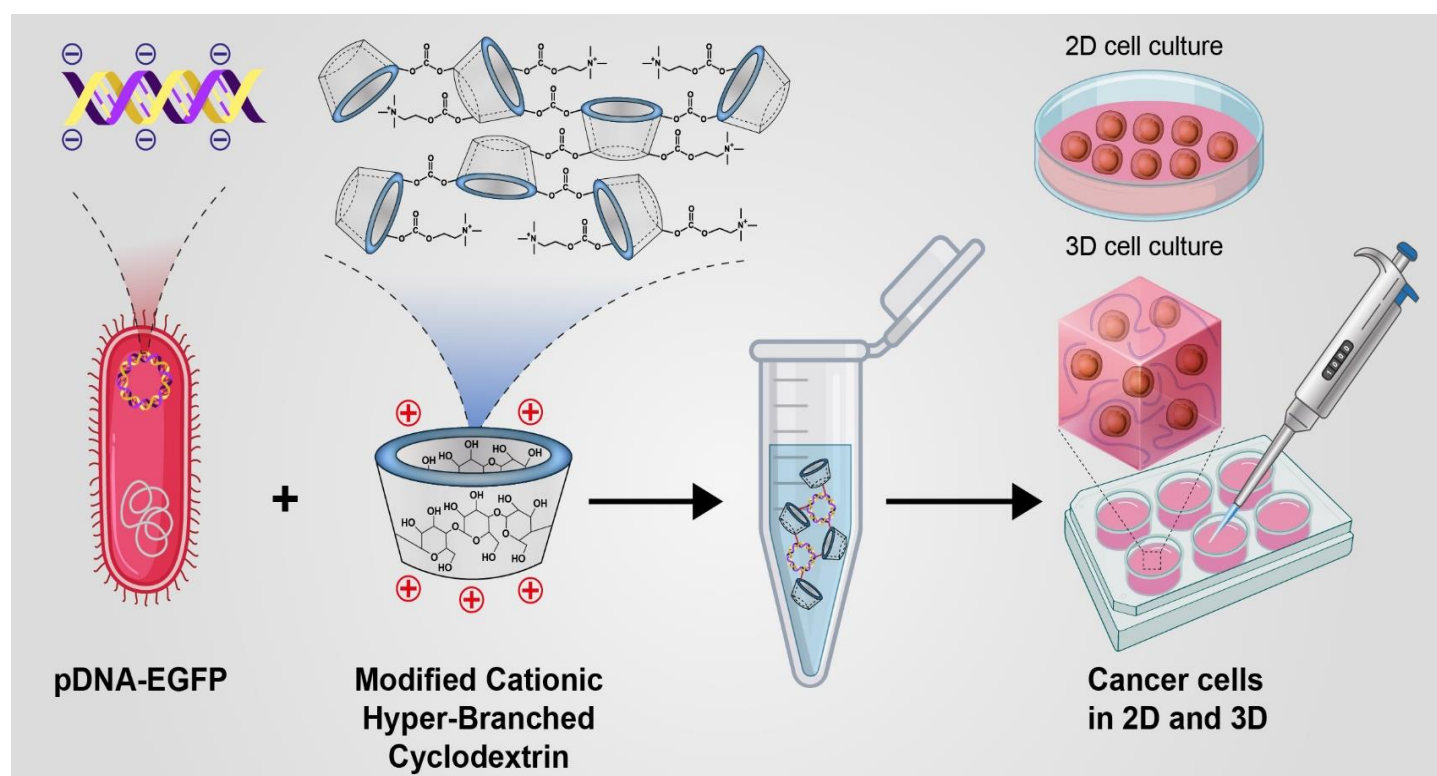
The degree of tissue injuries was evaluated semi-quantitatively according to the method[217]. Histological damages were scored as follows: 0: absent; +: slight; ++: mild; +++: moderate; and ++++: severe.

Finally, is important to mention that these histopathology damages are reversible in these groups, after stopping these agents' administration those mice could be recovered of those damages.

To sum up, a dosage of 0.05mg KYNA/gr/day was orally administered in formulations showing a higher bioavailability when the nutraceutical was complexed with β NS-CDI in comparison to HP β -CD and KYNA free, respectively. Several antioxidant statuses demonstrated a higher global antioxidant status perfectly related to the bioavailability. Finally, the complexed formulation of KYNA was able to reduce the temporal oxidative stress damaged in kidney and liver, being β NS-CDI the best formulation. These results suggest an important future application of cyclodextrin based nanosponges for the oral delivery of nutraceuticals and their stabilization.

Section 3: Cationic Hyperbranched Polymers for pDNA and mRNA Delivery

First Project: Hyper-branched cationic cyclodextrin polymers
for improving the plasmid transfections in 2D and 3D spheroid cells



Graphical Abstract

Aim of study:

Over the past decades, viral vectors have been used as the gold standard vehicle to transfect the genes with the high level of expression. But their safety issue has remained as the main restriction. Therefore, recently, instead of viral vectors, the non-viral carriers such as inorganic nanoparticles (NPs), liposomes, and cationic polymeric NPs have been emerged. While, their low transfection efficiency has been suggested as the main problem of these delivery systems. Therefore, in this study we have aimed to address this problem by using the new modified cationic hyper-branched cyclodextrin-based polymers (Ppoly) to deliver the pDNA. We used the 2D and 3D multicellular spheroid model to increase the understanding of the gene delivery process of new modified cationic hyper-branched cyclodextrin-based polymers loaded plasmid encoding Enhanced Green Fluorescent Protein (EGFP).

3.3.1.1. Introduction

In 1990, Felgner and colleagues demonstrated in science that so-called "naked DNA," or plasmid DNA that was not prepared in transfecting agents, could be directly injected into muscle with the consequent production of the encoded protein by myocytes [246]. The discovery was notable since, up until that point, a great deal of work had been put into developing formulations to transfer DNA in vivo, and many of these substances had been employed for in vitro transfection. The approach's unexpected simplification sparked a great deal of interest, and when it was pretty shortly demonstrated (in 1993) that plasmid DNA coding for a conserved internal influenza protein could produce protection in a pre-clinical mouse model against influenza challenge with a strain that was very different from the strain from which the protein antigen was sequenced[247], many groups started working on plasmid DNA for vaccines, cancer immunotherapies, and immune interventions for various diseases[248-250]. While, the safety issues have been remained as a major concern [251-253] due to their ability to induce significant immunogenicity. Recently, instead of viral vectors, the non-viral carriers such as inorganic nanoparticles (NPs), liposomes, and cationic polymeric NPs [254-258] have attracted the scientific community due to their capacity for large-scale easy production with less immunogenicity and their ability to transfer the large DNA plasmids and RNAs [253, 259, 260]. Meanwhile, their low transfection efficiency has been suggested as the main problem of these systems[261,

262]. Among different nanocarriers, cyclodextrin (CD) has been recognized as a promising and efficient platform for pharmaceutical drug delivery, in this case the oligonucleotide-based therapy [32, 263, 264]. Cyclodextrins (CD) have been used as a plasmid DNA delivery system in 1999 and then developed as a siRNA carriers[265]. However, they could not be directly interacted with nucleic acids due to their weak interactions with these agents [37, 38]. Thus, modifying the CD structure was necessary and to follow this purpose, the CD-modified polycations have illustrated valuable biocompatibility with a good abilities to form a stable polyplexes with therapeutic nucleic acids to protect the therapeutic cargo, even in macromolecular structures [263, 266].

Typically, the conventional 2D cell culture models is being used to understand the efficiency of gene delivery systems prior to investigate in-vivo experiments. In despite, the well-deserved transfection efficiency of genes through non-viral systems in monolayer two dimensional (2D) cells, but they have not be success to provide therapeutic benefits in-vivo due to the complex microenvironment in vivo, such as cell differentiation, proliferation, and gene expression which cannot be fully demonstrated by 2D cell culture [267, 268]. Therefore, there is need to introduce the newly developed methods to fill this large gap between an in vitro 2D cell culture and in vivo animal (or human) models [269, 270].The supposed bridge to fill this gap between conventional 2D culture and animal models is three dimensional (3D) spheroid cell culture that illustrated a better realistic cell-cell interactions, biochemical and cellular parameters than 2D cell cultures for the purpose of

gene delivery systems evaluation before starting the pre-clinical in vivo studies [271-274]. The differences among 3D spheroids and 2D cell culture behavior has been proved, [275-277] as a cases, some drug candidates have shown a good activities in 2D culture, while they were not active in 3D cell culture [268].

We have set the aim of present study to investigate the capacity of our new synthesized cationic hyper-branched cyclodextrin-based polymers to be used as a gene delivery tool, in this case plasmid DNA encoding the Enhanced Green Fluorescent Protein (EGFP) in 2D (HT-29) and multicellular 3D spheroid cell cultures, in comparison with lipofectamine capacities. Bearing the above cited in mind, the objectives were:

- To study the ability of positive charge NPs to retard the pDNA-eGFP at proper N/P ratios
- To achieve enhanced the transfection efficiency and cellular uptake of the pDNA-eGFP in complex with positive charge- CD-NSs and comparison in 2D and 3D cell cultures
- Understand the safety of NPs in both 2D and 3D cell cultures
- Understand the differences among 2D and 3D spheroid cells conditions for gene delivery

3.3.1.2. Results and Discussion

3.3.1.2.1. Synthesis and characterization of hyperbranched cationic cyclodextrin-based polymer

Several routes have been reported to obtain soluble cyclodextrin-based polymers, such as i) grafting of cyclodextrins on previously synthesized polymers, ii) polycondensation of difunctionalized cyclodextrins monomers obtained by selective modification of the cyclodextrins' hydroxyl functions, iii) quenching of cross-linking process, iv) Inhibition of the gelation process in a sol-gel cross-linking reaction [31, 278, 279]. According to Flory's theory gelation occurs when the branching coefficient (α) is higher than a critical value (α_c) defined by the following equation:

$$\alpha_c = \frac{1}{f-1} \quad (4)$$

where f is the number of functional groups in the polyfunctional monomer.

In this regard, the possibility to synthesize cationic hyper-branched cyclodextrin-based polymers, avoiding the production of cross-linked structures, was demonstrated. With the purpose to obtain a β CD-based polyelectrolyte, CHO was used to impart to the final product positive charges. The presence of the positive charge and of CHO itself could increase the capacity of the material to bond DNA[280] . Moreover, CHO has been extensively reported for its non-toxicity, and especially during the last years, it has been widely exploited for the preparation of the so-called NADES (natural deep eutectic solvents) [281-283]. As reported in Figure. 47A, the presence of a hydroxyl

function in its structure, allowed CHO to display a similar reactivity of that displayed by β CD towards the linker, which in the reported case was CDI. CDI allowed to achieve the formation of carbonate bridges by linking i) β CD units together or ii) β CD to CHO molecules. As a result of the above-mentioned reactivity paths, the formation of a cationic hyper-branched polymer structure was hypothesized. The formation of a polymer structure was at first observed by the presence of a product after the purification step, performed using ultrafiltration technique with a 5k Da cut-off membrane. The amount of purified polymer (PPoly) obtained after freeze-drying of the retentate was expressed as mass balance and resulted roughly 85 wt %, with respect to the weight of the polymer before the ultrafiltration process. The molecular weight of PPoly was subsequently studied via size exclusion chromatography. As a result, a molecular weight of 22 kDa was observed. Furthermore, the FTIR-ATR spectra of PPoly (Figure. 47B) clearly displayed a peak centered at 1747 cm^{-1} associated to the presence of carbonyl carbons, belonging to carbonate bridges (1255 cm^{-1}), as hypothesized in Fig. 47. Also, a peak centered at 1479 cm^{-1} which was associated to the presence of quaternary ammonium functions, was a further proof of the mechanism previously described. Subsequently, the presence of quaternary ammonium pendants was studied via elemental analysis since the presence of nitrogen atoms within the polymer structures can be related only to the presence of CHO pendants. As reported (Table 13), the presence of ammonium pendants was

supported by elemental analysis results. Furthermore, by assuming that all the CHO introduced during the synthesis ended up bound within the polymer network and comparing it with the nitrogen content obtained via elemental analysis, it was possible to calculate the actual fraction of CHO present in the final product compared with the theoretical 100% value. The amount of CHO bound to PPoly resulted roughly 80% of the amount introduced during the synthesis. This feature might be related to either non-reacted CHO or CHO bound to oligomer chains characterized by molecular weight lower than 5k Da, thus separated from the purified product during the ultrafiltration step. Also, the thermal stability of PPoly was studied via TGA. As reported in Figure 9, PPoly displayed a Ton set at approximately 150 °C, followed by a two-step degradation taking place firstly between 150 °C and 250 °C, and then from 250 °C to 450 °C, giving a final carbon residue corresponding to approximately the 20 % of the initial weight (Figure. 47C). Furthermore, the molecular weight of PPoly was studied via size exclusion chromatography. With the aim to simulate the presence of amino groups along the polymer structure, proteins with known molecular weight have been used as the references, being characterized by high molecular weight structures, and charged functions. Lysozyme from chicken egg white with molecular weight of 14600 Da, albumin from porcine serum with molecular weight of 65000 Da, and bovine serum albumin with molecular weight 66000 Da, were used for this purpose. Both proteins and PPoly were at first solubilized in a 0.1 M sodium nitrate solution and subsequently

analysed. As a result, PPoly appeared to be composed by different molecular weight fractions, comprised between 14600 Da and 66000 Da. The presence of different molecular weight fractions was further evaluated via ultrafiltration technique. PPoly was solubilized in deionized water, filtered with membranes characterized by cut-off of 10k Da, 30k Da, and 50k Da, freeze-dried to obtain a dry powder, and stored in a desiccator. Doing so, four different molecular weight fractions were collected: (i) molecular weight higher than 50k Da, (ii) molecular weight comprised between 30k Da and 50k Da, (iii) molecular weight comprised between 10k Da and 30k Da, and molecular weight comprised between 5k Da and 10k Da. Considering the amount of polymer recovered after each filtration step and comparing it to the initial amount, PPoly resulted composed by 73.9 ± 4.5 wt. % molecular weight higher than 50k Da, 9.8 ± 3.4 wt. % molecular weight comprised between 30k Da and 50k Da, 8.9 ± 1.9 wt. % molecular weight comprised between 10k Da and 30k Da, and 7.4 ± 3.7 wt. % molecular weight comprised between 5k Da and 10k Da.

Table 13. Elemental analysis of PPoly

Element	%
C	3 ± 0.32
H	26 ± 0.55
N	5 ± 0.16

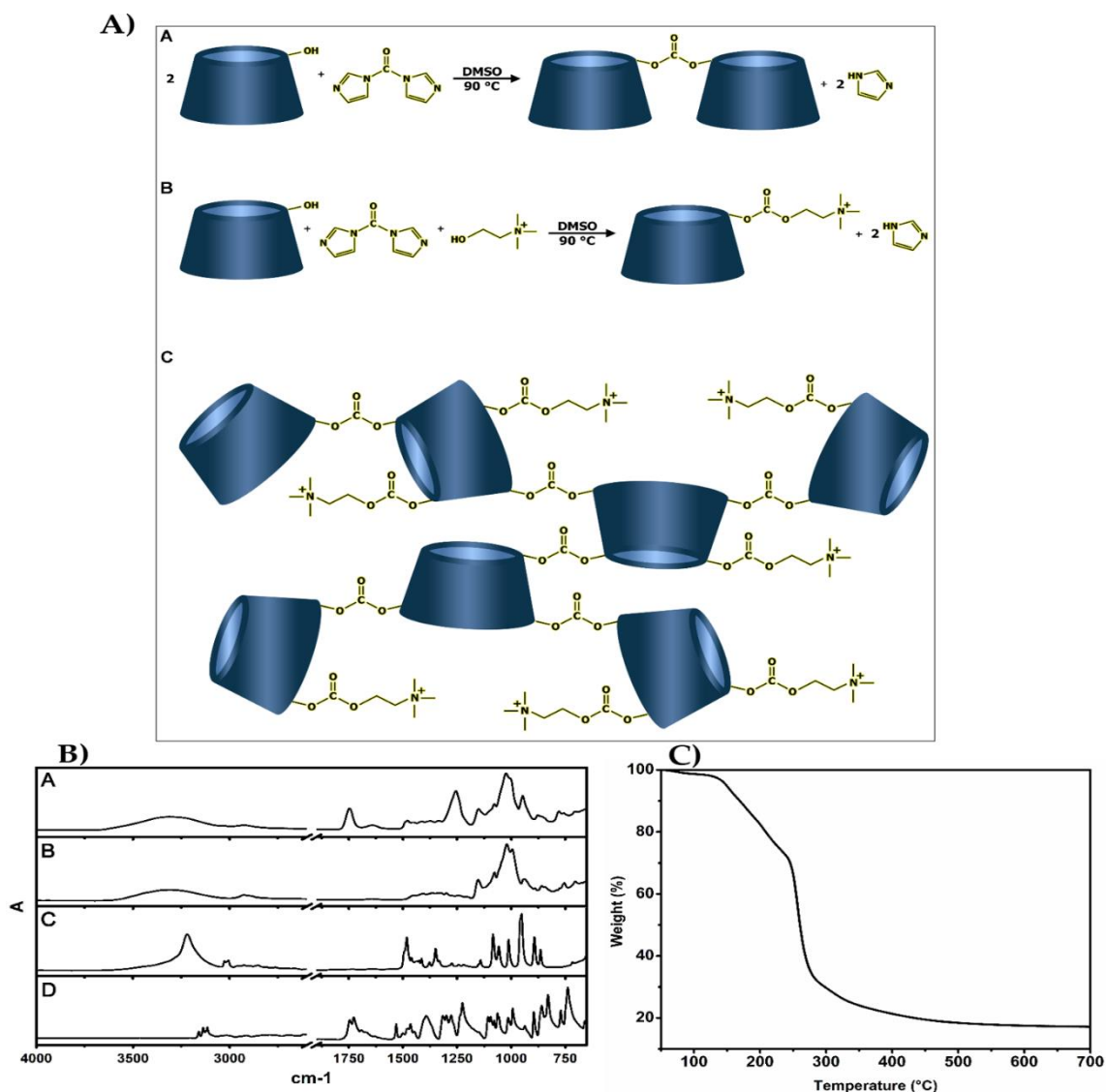


Figure. 47. A; (A) Reaction occurring between β CD and CDI, (B) Reaction occurring between β CD, CDI and CHO, (C) Structure of the resulting hyper-branched polymer. B; FTIR-ATR spectra of (A) Purified polymer, (B) β CD, (C) CHO, (D) CDI. C; TGA of PPoly.

3.3.1.2.2. Preparation and characterization of Ppoly/DNA noncomplexes

DNA condensation capability is a prerequisite property for consideration the vector as an efficient gene delivery agents [284]. Thus, the electrophoretic mobility shift assays (EMSA), particle size and zeta potential were measured, in addition, SEM imaging, were used to confirm the DNA condensing ability of the prepared polymer nanoparticles. The CDNS/DNA noncomplexes were made through electrostatic interactions of negatively charged pDNA with positively charged CD polymer by CHO [280, 285]. As shown in Figure 48A, the plasmid DNA was not completely condensed by CD-NS at lower N/P ratios, while by increasing that, the migration of DNA was completely retarded when the N/P ratio was 25:1, which indicates the complete complexation of all DNA by CD-NS through ionic interactions [286]. The hydrodynamic size and surface charge of non-viral carriers are the most important factors to consider them for drug and gene delivery, therefore the average particle size and z-potential of the CD-NS /DNA complexes were measured by dynamic light scattering (DLS). As can be seen in Fig. 48B, by increasing the N/P ratios, the average particle size and zeta potential of CD-NS/DNA complexes decreased and increased gradually, respectively. At low N / P ratios of 0.5: 1 and 1: 1, the particle sizes were higher at 360.5 ± 21.9 and 330 ± 25.4 , respectively; a possible explanation for this phenomenon may be due to incomplete DNA complexation with CD-NS at these ratios. The results indicated that when the polymer/pDNA weight ratio reached 25:1, the CD-NSs could effectively condense pDNA into a lower size particle (<200 nm) with a positive ζ -potential (approximately +15 mV), notably, there have been reported the polymeric NPs with a

diameter of 100–200 nm have the best properties for preventing phagocytic uptake [287]. Also, the particle size and morphology of Ppoly /DNA complex at that ratio was further characterized by SEM (Figure. 49). SEM image also showed that the particle sizes of Ppoly /DNA complexes were around 37-58 nm with spherical in shape, which were well correlated to the DLS data. In addition, the FTIR spectra for N/P ratio 25:1 confirmed the effective loading of DNA–polymer complexes inside the CD-NP (Figure. 48C): For example, the abundant peak on 3500 nm^{-1} (-O-H) is removed for the complexation. In addition, the PO_3^{2-} peak of DNA (around 1250 nm^{-1}) or base/in-plane vibration (around 1600 nm^{-1}) are shifted [288]. Therefore, according to obtained data the formulation at N/P ratio of 25:1 was selected for cellular transfection analysis.

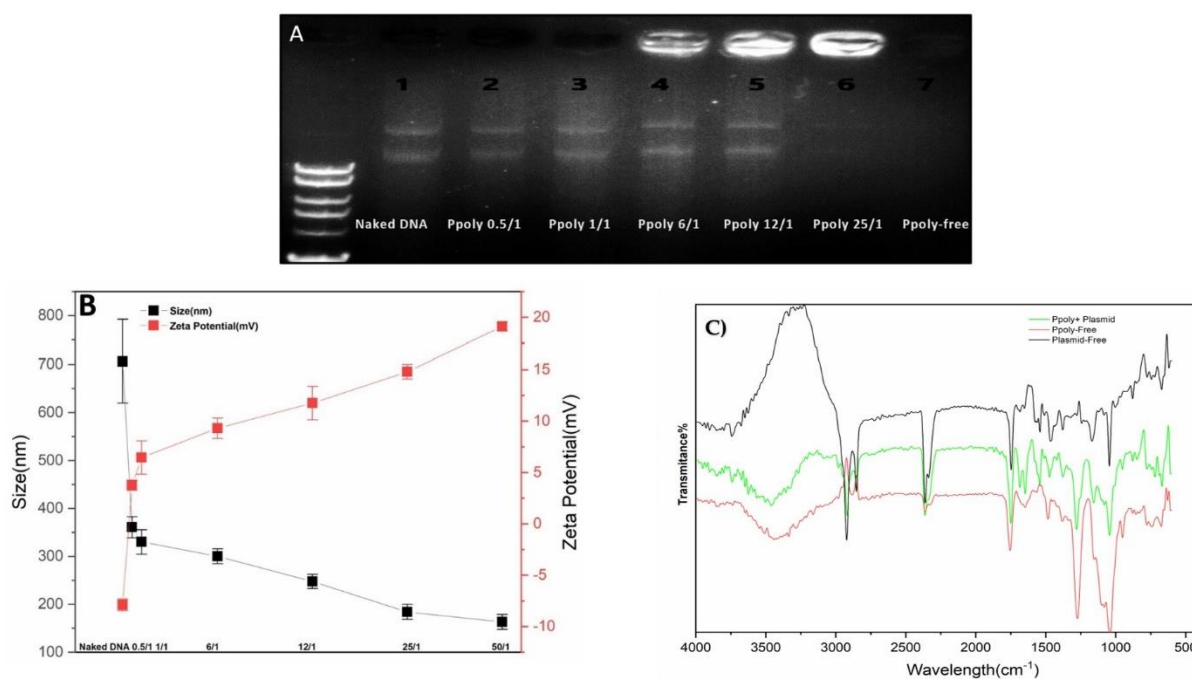


Figure. 48. Characterization of SD/DNA nanocomplexes. A) Agarose gel electrophoresis of Ppoly /DNA nanocomplexes; B) Size of Ppoly /DNA nanocomplexes at different N/P ratios and zeta-potential of DLS; C) The FTIR spectra of CD-NS free, DNA free and Ppoly /DNA nanocomplexes. The results were expressed as means $\pm$ SD.

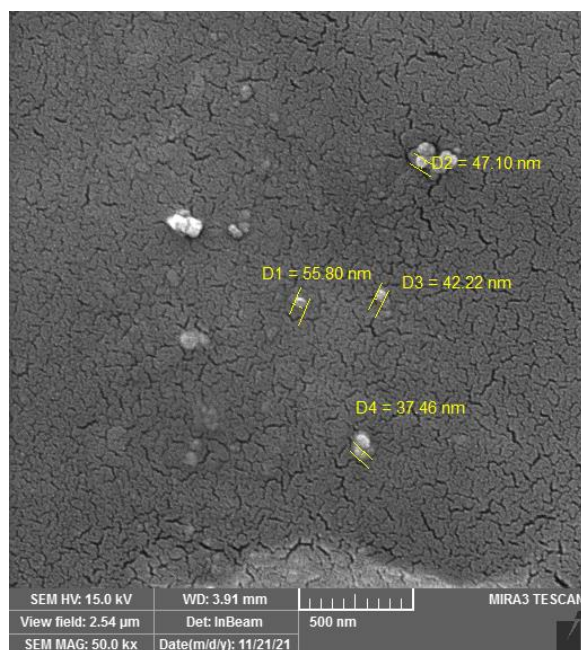


Figure 49. SEM image of Ppoly/pDNA at N/P ratio of 25:1.

3.3.1.2.3. Encapsulation efficacy and hemocompatibility

The encapsulation efficiency of plasmid DNA inside the Ppoly at 25:1 ratio was about %85, which might be the reason for the pDNA complete condensation by provided nanoparticle at that ratio. Another important concluded result was on evaluation the hemocompatibility of NP formulations as a crucial parameter to use of them in an in-vivo experiments [289, 290]. The supporting hypothesis for hemolysis activity of nanocarriers is that , when blood contacts with cationic macromolecules, the initial adsorption by plasma proteins led to adhesion and activation of platelets, leading to that cause to hemolysis, thrombosis and embolization[291]. The formulations free showed the hemolytic activity from about 1.2 to 5.5% at polymer concentrations 125 to 1000 µg/ml (Figure. 50), while this values were reduced to less than 0.8 to 4% when the nano-formulation was complexed with pDNA at ratios 1:1 to 25:1, The expected explanation may be that electrostatic interactions between the polymer and pDNA lead to a decrease in the net positive charge of the polymer to reduce the positive charge interaction of the polymers with the negatively charged red blood cells, leading to a decrease in hemolysis[292]. Thus, the results of presented experiment illustrated that our NPs are hemocompatible with rats' blood.

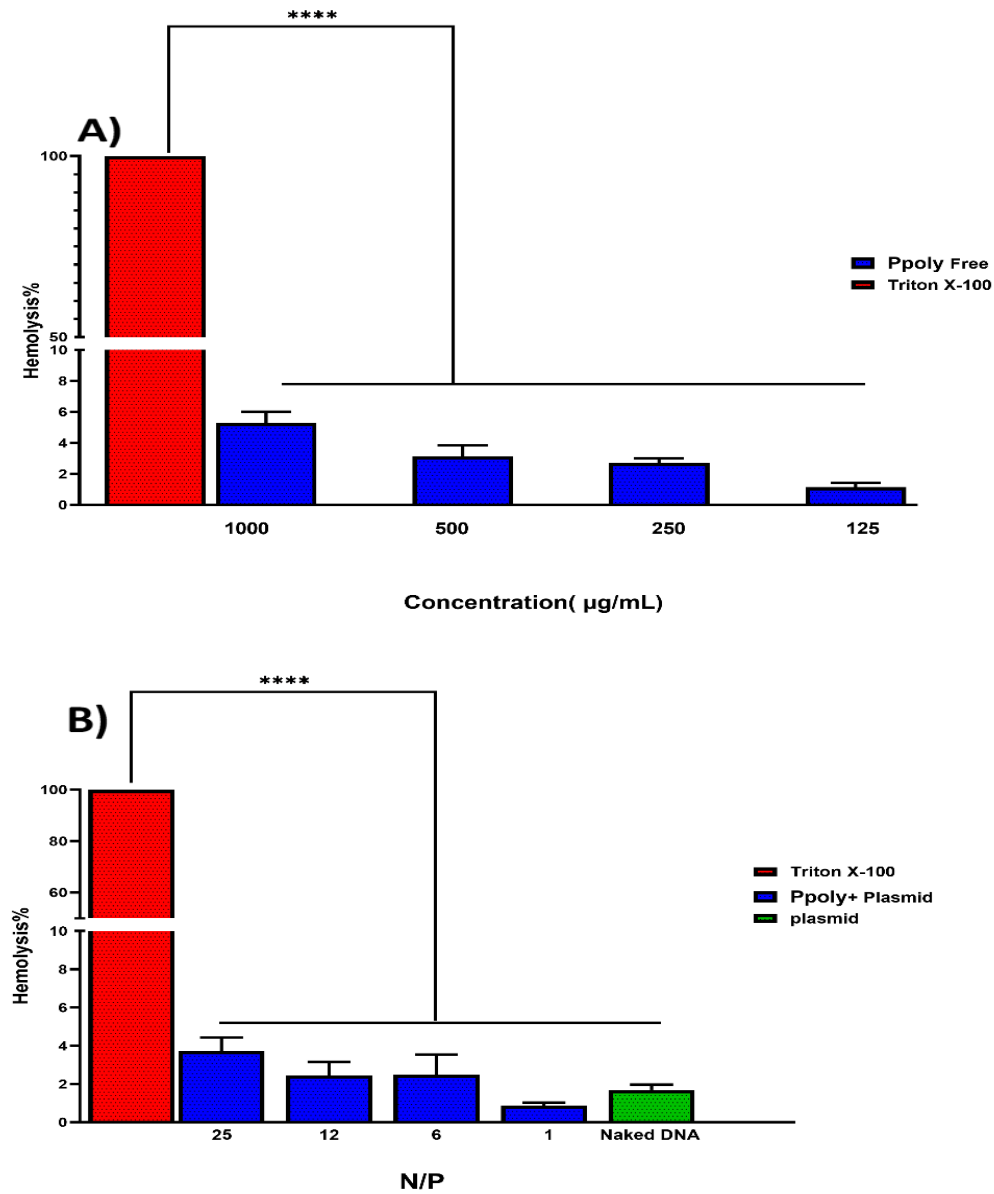


Figure. 50. A) Hemolysis percentages of RBCs at the concentration range of 125–1000 µg/mL of Ppoly free. PBS and Triton X-100 were used as negative and positive control, respectively. B) The Hemolysis percentages of Ppoly/pDNA with different N/P ratios (1, 6, 12, 25) and free pDNA. The data is provided as mean $\pm$ S.D. (n = 3). ****P<0.0001 (n = 3).

3.3.1.2.4. MTT assay in 2D cell culture

Safety is one of the most important concerns for the application of the non-viral gene delivery vehicles, so that the cytotoxicity effect of free- Ppoly and complexes with pDNA on HT-29 cell line was evaluated by MTT assay. The cells were treated with same N/P ratios and concentrations used in hemolysis test. HT-29 cells treated with different concentrations of free c did not show the significant cytotoxicity (62.5, 125, 250 and 500 $\mu\text{g} / \text{mL}$), even lower than $\beta\text{NS-CDI}$ 1:4 in the same cell line[33], while the cytotoxicity at 1000 $\mu\text{g}/\text{mL}$ concentration was observed around 12% (Figure. 51A). On the other hand, the cells treated with polyplexes at low N/P ratios indicated a negligible (around 4%) cell toxicity and around 10 % at 25:1 ratio (Figure.51B). The results indicated that, this nanoparticle at selected ratio (25:1) for biological activity is safe with a negligible cytotoxicity, although they had shown more cytotoxicity about 42 and 35% for free- Ppoly and complexes with pDNA, respectively, at ratio 50:1 and 2000 $\mu\text{g}/\text{mL}$. This elevated cells death rate was higher by increasing the amount of total positive charge of the carrier, as there has been reported the cytotoxicity of Tetraiodothyroacetic acid conjugated polyethylenimine (PEI) was increased by rising the positive charge density on the surface of PEI [293] that led to induce more cytotoxicity, and this issue may merely be explained by more interaction between the negatively charged components on the cell membrane

with the high positive charge of the polymer which leads to the disruption of plasma membrane and consequently cell death [291, 293-297].

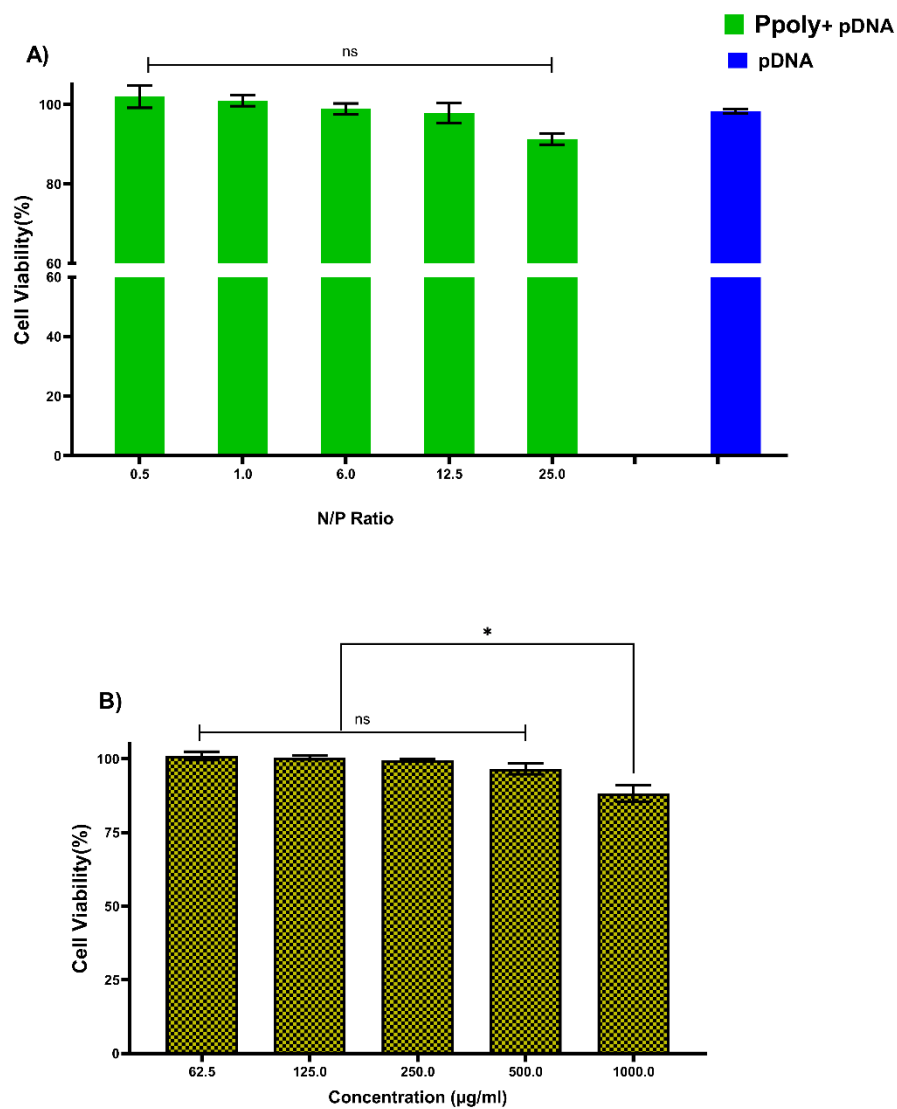


Figure. 51. Measuring the viability of HT-29 cells using MTT assay in a 2D culture system after 24 h. **A)** PPoly in complexes with pDNA at different N/P ratios. **B)** PPoly free at concentrations 62.5 to 2000 µg/mL. One-way ANOVA with Tukey post hoc analysis was used. *** $p < 0.001$ and ns (Not Significant).

3.3.1.2.5. LDH assay in 2D and 3D cell cultures

Various studies indicated that 3D and 2D cell cultures can show different sensitivities to induced toxicity [298-302], because the 3D cell culture and tumoroid formation can show the in-vivo behavior of cells to some extent, but not completely. Therefore, in the next step, the toxic effects of NPs in complex with pDNA at different ratios were evaluated quantitatively utilizing lactate dehydrogenase (LDH) in 2D and 3D cell culture. LDH release is considered as an indicator for understanding plasma membrane cell damage. This extracellular protein assay was evaluated in supernatant of both 2D and spheroid cultures after 24 exposures to the NPs in complex with pDNA at different ratios. The data showed the upward trend for LDH release by increasing the NPs concentration that the amount of LDH released in two-dimensional cells increased from about 10 to 60% in the ratios of 12.5 to 100 N: P of the complexes, respectively (Figure 52A). In addition, the LDH release in 3D cell culture was slightly increased from 1 to 12% in the ratio 12.5 to 100, respectively. These data indicated that the LDH leakage in 3D cell culture was lower about five times at all ratios compared to the 2D cells ($p < 0.0001$), however, this large gap between the 2D and 3D spheroid cells sensitivity to different ratios of complexes to release the LDH content

was expected due to the nature of the different cell phenotypes. For example, the cells in 2D culture are more contacted to the solution, while approximately all portion of cells in spheroid culture are enclosed by the outer layer of cells and they would not make a direct contact with the solution interface, therefore 2D cells are more sensitive to leakage their cytosolic contents into the media[272, 303]. Another study results showed that spheroid cells were considerably less sensitive than 2D when the cells were treated with chemotherapeutic agent Taxotere [304]. Furthermore, the integrity of spheroid HT-29 cells were examined in present defined ratios like LDH assay to understand the complex effect on the cell's density and integrity after 24 h exposure. The results showed that only at the last ratio did the spheres begin to disintegrate slightly and lose their central density compare to control (Figure 52B). One explanation could be that placement of HT-29 tumor cells with complexes in a specific ratio of 100 can lead to a weakening of structural consistency and help reduce cell-to-cell attachment and intercellular attachment, thereby increasing the LDH leakage of three-dimensional spheres at this ratio than others [305].

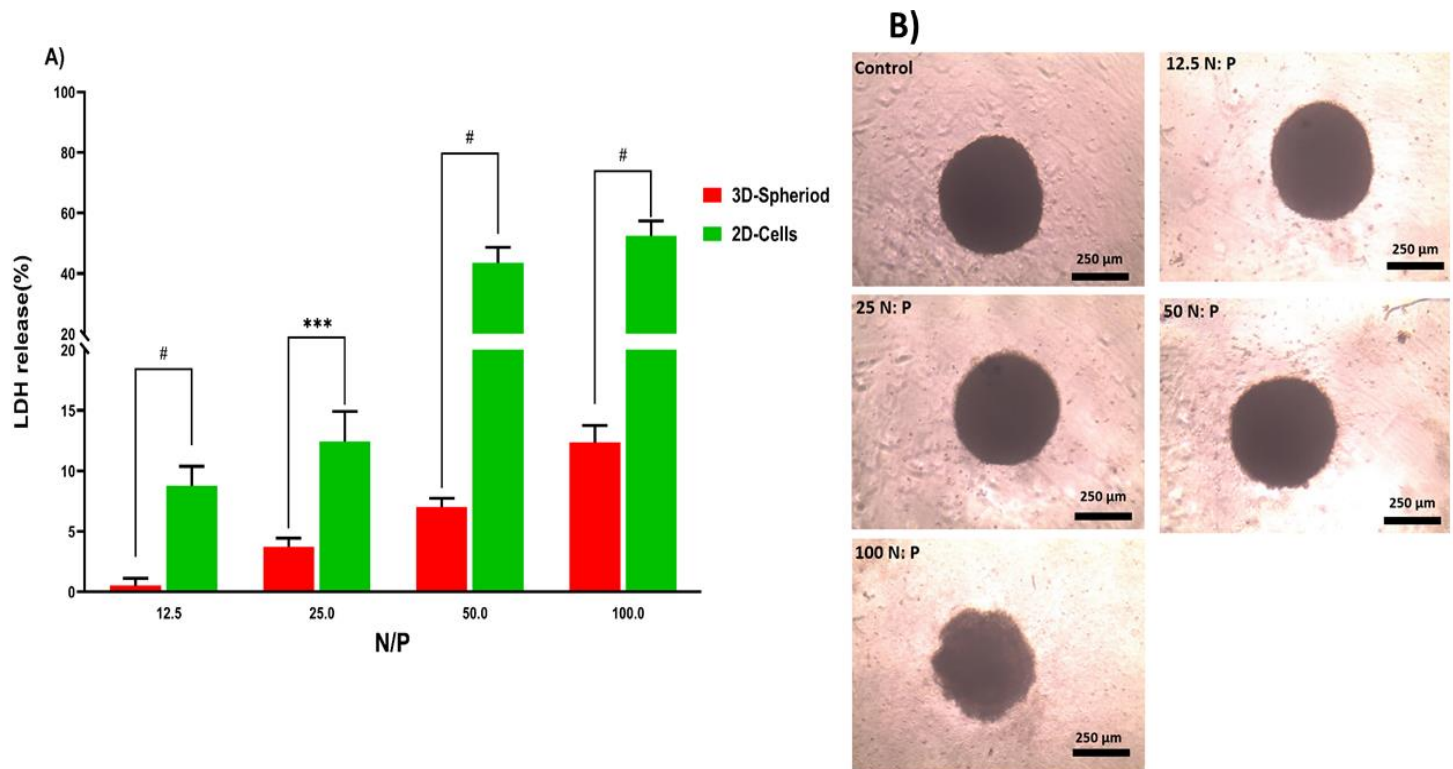


Figure. 52. A). Evaluation of LDH release from HT-29 2D and 3D spheroid cells after 24 h exposure to PPoly-pDNA complexes at 12.5,25,50 and 100 N: P ratios, **B).** The incubation of spheroids with different ratios PPoly-pDNA complexes of contributed to the loss of spheroid integrity and structure. One-way ANOVA with Tukey post hoc analysis was used. *** $p < 0.001$ and # $p < 0.0001$.

3.3.1.2.6. Cellular uptake and transfection analysis in 2D culture

Quantitative and qualitative analyzes were performed to analyze intracellular transfection and localization in vitro 2D monolayer cells. Two-dimensional cells were incubated with complexes containing 1 µg / ml pDNA-eGFP at N / P ratio of 25:1 for 2, 4, 6 and 24 hours. As shown in Figure 53A, B, strong green fluorescence related to EGFP protein expression

was detected at all times in cells transfected with CD-NSs-NP formulation with pDNA compared to free pDNA and lipofectamibe as a postive control (Figure 15C), that showed the moderate EGFP protein expression, by microscopic fluorescence. Of noting that, this strong green fluorescence inside the cells is related to the ability of cells to uptake our NPs. The quantitative uptake of complexes contains 1 μg / ml pDNA-eGFP at a N / P ratio of 25:1 was assessed by flow cytometer (FACS) analysis (Figure. 53, 55) after incubation at determine times like microscopic fluorescence and the efficacy of uptake was obtained. While, free pDNA showed low cell uptake of less than 3% for 2, 4 and 6 hours and about 12% after 24 hours in HT-29, CD-NS obtained about 40, 50, 55 and 65% at 2, 4, 6 and 24 hours, respectively. In addition, lipofectamine illustrated about 70% efficacy of uptake after 24 h incubation, but this transaction percentage was less than CDNSs at the same time (Figure 53, 56). These data showed that CD-NPs loaded with DNA plasmid revealed transfection efficiency, which increased over time by prolonging the plasmid release of nanoparticle compared to free pDNA and lipofectamin (Figure. 53D).

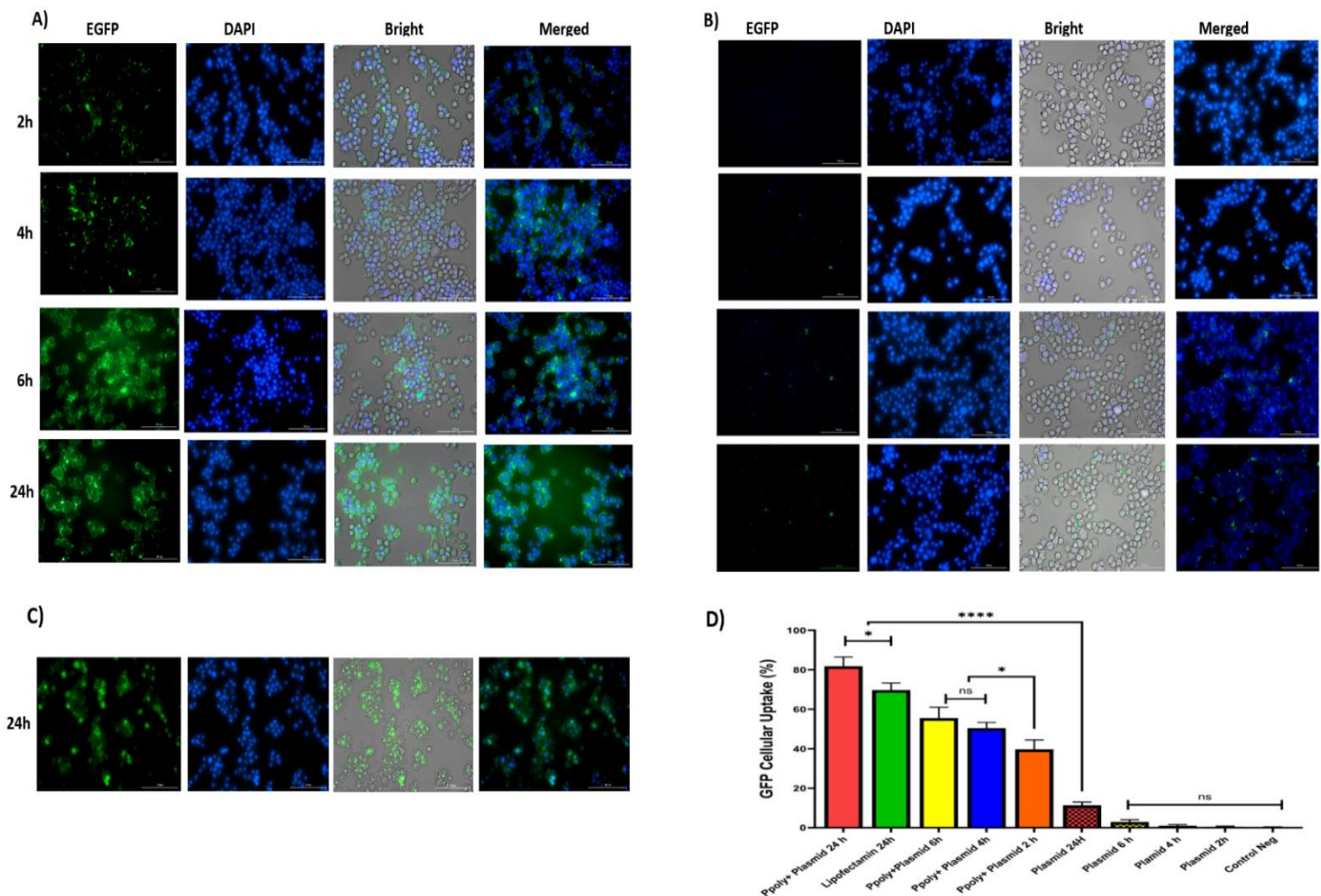
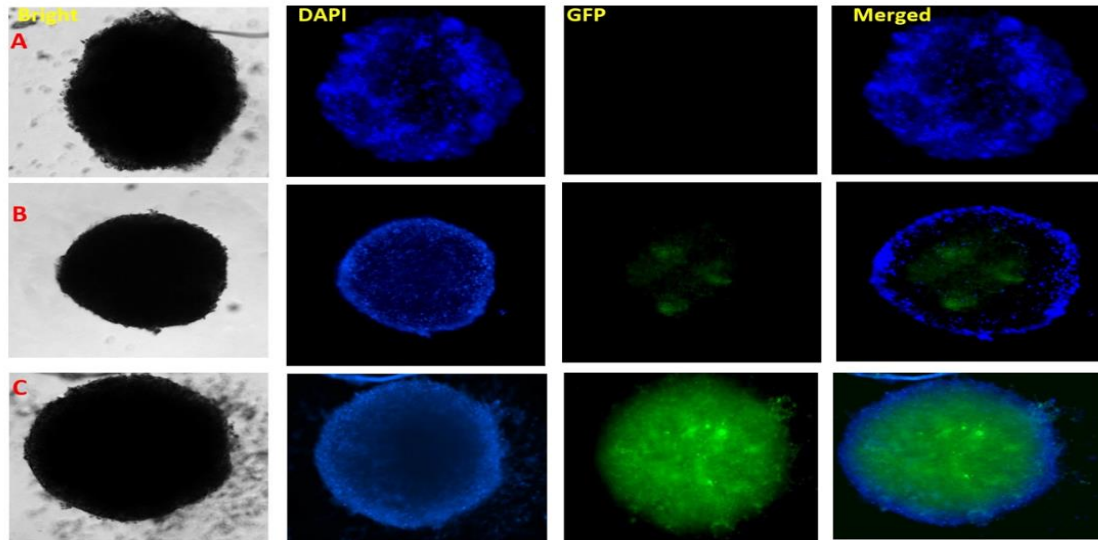


Figure 53. A; Microscopic fluorescent images of 2D cells after 2, 4, 6 and 24 h incubation with PPoly/pEGFP nanocomplexes at N/P ratio 25:1. **B;** Microscopic fluorescent images of 2D cells transfected with free pDNA after 2, 4, 6 and 24 h. **C;** Microscopic fluorescent images of 2D cells transfected with Lipofectamin/ pEGFP at ration 3:1 after 24h. **D;** Quantitative transfection efficacy (%) determined by the EGFP content as assessed by FACS analysis of treated 2D cells with PPoly/pEGFP nanocomplexes at N/P ratio 25:1 compared to free pDNA after 2, 4, 6 and 24 h, and lipofectamine after 24 h incubation. Representative measurements of three independent experiments have been reported; # indicates P-values of <0.0001.

3.3.1.2.7. Cellular uptake and transfection analysis in 3D spheroid culture

In the next step the capability of our NPs to penetrate into a 3D spheroid structure was evaluated. Spheroids were made by mixing the HT-29, HUVEC and Fibroblast cells as a model for the study of uptake and transfection of our pDNA loaded CD-NPs in comparison with free pDNA and lipofectamin. The tumorous spheroid cells were exposed to the complexes containing 1 µg /ml pDNA-eGFP at N / P ratio of 25:1 for 24 hours as well as the free pDNA with same concentration, and lipofectamine was used at ratio 3:1 (DreamFect/pEGFP) at the similar time. Qualitative observations using fluorescent microscopy revealed that our NPs showed a much better capacity to penetrate the spheroid structure, and to induce transfection in comparison with free pDNA and lipofectamine after 24 hours incubation (Figure. 54A).

A)



B)

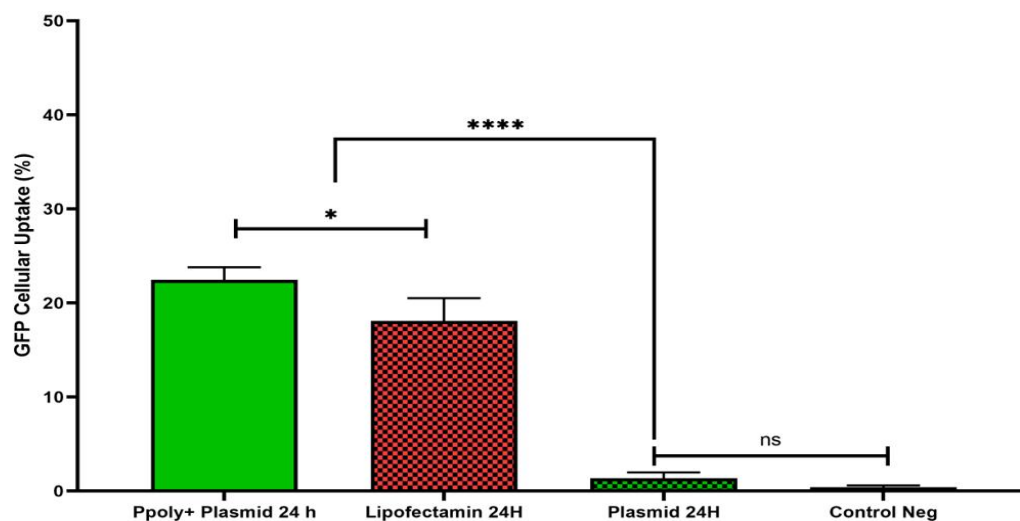


Figure. 54. A; Microscopic fluorescent images of 3D spheroids PPoly/pEGFP nanocomplexes transfection at N/P ratio 25:1(c) in compared to free pDNA(a) and lipofectamine(b) after 24 h incubation. **B;** Quantitative transfection efficacy (%) determined by the EGFP content as assessed by FACS analysis of treated 3D cells with PPoly/pEGFP nanocomplexes at N/P ratio 25:1 in

compared to free pDNA and lipofectamine after 24 h incubation. Representative measurements of three independent experiments have been reported; indicates P-values of * <0.05 , *** <0.001 and ns (Not Significant).

The quantitative uptake of complexes contains 1 μg / ml pDNA-eGFP at a N / P ratio of 25:1 was determined by flow cytometer (FACS) analysis (Figure. 54B, 56) after incubation at determine times like microscopic fluorescence and the efficacy of uptake was found to be about 23% and 17% at 24 hours for pDNA loaded on CD-NPs and lipofectamine, respectively. Although, free pDNA did not show cell uptake after 24 hours incubation with the spheroid cells. These data revealed that the EGFP expression in 3D spheroid cells transfected by CD-NS-pDNA complexes at determined time was lower than 2D cells, due to the extracellular matrix (ECM) and high cell density multicellular barriers in the deep regions of the 3D spheroids to inhibit the gene delivery agents' penetration inside the spheroid cells like other studies results on gene delivery in 3D spheroid cells showed a similar low transfection efficiency [306-309]. These cells are commonly known to exhibit extensive cell contact, with simultaneous increases in interstitial pressure and significant resistance to chemotherapy and radiotherapy [310]. This may also be true for the non-viral gene delivery systems. It is a known problem that transfection efficiency and cytotoxicity of non-viral gene delivery are closely related, suggesting that higher transfection efficiencies may be related to higher cytotoxicity. In our study, we observed this phenomenon and showed that it is true for transfection in both two-dimensional and three-dimensional spheroids. Our NPs demonstrated well transfection efficiency when examined

in conventional 2D monolayer cells (HT-29), while they showed low transfection efficiency in 3D spheroids. The possible explanation would be that the only parts of the peripheral cells (outer most cell layers) in 3D spheroids were transfected by Ppoly/pDNA complexes (N/P ratio 25:1). These results are consistent with the results of other studies on the in vitro 3D spheroid model for gene delivery [309, 311] and it also justifies clinical results that have shown low non-viral gene delivery efficiency in solid tumors or three-dimensional architectural tissues in vivo[311].

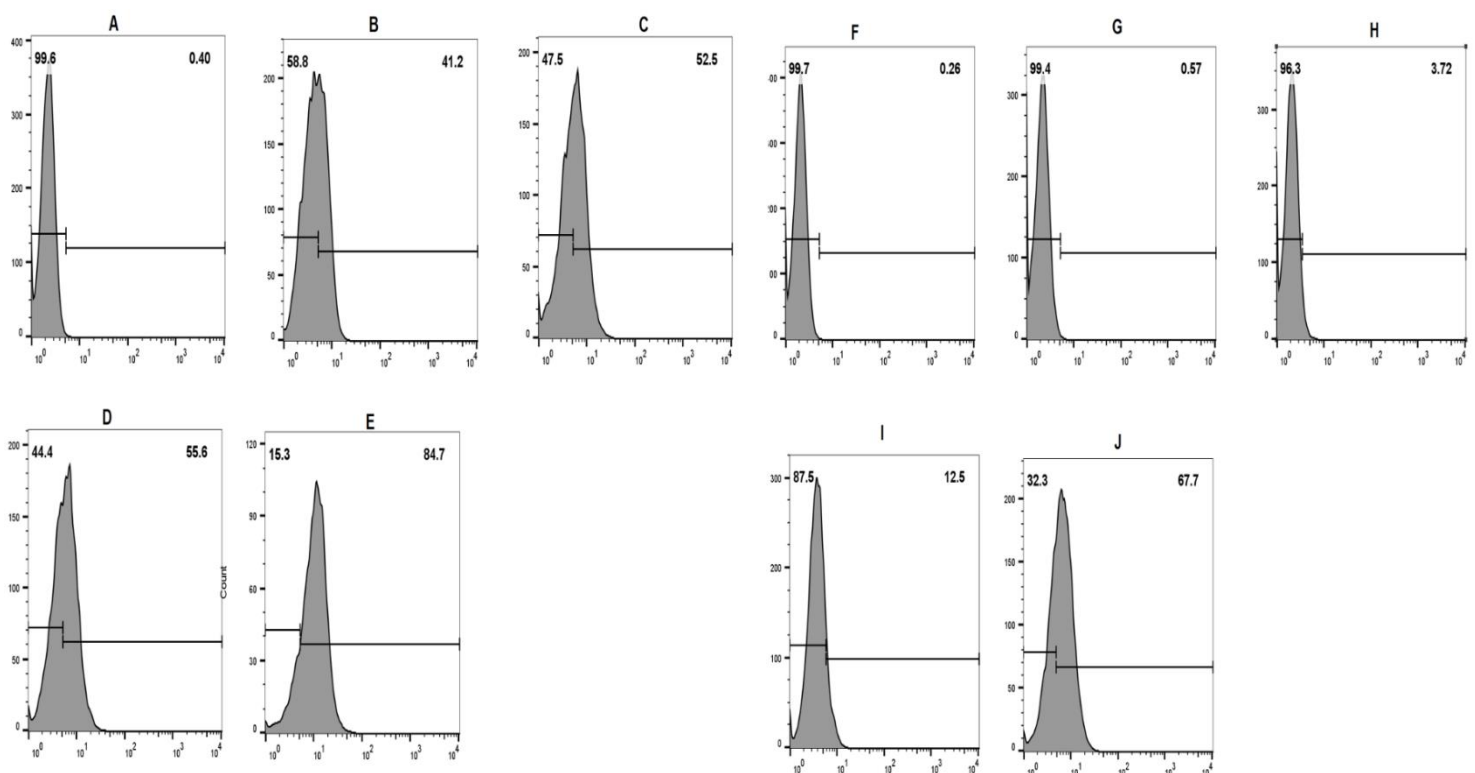


Figure 55. Quantitative transfection efficacy determined by the EGFP content as assessed by FACS analysis of treated 2D cells with Ppoly /pEGFP nanocomplexes 2(B), 4(C), 6(D) and 24(E) hours at N/P ratio 25:1 in compared to free pDNA after 2(F), 4(G), 6(H), 24(I), and Lipofectaim/ pEGFP 24(J) hours incubation and control (A).

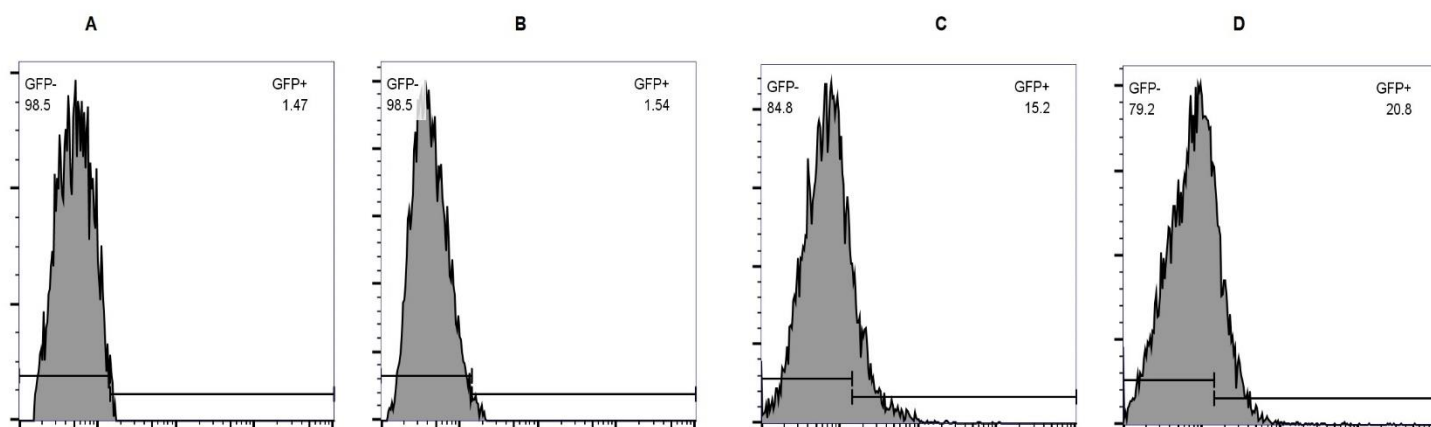


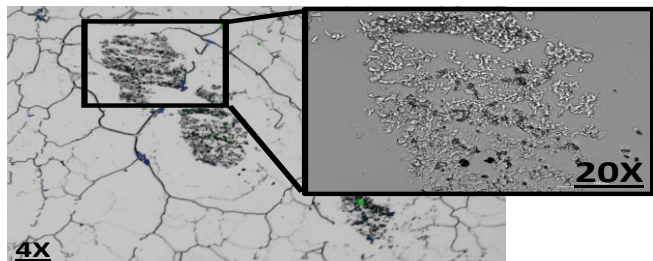
Figure 56. Quantitative transfection efficacy determined by the EGFP content as assessed by FACS analysis of treated 3D cells after 24h. Control(A), pDNA after(B), Lipofectaim/ pEGFP 24 (C), and Ppoly/pEGFP nanocomplexes at N/P ratio 25:1 (D).

3.3.1.2.8. Evaluation the penetration capacity of Ppoly/pDNA complexes inside the 3D spheroid cells on paraffin-embedded cell block

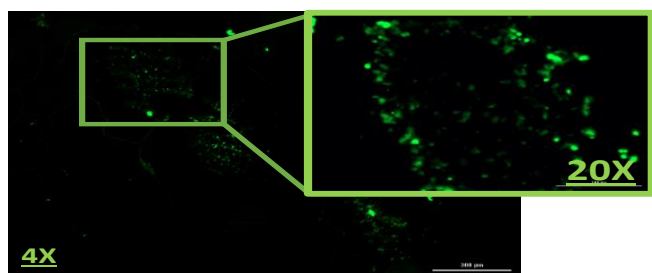
Furthermore, the histopathology method was used to evaluate the pDNA-polymer penetration in 3D spheroid cells by incubating the cells with 2 μ g pDNA-eGFP in complexes with CD-NS at N/P ratio of 25:1 for 48 h. As shown in Figure. 57, the EGFP fluorescent was seen which is indicated the capacity of our NPs to be penetrated into inner layers of the spheroid cells, while DAPI dyes were remained in outliers' cells due to its disability to be penetrate inside the spheroid cells, this result supported our previous experiments and

demonstrated the ability of our NPs to carry out the pDNA inside the cells in 2D and 3D cultures.

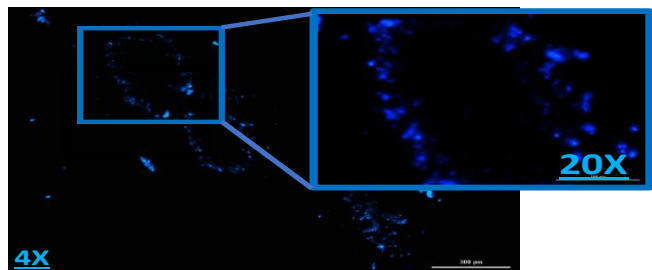
Bright Field



EGFP



DAPI



Merged

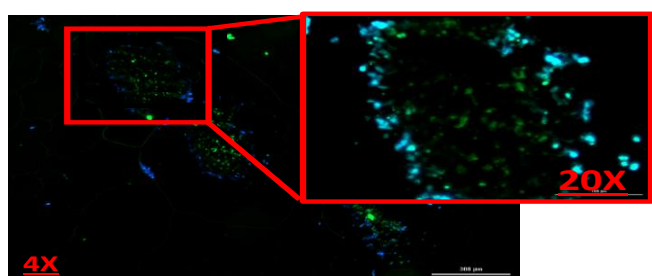
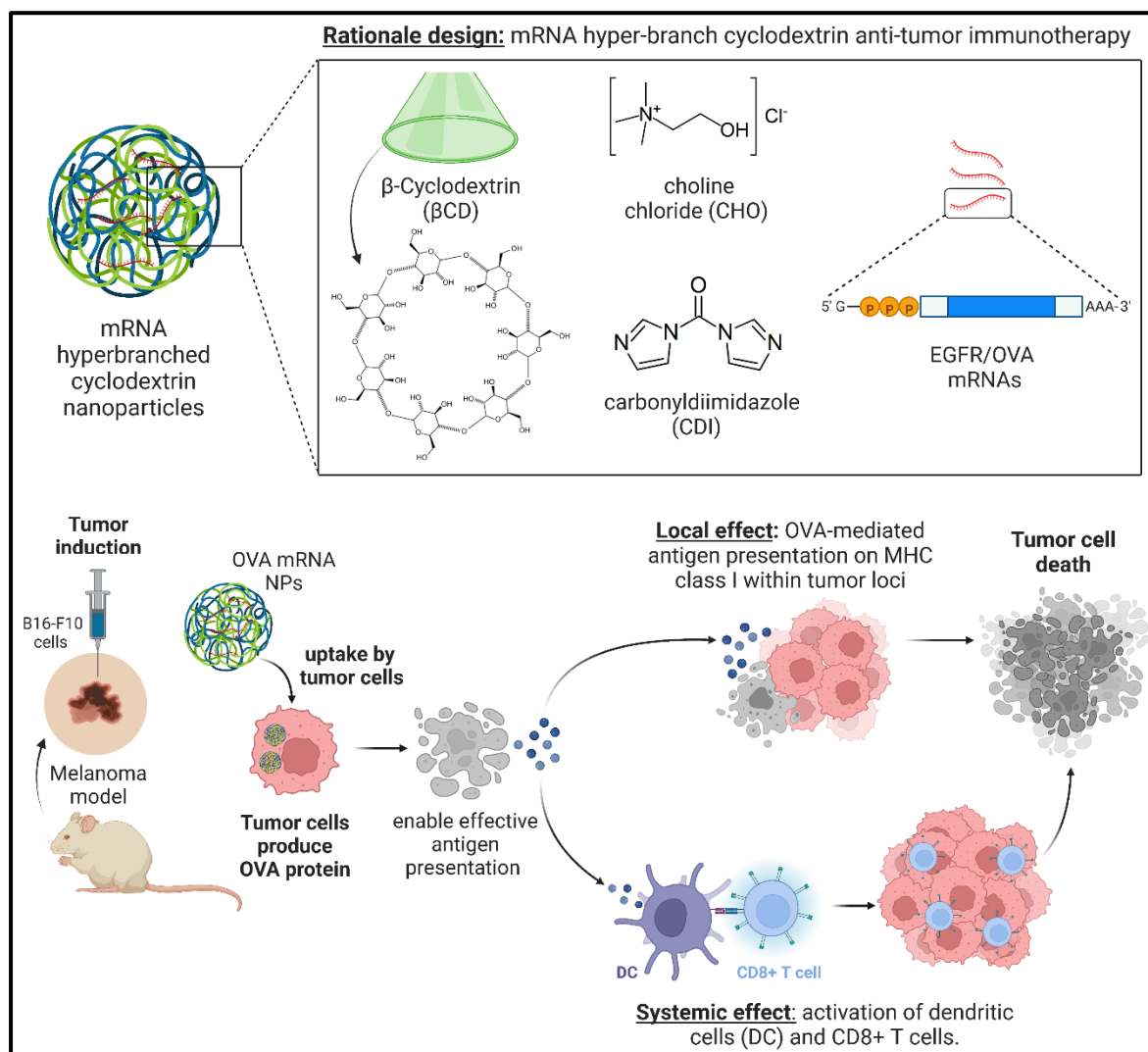


Figure. 57. Evaluation the 3D spheroid cells on paraffin-embedded cell block to confirm the spatial expression of EGFP delivered by Ppoly inside the spheroids. Scale bars: 4X and 20X. DNA refers to the pmaxGFP plasmid.

To sum up, we have synthesized the new cationic hyper-branched cyclodextrin-based polymers, encapsulating plasmid DNA encoding EGFP proteins as a model nucleic acid to demonstrate the uptake and transfection efficiency of nanoparticles and their application by two-dimensional and three-dimensional cell cultures. PPoly loaded pDNA were effectively taken up more than 80% and 20% by cells in 2D and 3D cell culture models, respectively, meanwhile, pDNA cellular uptake at both 2D and 3D cells was better in complexes with PPoly compared to lipofectamine after 24h. The results of cytotoxicity (MTT and LDH) assays illustrated that our NPs were non-cytotoxic at define N/P ratio (25:1), in specific in 3D spheroid culture, furthermore the hemolysis results indicated the complexes of Ppoly/pDNA were hemocompatible when they were exposed to the red blood cells of rat with less than 4% lysis rate. In addition, the results of the histopathology confirmed the ability of our PPoly to penetrate into and allow the transfection of a 3D spheroid. Taken together, thanks to our NPs properties; biocompatibility, hemocompatibility and considerable cellular uptake to transfect the pDNA inside the cells, might be considered as an efficient and promising platform for gene delivery in 2D and 3D mediums.

Second Project: Intratumorally delivery of mRNA loaded on positive charge Hyper-branch Cyclodextrin polymer induced therapeutic anti-tumor immunity against melanoma animal model



Aim of study:

mRNA technology has shown an exciting capacity to be used as effective cancer immunotherapy. However, inefficient in vivo mRNA delivery along with a requirement for immune co-stimulation present major hurdles to achieving anti-tumor therapeutic efficacy. Therefore, in this study, we used the modified cationic hyper-branched cyclodextrin-based polymers (Ppoly) to increase the mRNA delivery in both in-vitro and in-vivo melanoma cancer by increasing the immune system responds.

3.3.2.1. Introduction

The frequency of metastatic melanoma, a highly deadly malignancy, is rapidly rising worldwide. The condition spreads and is potentially fatal if it isn't detected early and surgically removed. Irradiation and chemotherapy are both unsuccessful[312]. Thus, new treatments that focus on oncogenic drivers have resulted in some advancements. Recently, messenger RNA (mRNA) injections have emerged as one such treatment that provides low mutational risk, good safety, and adaptive modularity to express a variety of diverse therapeutically useful proteins[313-316]. Research in molecular medicine now focuses on mRNA. This is especially true when it comes to immunization. Many mRNA vaccines, including those for cancer immunotherapy and those against viral infections, have entered clinical trials[317, 318]. The success of mRNA-based therapy has been demonstrated in numerous applications, including gene editing, protein replacement therapy, cancer immunotherapy, and immunization against the SARS-CoV-2 virus[314, 317, 318].

Given that mRNA is vulnerable to rapid destruction by common RNases, it has long been thought to be poorly stable for pharmacological uses. In the past 30 years, significant efforts have been undertaken to improve the stability, translational efficiency, and absorption of mRNA within cells. These improvements were made by modifying the coding region [319], as well as the non-coding elements (5' cap

structure and capping efficiency[320, 321], 5' and 3' untranslated regions (UTRs)[322-324], and 3' poly(A) tail[325] . They were also made by continuing to develop transfection and formulation technologies (Figure 58). Based on these developments, synthetic mRNA has become a flexible genetic information delivery technology that encourages the cell creation of peptides and proteins.

Synthetic mRNA is produced by in vitro transcription (IVT) from a linear DNA template and is single-stranded (ss), has a 5' cap, UTRs embracing the coding area, and a 3' poly(A) tail, mimicking naturally occurring processed mature mRNA molecules. Exogenous mRNA either enters the cell directly through the cytoplasmic membrane (such as when electroporated) or through endocytosis and endosomal escape (if delivered as naked or formulated mRNAs). mRNA does not incorporate further into the genome or get to the nucleus. Translation takes place in the cytosol, and it is impossible to tell the difference between endogenous and synthetic mRNA-derived proteins. The protein is subjected to post-translational changes and is transported via targeting sequences or transmembrane domains to subcellular compartments such the secretory pathway, the cell membrane, the nucleus, mitochondria, or peroxisomes. Peptides are subsequently displayed on major histocompatibility (MHC) complexes when the protein is destroyed. Exogenous mRNA imposes its activity as a natural ligand of endosomal toll-like receptors (TLRs) 3, 7, and 8 or of retinoic acid-inducible

gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) in the cytoplasm, which leads to the release of type I interferon (IFN) and pro-inflammatory cytokines (reviewed in Pastor et al[326]. The most sophisticated use of mRNA in cancer immunotherapy is therapeutic vaccination, which takes advantage of the both mRNA's ability to transmit genetic information and its built-in immunostimulatory activity. When cancer-related self-antigens are attacked, the latter is crucial for overcoming immune tolerance. Notwithstanding, IFN-stimulated genes (ISGs), including 2',5'-oligoadenylate synthetase (OAS) with subsequent RNase L expression and IFN-inducible double-stranded (ds)RNA-activated protein kinase (PKR), start an anti-viral protection that is described by stalled mRNA translation and enhanced targeting of mRNA for breakdown[327, 328]. Programming T cells and natural killer (NK) cells containing antigen receptors is another way that mRNA is used in cancer immunotherapy. It is also used as a template for immunologically active proteins in a variety of immune and non-immune cells high area under the curve (AUC) of mRNA translation is necessary for many of these applications, and translation that is halted is undesirable. Pseudouridine, N1-methylpseudouridine, 2-thiouridine, 5-methylcytidine, or N6-methyladenosine are examples of modified nucleosides that can reduce the immunostimulatory activity of mRNA [324, 329], as does the removal of dsRNA (Figure 59). Rapid-response vaccine development has been accelerated by the COVID-19 pandemic threat, and mRNA-based therapeutics have proven to live up to

their promise by being effective at relatively low dose ranges, developing quickly, and easily scaling up GMP-compliant manufacturing processes for quick availability of large numbers of doses[330, 331]. It is envisaged that the development of mRNA-based cancer immunotherapies will advance thanks to the lessons discovered during the creation of the COVID-19 vaccine.

While mRNA therapies have demonstrated significant promise, the stability of naked form remains a barrier since naked mRNAs are vulnerable to common ribonucleases, they are unable to penetrate plasma membranes effectively, and they are unable to escape from endosomes[332]. A solution to this issue is to encapsulate mRNA in nanoparticles since it not only protects mRNA from being broken down by nucleases but also makes it simpler for cells to take the mRNA up and transport it to other areas of the body. Indeed, a lot of research teams have recently expressed interest in mRNA nanoparticle delivery [333-335]. Furthermore, recent developments in cancer immunotherapy have been remarkable thanks to mRNA nanoparticles with therapeutic efficiency for tumor vaccination [336-338]. As a result, one of the most pressing challenges is the requirement for effective delivery, which mandates the imperative need for sophisticated and target delivery methods [333, 339, 340].

On the other hand, polymers have drawn interest as a platform for gene delivery because of their desired qualities, such as non-integration, scalable manufacturing,

and extensive chemical structural flexibility[263, 341, 342]. Cyclodextrin (CD) has been recognized as an effective and efficient oligonucleotide delivery technique among polymer delivery technologies[32, 263, 342, 343]. They were used as siRNA carriers after being employed for the first time as a plasmid DNA delivery device in 1999 [265]. The CD-modified polycations have shown beneficial biocompatibility and a good capacity to create stable polyplexes with therapeutic nucleic acids to keep them stable[266]. Due to their relatively high transfection effectiveness and adaptability of modification, CDNSs and its derivatives have also been identified as being among the documented mRNA vaccine delivery systems that are frequently used[257-259].

There has been shown that local immunotherapy ideally eliminates the toxicities connected with systemic treatment while promoting immune responses against cancers. However, negative consequences like off-targeting or anti-vector immunity limit current methods for tumor-targeted, gene-based delivery[344-346]. Therefore, the goal of the current investigation was to determine whether Ppoly, which have been shown to be an highly efficient carrier for pDNA transport in both 2D and 3D spheroid cells, may overcome the challenges of mRNA delivery against melanoma cancer in vivo. Ppoly encapsulating oval albumin (OVA)-mRNA, a foreign antigen, was constructed and administrated intratumorally.

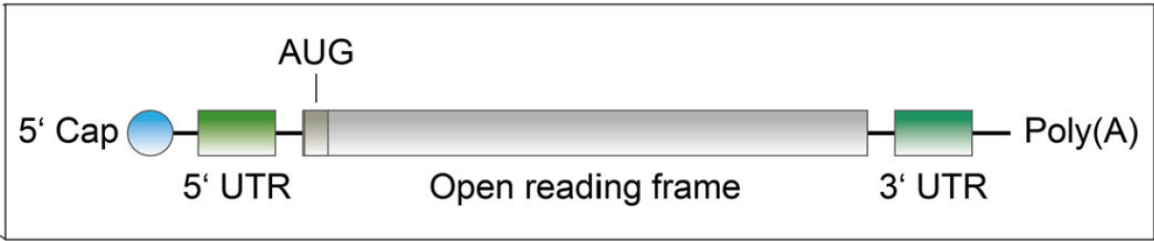


Figure 58. mRNA structure and characters[314]

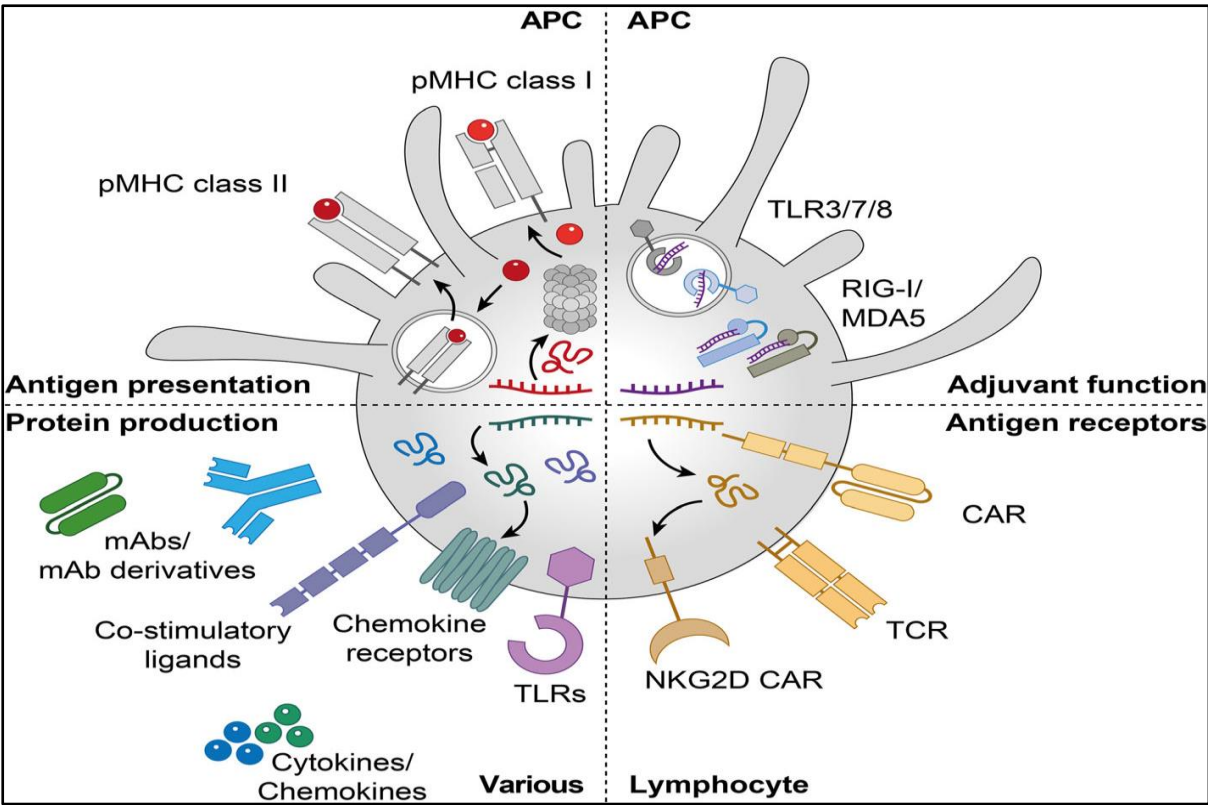


Figure 59. mRNA therapeutics in cancer immunotherapy. mRNA is used for anti-cancer vaccination, where it

delivers cancer antigens to APCs for the presentation on MHC class I and II (top left) and stimulates innate immune activation by binding to PRRs expressed by APCs (top right), introduces antigen receptors such as CARs and TCRs into lymphocytes (bottom right), and allows the expression of immunomodulatory proteins including TLRs, chemokine receptors, co-stimulatory ligands, cytokines, chemokines and different mAb formats in various cell subsets (bottom left). APC: antigen-presenting cell; CAR: chimeric antigen receptor; mAb: monoclonal antibody; MDA5: melanoma differentiation-associated protein 5; pMHC: peptide-major histocompatibility complex; NKG2D: natural killer group 2D; PRR: pattern recognition receptors; RIG-I: retinoic acid-inducible gene 1; TCR: T-cell receptor; TLR: Toll-like receptor[314].

3.3.2.2. Results and discussions

3.3.2.1.1. Characterization

To be considered as a successful gene delivery vehicle, the vector must be able to condense nucleic acids[283]. The electrophoretic mobility shift assays (EMSA), particle size, zeta potential, and SEM imaging were also performed to confirm the ability of the synthesized polymer nanoparticles to condense mRNA. The Ppoly/mRNA noncomplexes were created by electrostatic interaction between the positively charged CD polymer and negatively charged mRNA [284]. A gel retardation experiment was used to assess how well PPoly packed the mRNA[286]. The encapsulating efficiency increased from 55 to 90% as the N/P ratio was raised (Fig60 E), demonstrating improved complex formation between the polymer and mRNAs through ionic interactions. As can be seen in Figure 60A, even at the lowest N/P ratio,

both EGFP and OVA- mRNAs have been almost completely retarded by Ppoly. As displayed in Figure 22B, when the polymer/mRNA weight ratio reached 10:1, the Ppoly could successfully condense mRNAs into a particle size (300 nm) with a positive - potential (about +5 mV), therefore the particle size and shape of the polymer/mRNA complex at that ratio was further examined by SEM (Figure60C). Moreover, the SEM image demonstrated that the polymer/mRNA complexes were spherical in shape.

Additionally, the 10:1 N/P ratio FTIR spectrum showed that mRNA complexes were successfully incorporated into the Ppoly (Figure 60D).

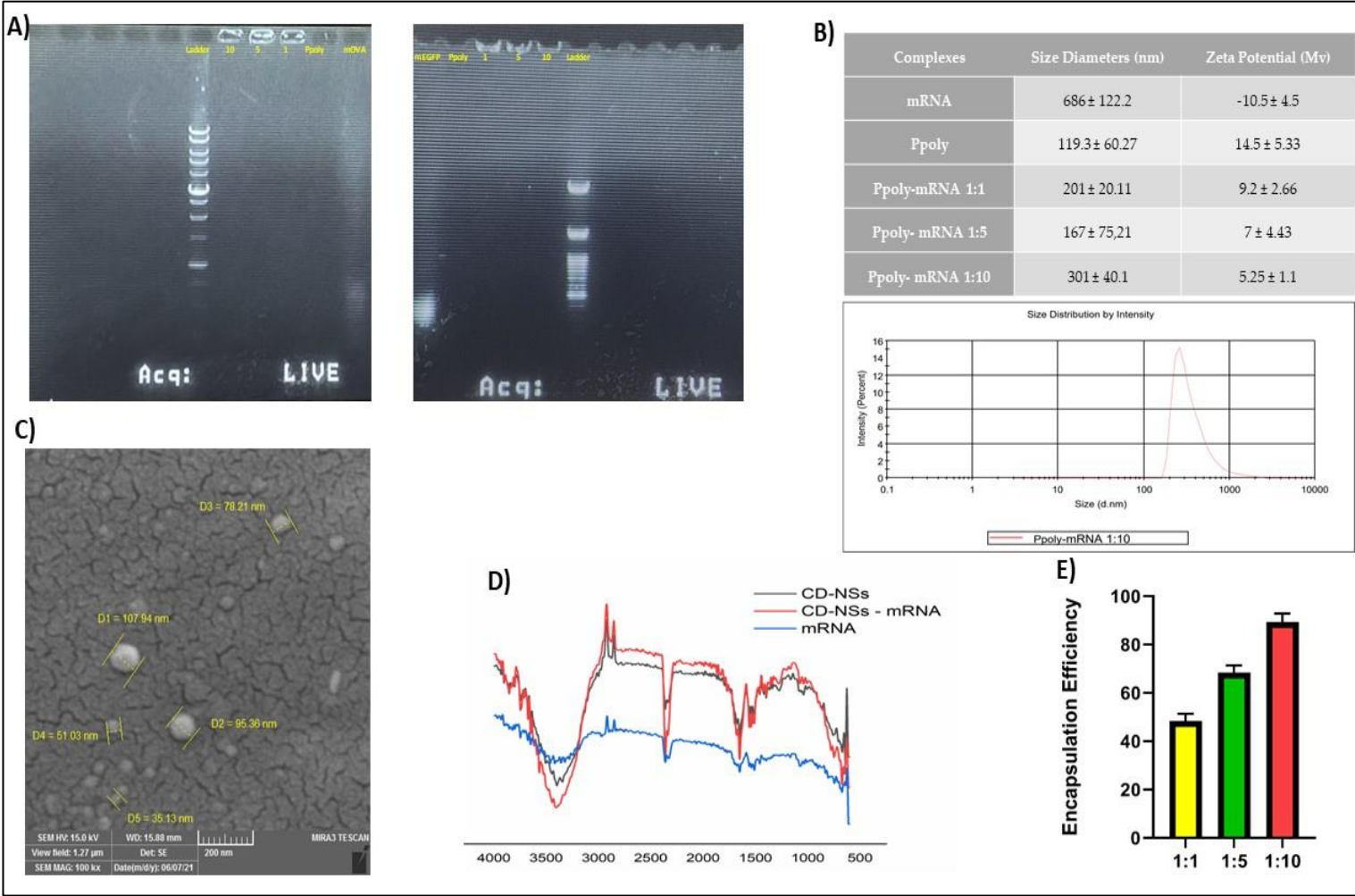


Figure 60. (A) Gel retardation assay for OVA and EGFP-mRNA, (B) DLS of complexes at different ratios, (C) SEM Image of mRNA Ppoly at ratio 10:1, (D) FTIR of complexes, (E) Encapsulation efficiency of Ppoly for mRNA.

3.3.2.1.2. Cell cytotoxicity and cellular uptake analysis in 2D and 3D spheroid culture

The cytotoxicity effect of different ratios 1:1, 1:5, and 1:10 of free Ppoly free and complexes with mRNA in compared to lipofectamine at 3, 6 and 12 μ L (routinely amount used for gene delivery) in free and complex with mRNA was assessed. The results illustrated the Ppoly at different ratios did not show cytotoxicity, while the lipofectamine at 6 and 12 μ L showed about 32 and 52% toxicity, prospectively, but interestingly, this effect was dramatically decreased at both ratios when lipofectamine was complexed with mRNA which is might be related to decrease the positive charge of Ppoly and lipofectamine (Figure 61 A-C). These findings emphasize the benefits of modified cationic polymers in terms of safety and effectiveness, which may help to overcome delivery barriers and be useful for in vivo applications[114, 347].

In light of the characterization findings obtained, the formulation with a N/P ratio of 1:1, 1:5 and 1:10 were chosen for 2D cellular transfection study to evaluate the ability of the Ppoly positive charge to deliver the EGFP-mRNA inside the melanoma cancer cell lines (B16-F10) in compared to free-mRNA and complex with commercial lipofectamine agent (OZ-Bioscience) by using the fluorescent microscopy and flowcytometry. The strong green fluorescence related to EGFP protein expression was detected in cell treated with mRNA complexes with Ppoly at ratios 1:5 and 1:10

compared to lipofectamine (1:3 ratio) and also free-mRNA. It should be emphasized that the intensity of the green fluorescence within the cells reflects the capacity of the cells to uptake our NPs (Figure 61 D). The quantitative uptake of complexes contains 1 μ g mRNA-eGFP at different N / P ratio was assessed by flow cytometer (FACS) analysis after incubation at determine time and the efficacy of uptake was found to be about 77, 72, 26%, and 58% for the mRNA in complex with the Ppoly 1:10, 1:5, 1:1, and lipofectamine, respectively (Figure 61 E). While, naked mRNA showed a negligible level of EGFP expression due to the inefficiency in the cellular entry of mRNA. These findings, which were in line with the literature[284, 347-349], indicated that Ppoly loaded with mRNA enhanced transfection efficiency by postponing the release of mRNA from nanoparticles relative to free mRNA.

The ability of our NPs to enter a 3D spheroid structure was assessed in the following stage. To evaluate the uptake and transfection of the mRNA-loaded Ppoly in comparison to free mRNA, spheroids of B16-F10 cells were created. The outcomes showed that after 24 hours of incubation with the spheroid cells, free mRNA was unable to be taken up by 3D cancer cells (Figure 62), whereas EGFP expression in 3D spheroid cells transfected by Ppoly-mRNA complexes at determined times was significantly higher than lipofectamine (Figure 62). Our NP showed highly transfection efficiency in typical 2D monolayer cells (B16-F10), while these values in 3D spheroids were lower, as other studies on gene delivery in 3D spheroid cells

showed the similar low transfection efficiency[306-309]. The extracellular matrix (ECM) and high cell density multicellular barriers in the deep regions of the 3D spheroids are main barriers to inhibit the gene delivery agents' penetration inside the spheroid cells. These cells are known to have extensive cell contact, increase interstitial pressure simultaneously, and display significant resistance to chemotherapy and radiation therapy [310]. We noticed this phenomenon in our study and demonstrated that it holds true for transfection in both two-dimensional and three-dimensional spheroids. The outer cell layers in 3D spheroids were the only areas of the peripheral cells that were transfected by Ppoly/mRNA complexes (N/P ratio 10:1), which could be the explanation. These findings support those of other studies using the in vitro 3D spheroid model for gene delivery[309, 311], additionally, it supports clinical findings that indicated poor non-viral gene transfer effectiveness in vivo in tissues with three-dimensional architecture or solid malignancies.

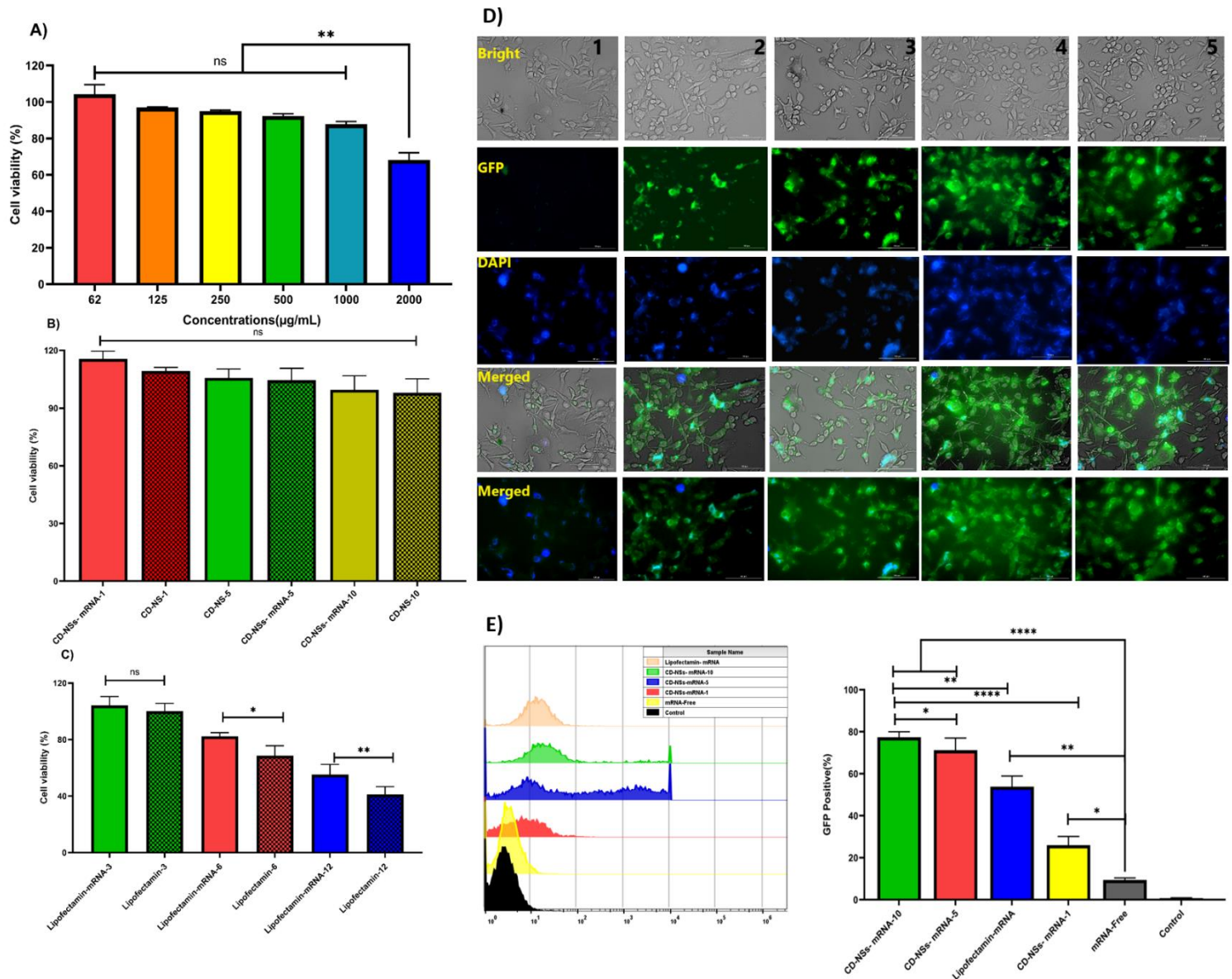


Figure 61. A; Cytotoxicity results for different concentrations of Ppoly, B; Different ratios (1:1, 1:5 and 1:10) of Ppoly free and complexes with EGFP-mRNA, C; Different volumes of Lipofectamine (μ L) free and complexes with mRNA against melanoma cancer cell lines. D; The fluorescents microscopy results for the EGFP-mRNA-free and in complexes with Lipofectamine and Ppoly. 1; mRNA-Free, 2; Lipofectamine, 3; 1:1(mRNA /Ppoly), 4; 1:5, 5; 1:10. E; The Flow cytometry results for the EGFP-mRNA-free and in complexes with Lipofectamine and CD-NSs.

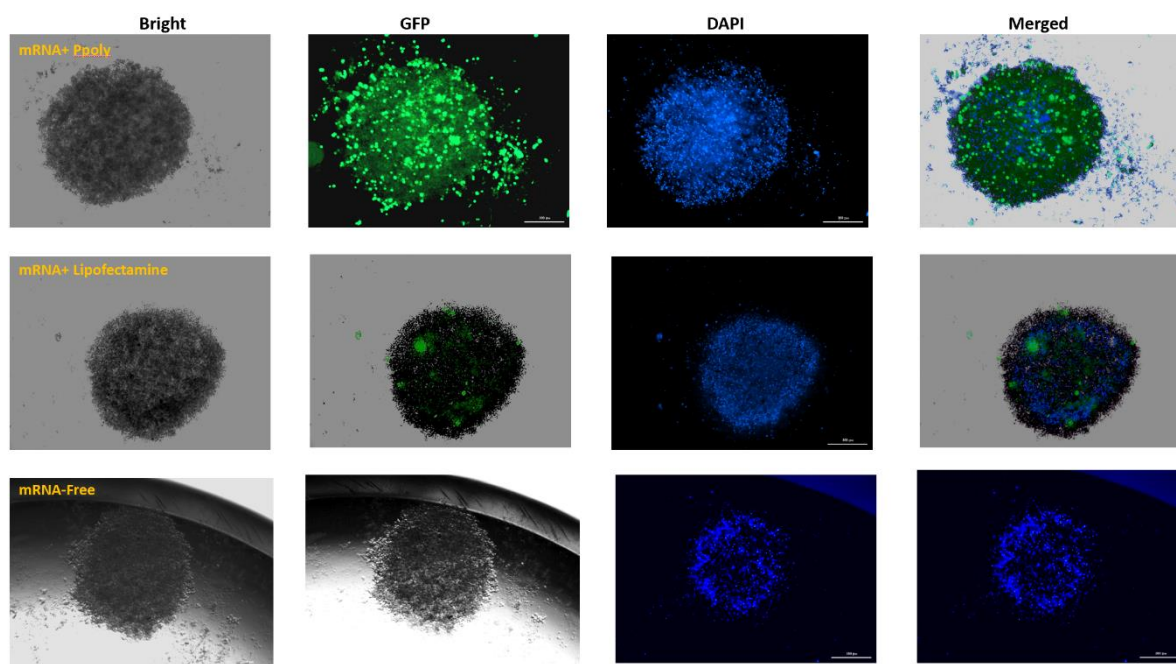


Figure 62. Microscopic fluorescent images of 3D spheroids (B16-F10 cells), mRNA/Ppoly nanocomplexes transfection at N/P ratio 10:1 in compared to free mRNA-free and lipofectamine after 24 h incubation.

3.3.2.1.3. In vivo biodistribution of EGFP-mRNA complexes with Ppoly

To evaluate in vivo delivery efficacy of mRNA in complexes with Ppoly, 50 μ l of EGFP-mRNA-free (10 μ g) and in complex with Ppoly at ratio 1:10 were injected intratumorally into female C57BL6/J induce the melanoma cancer mice. To evaluate the ability of eGFP-mRNA-loaded Ppoly expression in melanoma tumor site, the fluorescent intensity was observed at 2, 4, 6 and 24 hours after intratumorally injection through an in vivo imaging system (Kodak live animal imaging) with an excitation wavelength of 480 nm and an emission wavelength of 510 nm (Figure 63). The results showed

that the fluorescence intensity in group received eGFP-mRNA-Ppoly was significantly increased in a time depending manner in compared to free-mRNA, a group received PBS was considered as a control. This is indicating EGFP-mRNA encapsulated with Ppoly could be efficiently delivered and concentrated in tumor site that was in the same line with other studies results [350-352].

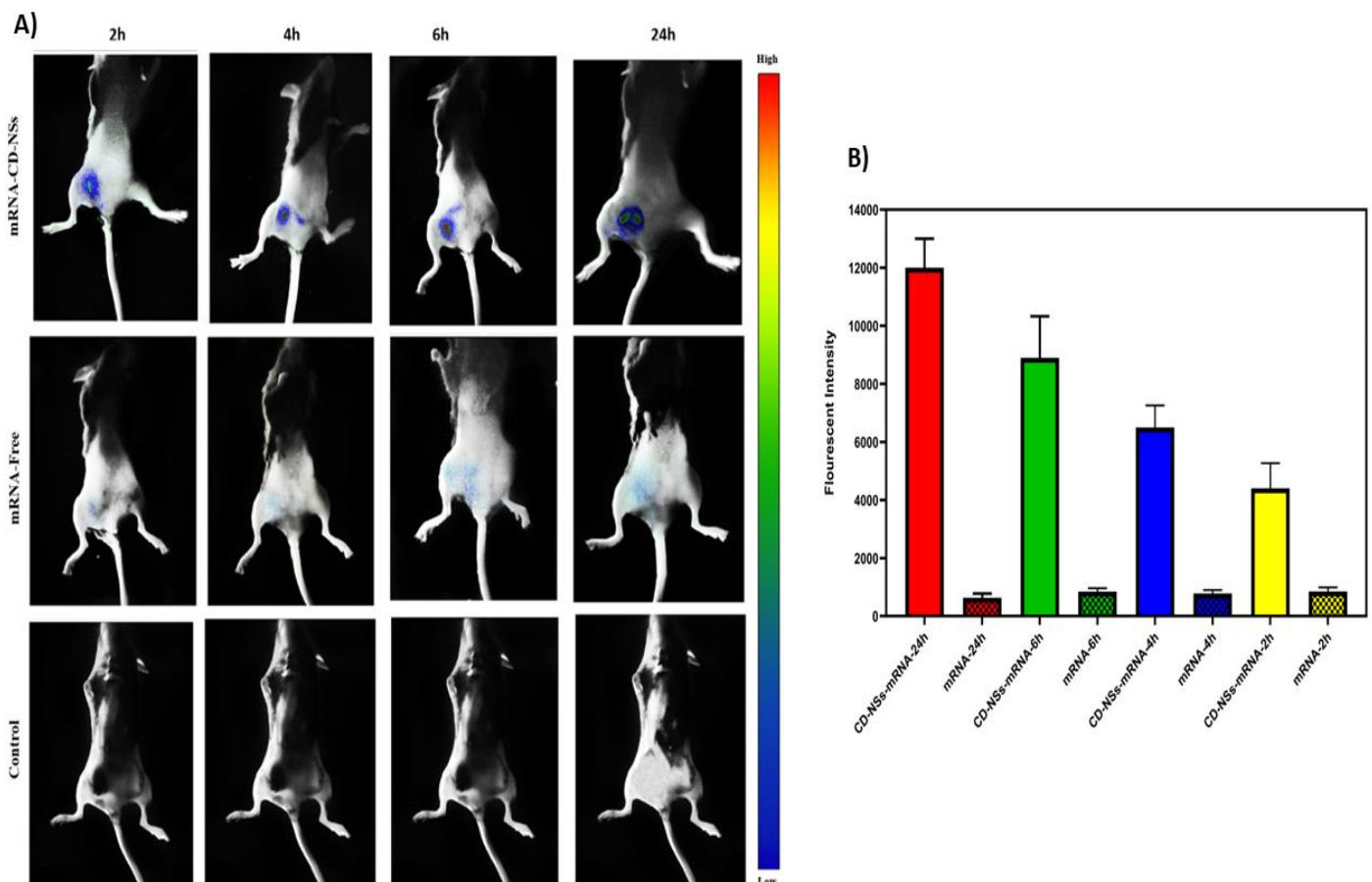


Figure 63. A, Typical *in vivo* images of female C57BL6/J mice intra-tumoral injection of saline (control group), free mRNA, and mRNA in complex with Ppoly with a dosage of 8 μ g EGFP-mRNA, respectively. b, Quantitative assay of the fluorescence intensity from the tumors of different groups based on *in vivo* images.

3.3.2.1.4. In vivo immunotherapeutic efficacy of OVA-mRNA in complexes with Ppoly

The *in vivo* anti-cancer activity of OVA-mRNA free and in complexes with Ppoly in an induced melanoma animal tumor model was the next thing we looked at. C57BL/6 mice were treated four times in 4 days interval when the melanoma tumor was palpable (n = 4 mice) (Figure 64 A). When tumors became visible and palpable, the size was measured every other day using a vernier caliper. Mice treated with PBS and Ppoly-free developed extensive tumors; in contrast, those treated with mRNA-OVA had a negligible slower tumor growth and complexes with Ppoly showed approximately 3 times more suppression compared to untreated group, demonstrating the potential of the mOVA-Ppoly therapy to prevent malignant tumors. In addition, treatments did not significantly affect the body weights of the mice (Figure 64B-C).

Interestingly, there were no differences in average tumor weight among PBS and free-Ppoly. While, the average tumor weight for mOVA+ Ppoly was significantly lower compared to all other groups (Figure 64D). Notably, the mice in the PBS groups were sacrificed at day 25 because the tumors were expanding too quickly and were too big. These data demonstrating the potential effect of the Ppoly-mOVA therapy to prevent

malignant tumors. Additionally, therapies had little impact on the mice's body weights (data not shown).

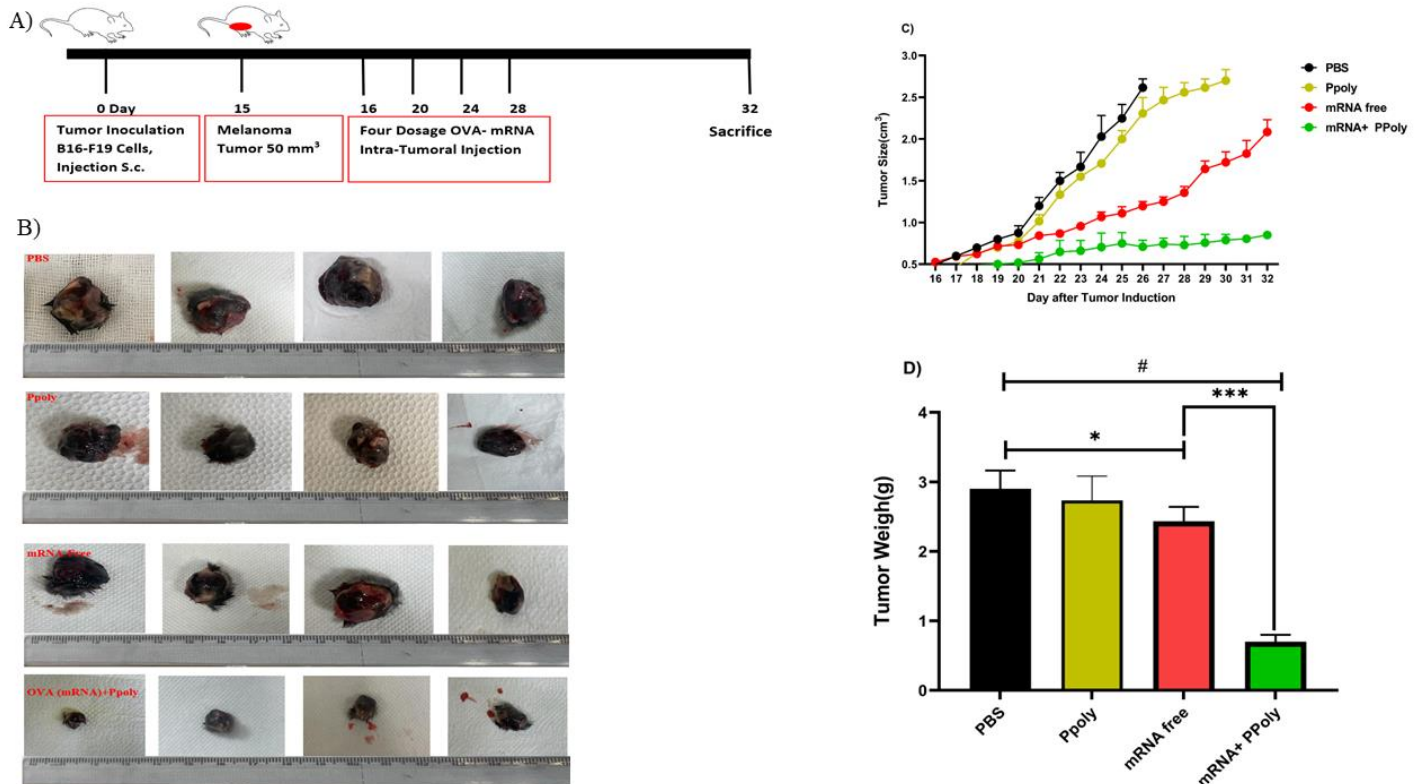


Figure 64. The anti-tumor growth effect of mOVA free and in complexes with positive charge Ppoly. (A) Scheme of melanoma tumor treatment with PBS ($n = 4$), mOVA free ($n = 4$), free-Ppoly ($n=4$), and mOVA +Ppoly ($n = 5$) and tumor inoculation by injecting B16-F10 cancer cells subcutaneously in the C57BL/6 black mice. (B) The mice of untreated, mOVA, free-Ppoly, and mOVA + Ppoly groups and their excised tumors were imaged at the terminal day 25 and 32 post tumor induction, respectively. (C) The mice were monitored daily and tumor measurement was started as soon as the tumors were first palpable. Tumor measurements were performed daily using calipers and the average tumor volume was calculated as $\frac{1}{2}$ (length $\times$ width $\times$ height), (D) Represents average tumor weight

and statistical size differences amongst the groups. (Error bars represent the SD and significance was determined using One-Way ANOVA test (ns, Not Significant, and ***P < 0.001).

3.3.2.1.5. Cellular and humoral Immune system responses

The initial activation of the adaptive immune activity is necessary for the formation of efficient and long-lasting anti-tumor immunity [353-355]. In addition to humoral immune responses, cellular immune responses triggered by mOVA-Ppoly were assessed using isolated splenocytes and tumor single cells from the treated mice after the treatment duration was complete to detect the variations of immune-cell populations. This was performed to confirm whether the effective anti-tumor effect of mOVA-Ppoly was due to an endogenous host immune-cell response. The harvested tumor and spleen single cells were stained with anti-CD8a (53-6.7, PE), CD4 (GK1.5, PE), CD19 (eBio1D3 (1D3), PE), CD11b (M1/70, PE), and NK1.1 (PK136, FITC). In comparison to the control group, mice given mOVA-Ppoly complexes had higher percentages of almost all subtype of leukocytes in the tumor (Figure 65A) and spleen mononuclear cells (Figure 65B), including CD19+ B cells, CD11b+ macrophages, CD4+ T cells, CD8+ T cells, and NK cells.

In theoretically, the immunoproteasome first converts the OVA mRNA into OVA protein, which is then processed to enable effective antigen presentation by the antigen presenting cells (APC) to CD8+ T lymphocytes [355]. The mRNA molecules

reach to cytoplasm after vaccination, where they are translated into proteins. Cytosolic proteasomes break down dendritic cells (DC) produced proteins, and the resultant epitope peptides are then delivered via the MHC class I pathway, which triggers an antigen-specific CD8⁺ T cell response [356]. Moreover, uptake of the extracellular antigens by DCs will induce a CD4⁺ helper T cell response[357]. Another fact is that mRNA vaccines have been shown to stimulate cellular and humoral immune reactions [358, 359]. Therefore, we have quantified the OVA antigen in tumor and spleen cells, consists of a variety of immune cell populations,[360, 361] excised from each group of the treated mice, by using the flow cytometry.

Interestingly, the mice treated with the mOVA-Ppoly complex had expressed approximately 11% MHC class I -OVA peptide in tumor (Figure 66A) and 4.5% in spleen cells, respectively. This suggests that the Ppoly is able to efficiently deliver and present a passenger antigen on MHC class I in the tumor loci. Furthermore, the expansion of OVA-specific CD8⁺ T cells was evaluated by using tetramer⁺ (SIINFEKL-H2Kb APC) and CD8a for detection of H-2Kb/SIINFEKL (OVA)-specific CD8-T⁺ cells [353]. As shown in Figure 66 A and B, compared with untreated mice, mOVA complexes with Ppoly resulted in significant increase of CD8⁺Tetramer⁺ T cells in tumor and spleen mononuclear cells (0.023% vs. 0.8%, and 0.010% vs. 0.4%, respectively).

The increased potency of mOVA complexes with Ppoly in comparison to mOVA alone was abundantly verified by tumor growth data. Increased ovalbumin-specific CD8+ T cell numbers in mOVA-Ppoly group are probably capable of preventing tumor growth. This could be as a result of cytotoxic T lymphocytes (CTLs) specifically recognizing tumor cells' antigens, which causes the activation of apoptotic cell death as well as CTL multiplication [362, 363].

Moreover, the serum samples were taken, and levels of induced OVA-specific total immunoglobulin G (IgG) and IgG subclasses (IgG1 and IgG2a) were measured in order to confirm the humoral immune responses to various formulations. According to the results of Figure 67A, mOVA-Ppoly significantly increased levels of total IgG, IgG1, and IgG2a compared to naked mRNA. Empty Ppoly did not induce humoral responses, confirming that Ppoly alone cannot induce the antigen specific immune responses. It is noteworthy to note that naked mRNA injection could produce moderate humoral immune responses. This effect was previously observed, and investigations have since demonstrated that various humoral responses can be partially induced by mRNA injection[353]. These results suggested that Ppoly formulation could indeed enhance the cellular delivery of mRNA and resulted in stronger humoral immune responses.

Both successful antigen presentation and appropriate immunostimulatory signals must be produced for immunotherapy to be effective. Therefore, the serum levels of proinflammatory cytokines were assessed to confirm the immune stimulating effect of

mOVA-Ppoly. Enzyme-linked immunosorbent assay (ELISA) was used to evaluate serum levels of tumor necrosis factor (TNF- α) and interferon gamma (IFN- γ), which are important markers of a strong immune response. Interestingly, mOVA-Ppoly complex induced higher IFN- γ and TNF- α levels compared to untreated and naked mRNA treated groups. This made abundantly clear the synergistic immune-stimulating effects of Ppoly encapsulating mRNA (Figure 69B). These findings demonstrated that, in comparison to mRNA alone, mOVA-Ppoly could boost cellular immune responses. Collectively, complexation between mRNA and Ppoly may be essential for the robust induction of Th1-related immune responses by mRNA-Ppoly complex system, which is expected to mainly contribute to immunotherapeutic effects in cancer model.

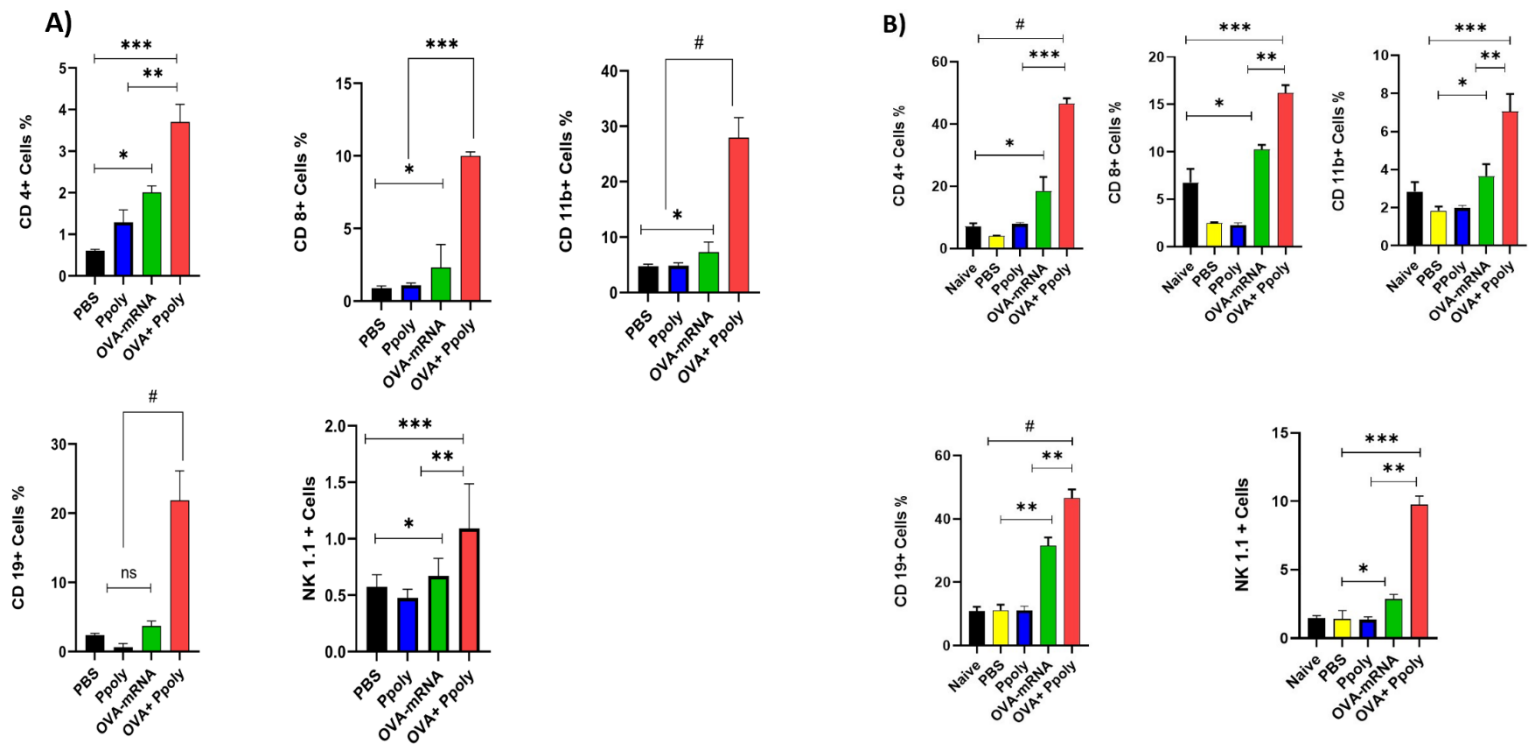


Figure 65. The cellular immune system response in C57BL/6 mice tumor and spleen tissues. On last days of treatment period tumors (A) and spleens (B) were excised, homogenized and immune cell infiltration analyzed by flow cytometry. (Error bars represent the SD and significance was determined using One-Way ANOVA test (ns, Not Significant, and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, # $P < 0.0001$)).

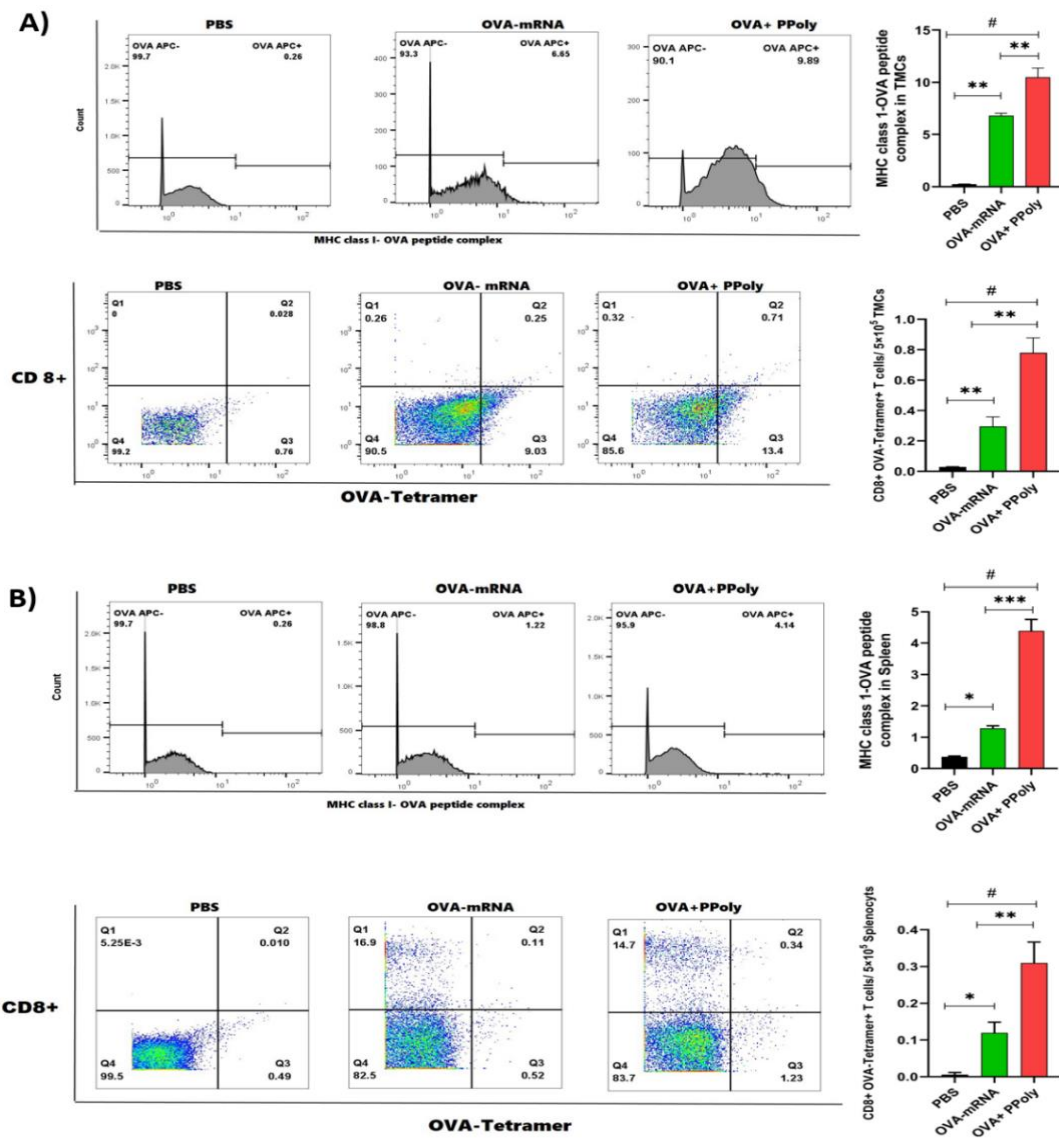


Figure 66. Quantification of H2-Kb SIINFEKL expression and antigen specific T cell responses and CD8+ T cells stained with tetramer (SIINFEKL-H2Kb PE), CD8a-FITC to determine SIINFEKL+ CD8+ T cells by flow cytometry. A, Tumor and B, Spleen cells.

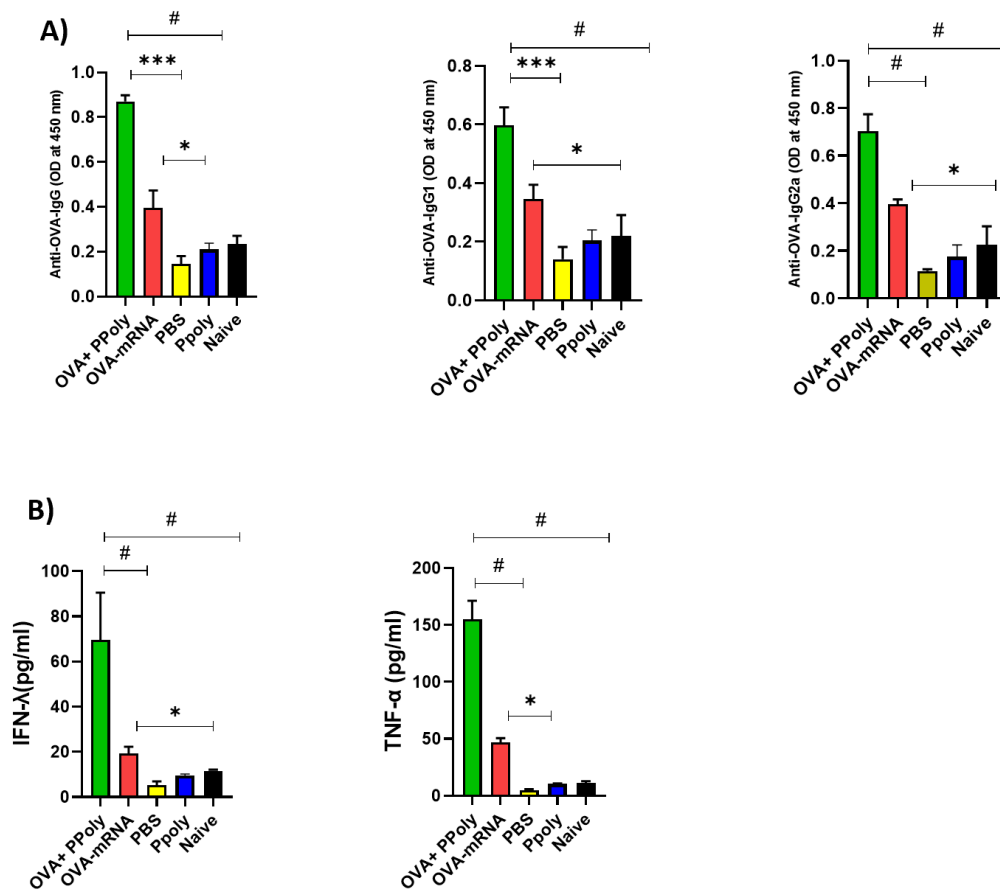


Figure 67. Antigen-specific antibodies induced by Ppoly-mOVA nanocomplexes. Mice were treated with different formulations intratumorally. A, after ending the therapy period, the OVA-specific antibodies including serum IgG antibodies, IgG1 antibodies, and IgG2a antibodies were examined in the serum. B, the serum level of TNF-α and IFN-γ were assessed in different groups.

3.3.2.1.6. Histopathology of tumor

The microscopic morphology of group treated with PBS exhibited solid sheaths of neoplastic cells. The tumor was fairly vascular, and many of the blood vessels were

congested. Tumor cells were most often arranged in irregular cords around a blood vessel. Tumor cells were characteristically devoid of pigment. The nuclei of neoplastic cells were large, ranging in shape from ovoid to round to rather irregularly polygonal. The mitotic index was above. Invasion of the tumor cells into surrounding tissues and blood vessels was appeared. In mRNA treatment group proportionate increase of vacuolar degeneration and necrotic areas of the tumor were seen. The cell displayed characteristics of degeneration, including swelling, vacuolization, rupture, and fragmentation. The cytoplasm of degenerated melanoma cells contained varying amounts of fine, light brown melanin pigment Mitotic figures was significantly decreased compared to untreated group, as malignant cell population was lower than this group. The tumor exhibited a remarkably small blood supply. In OVA-mRNA complexes with Ppoly treatment group the parenchyma of tumor mass consisted chiefly of necrotic areas. These necrotic areas contained tumor cells with pyknotic and karyorrhectic nuclei with eosinophilic cytoplasm, and cell debris. Viable malignant cell

population was lower than the other groups. Mitotic figures were significantly cell depressed in comparison with untreated group (Figure 68).

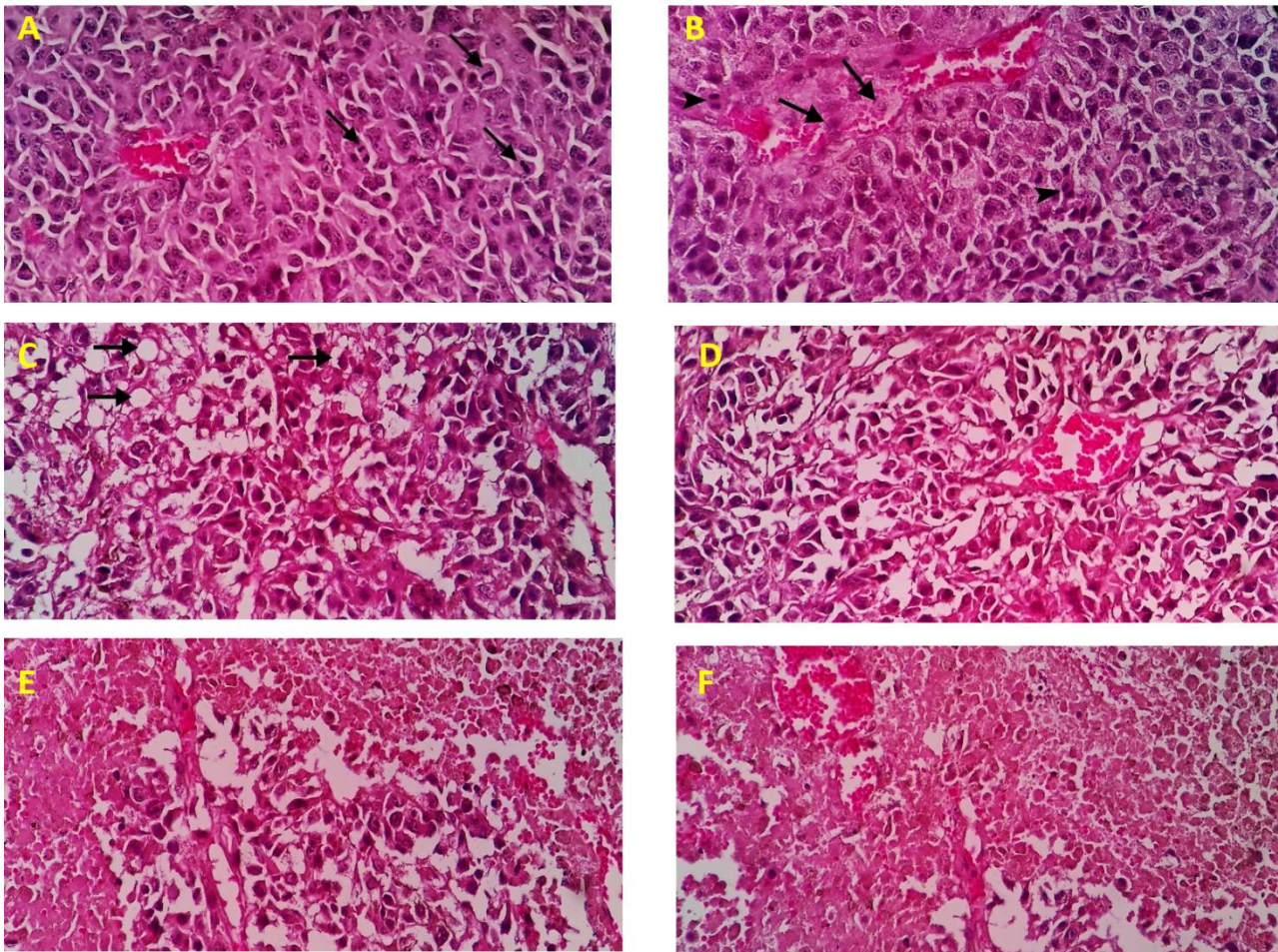


Figure 68. (A and B) Microscopic appearance of tumor mass from group treated with PBS. The tumor exhibits solid sheaths of neoplastic cells. Tumor cells are arranged in nests or as cords around the blood vessel. Mitotic figures (arrows) are prominent (H&E, 400×). B) The tumor exhibits a remarkably small blood supply as well as tumor cells are arranged in nests or as cords around the blood vessel with invasion of the tumor cells into blood vessel is obvious (arrows), mitotic figures (arrow heads) are prominent (H&E, 400×). C and D) Microscopic appearance of tumor mass from mOVA free treatment group. Extensive tumor cell vacuolation (arrows), swelling,

rupture and fragmentation are observed, and decreased cell population is prominent (H&E, 400×). D) Neoplastic tissue is rather degenerated, and characteristics of degeneration are prominent with decreasing in cell population (H&E, 400×). E and F) Microscopic appearance of tumor mass from treated group with mOVA in complexes with PPoly. E) Tumor tissue is rather necrotic, and characteristics of coagulative necrosis are prominent (H&E, 400×). F) Massive necrosis of melanoma cells is prominent (H&E, 400×).

3.3.2.1.7. Adjuvant ability of PPoly

To verify further the robust immunostimulatory effect mechanism of mRNA-PPoly complex, we examined whether Ppoly possesses adjuvanticity by intramuscularly immunising the normal mice with a mixture of OVA-protein and Ppoly. As the result, the production of OVA-specific IgG1 was increased in the presence of Ppoly although there was no change in that of OVA-specific IgG2c (Fig. 69A). The levels of antigen-specific IgG1 and IgG2c reflect the induction of Th2 and Th1 responses, respectively [364]. Therefore, these data suggest that Ppoly is able to increase the antigenicity of loaded OVA-protein compared to free-OVA by higher induction of Th2-related immune responses.

Given that many vaccinations are intended to be administered to healthy individuals, one of the most crucial qualities of vaccine adjuvants, in addition to their efficiency, is their safety. Traditionally, it has been considered that Th2 and IgE, an allergic antibody, responses are sequential [365]; we therefore checked the IgE secretion level in mice treated with OVA-protein-free and complexes with Ppoly because the ability

of an adjuvant to induce IgE may be a crucial risk factor affecting the allergenic potential of vaccines. The data suggested that Th2 induction does not always induce strong IgE production because PPoly at defined concentrations did not lead to the production of IgE in mice (Fig. 69B). Therefore, Ppoly may be a safer adjuvant than alum because it entails a lower risk of inducing potentially allergic IgE responses [364]. This result was consistent with the finding of the study by Hayashi et al., which demonstrated the effective adjuvant ability of Hydroxypropyl- β -Cyclodextrin complexes with the OVA-protein [365, 366]. As a result, these data suggest that Ppoly in complexes with OVA-protein has the ability to induce Th2-related immune responses in a safety pathway. In contrast, the mRNA-Ppoly complex induced not only Th2 but also Th1 responses, such as increased CD8⁺ T cells, antigen-specific IgG2a and IFN- γ production (Fig. 67A-C). Collectively, complexation between mRNA and Ppoly may be essential for the robust induction of Th1-related immune responses by the mRNA-Ppoly complex system, which is expected to contribute mainly to immunotherapeutic effects in the tumour model.

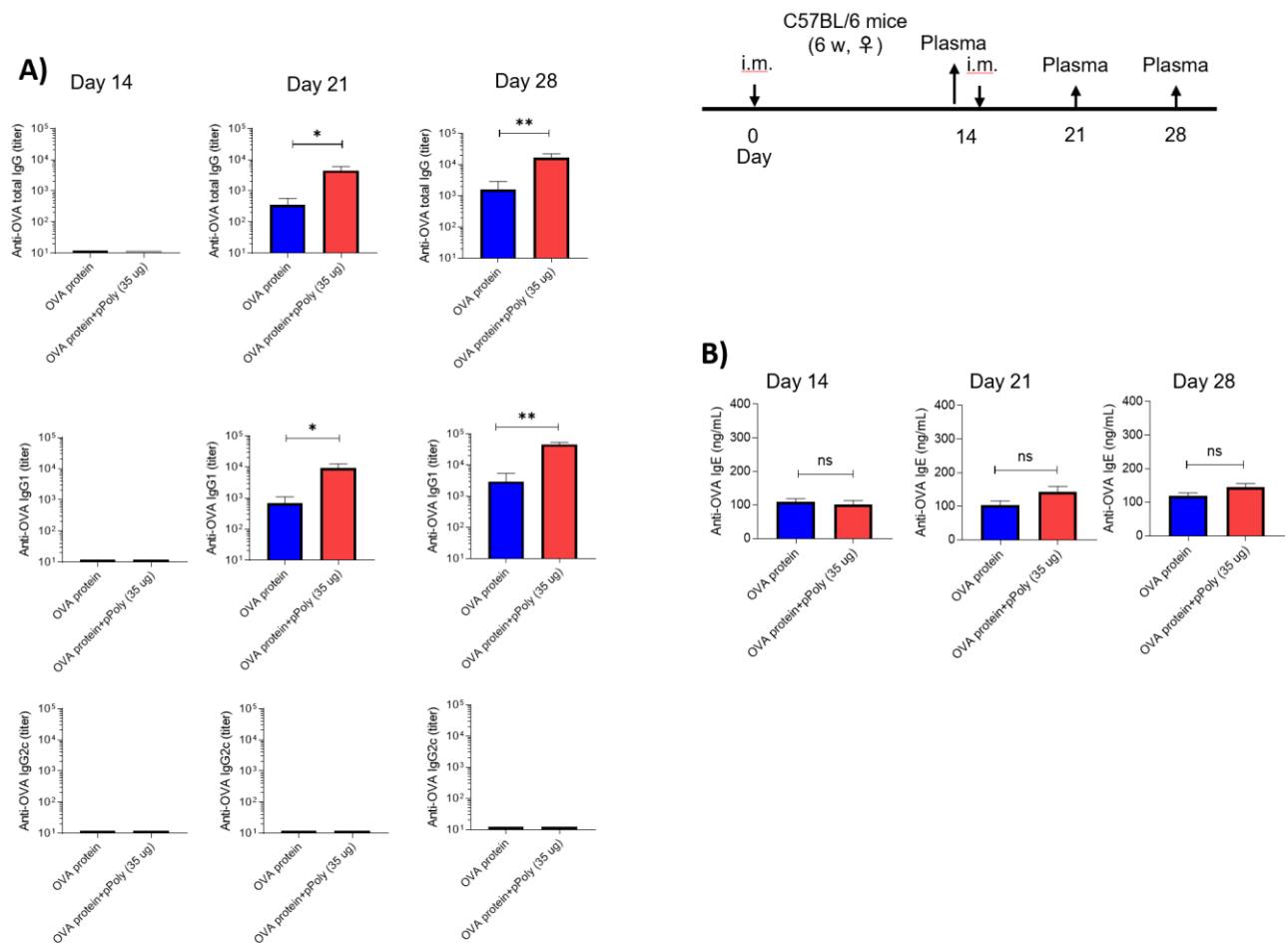


Fig 69. A, The humoral immune system response of immunised mice with OVA-protein free (10 µg) and complexes with Ppoly(35µg) by evaluating the OVA-specific antibodies including serum IgG antibodies, IgG1 antibodies, and IgG2a antibodies in the plasma. **B,** The IgE quantity production of immunised mice with OVA-protein free (10 µg) and complexes with Ppoly(35µg).

Herein, we demonstrated that a Ppoly was able to effectively deliver the EGFP-mRNA in both 2D and 3D melanoma cell lines compared naked mRNA, in addition, Ppoly did not show cytotoxicity. The Ppoly was demonstrated to be a powerful and secure mRNA delivery vehicle with in vitro proof when combined with the cytotoxicity assay. The

next step was injecting mRNA-EGFP intratumorally to evaluate its biodistribution in mice with induced melanoma cancer; the results showed that EGFP expression was time-dependent and higher in complexes with PPoly than free-EGFP. Then, the anti-tumor effect of intratumorally injected mOVA+PPoly was checked; the results showed a significant decrease in both tumor size and weight in group treated with mOVA in complexes with Ppoly compared to other formulations. Finally, we observed that mOVA, a foreign antigen, complexes with Ppoly strategy caused an efficient adaptive immune response by dramatically increasing the majority of leukocyte subtypes and OVA-specific CD8⁺ T cells in both spleen and tumor tissues *in vivo*, in comparison to untreated groups. Additionally, mOVA in formulation with Ppoly has significantly increased the humoral immune responses in mice with induced melanoma tumors. These facts were taken into account in the strategy, which resulted in an efficient anti-tumor immunity against melanoma cancer and significant inhibition of tumor growth (3 times reduction vs. control). The host immune system detects tumor cells that display non-self-foreign antigens as foreign or infected cells[352], which is why targeted delivery of foreign antigens via a polymeric conjugate can have anti-tumor effects. The results of this work may serve as proof of concept that intra-tumor administration of positive charge hyper-branch cyclodextrin polymer conjugated with a foreign antigen, mOVA, can be a novel and promising therapy to promote the immunological treatment of melanoma cancer.

Chapter 4

General Conclusions

4.1. General Conclusions

- ✓ We have used two types of β CDNS-PMDA and CDI, to deliver the peptide, Nisin-Z as an example to check the effect of these nanosponges to increase Nisin-Z antimicrobial and anti-cancer effects against different cancer cells and melanoma animal model. The results of these studies led us to conclude that PMDA and CDI-NSs have potential antimicrobial activities themselves, as well as the ability to increase nisin stability and antimicrobial activity, which is one of the most common attributes of this food preservative agent and suggests its potential application in the food industry. These results support the idea of a novel antimicrobial formulation based on CD-NS alone or in complexes with some antimicrobials. At another side, this non-toxic delivery system showed notable uptake into breast and colon cancer cell lines, and the higher toxicity and cell membrane damage were shown by Nisin-Z encapsulated with NSs as compared to that of free Nisin Z against both cancer cells in MTT and LDH assays. Furthermore, the in vivo anti-cancer effect of Nisin-Z loaded on CDNSs shows that incorporating nisin into nanosponges as a safe carrier increases the anti-tumor effect of nisin in melanoma cancer animal models by increasing apoptosis in collaboration with anti-oxidant mechanisms and could be used for future biomedical applications.

- ✓ In the next step, the oral supplementation of KYNA complexed in HP β -CD and β NS-CDI nanosponges was evaluated in mice. A complete comparison between kneading and classical inclusion complex preparation was carried out, showing exciting differences between both procedures, also creating a fast protocol to prepare complexes using ball milling. The results showed that, the complexed formulation of KYNA reduced the temporal oxidative stress damaged in kidney and liver, making β NS-CDI the best formulation. These results suggest an important future application of CD-NSs for the oral supplementation of nutraceuticals and its stabilization.

- ✓ Lastly, we have synthesized the new cationic hyper-branched cyclodextrin-based polymers (Ppoly) to deliver the nucleic acids including, plasmid DNA encoding EGFP proteins and intra-tumoral injection of messenger-RNA (mRNA) as a model to demonstrate the cellular uptake and transfection efficiency of nanoparticles and their application by two-dimensional, three-dimensional cell cultures, and animal cancer model study. Taken together, thanks to our Ppoly carrier properties; biocompatibility, hemocompatibility, and considerable cellular uptake to transfect the pDNA inside the cells, might be considered as an efficient and promising

platform for gene delivery in 2D and 3D mediums. Furthermore, the results of mRNA delivery study indicated that, this work may serve as proof of concept that intra-tumor administration of positive charge hyper-branch cyclodextrin polymer conjugated with a foreign antigen, mRNA-OVA, can be a novel and promising therapy to promote the immunological treatment of melanoma cancer.

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