ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCE

CARDIOMYOCYTE LOADED POLYMERIC MICROSPHERE DESIGN FOR TREATMENT OF CARDIAC REGENERATION AFTER MYOCARDIAL ISCHEMIA AND IN VITRO EVALUATION

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MSc. THESIS

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DECLARATION

I hereby declare that this thesis has been comprised by myself and it has not been submitted in any previous application for any other degree. Except collaborative contributions,the experimental work and the data analysis were entirely done by my own.References have been used for supporting literatures.

26 Jul 2024

Berna Ateş

PREFACE AND ACKNOWLEDGEMENT

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2 TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

MI	Myocardial Ischemia
DDS	Drug Delivery Systems
NPs	Nanoparticles
DM	Diabetes Mellitus
CNS	Central Nervous System
(mESCs)	Mouse Embryonic Stem Cells
PEG	Poly (Ethylene Glycol)
ECT	Engineered Cardiac Tissue
PLGA	Poly (Lactide-Co-Glycolide)
PCL	Poly(E-Caprolactone)
PDLA, PLLA	Polylactide
NH3+	Ammonium Group
COO-	Carboxylic Groups
SO-3	Sulfate Group
ECM	Extreacellular Matrix
RGD	Arginine-Glutamine-Aspartic Acid
LH-RH	Leutinizing Hormone Releasing Hormone
$CaCI_2$	Calcium Chloride
MS	Microphere
BBB	Blood-Brain Barrier
HD	Huntington's Disease
TED	Tissue-Engineered Dermis
CMP	Cardiomyocyte-Specific Peptide
LA	Lipoic Acid
ALG	Alginate
GA-MS	Alginate-Gelatin Microsphere

Gel-LA	A-Lipoic Acid -Gelatin
EDC-HCI	1-Ethyl-3-(3-Dimethylaminopropylcarbodiimide
NHS	N-Hydroxysuccinimide
RC	Regenerated Cellulose
DMSO	Dimethyl Sulfoxide

ÖZET

Miyokardiyal İskemi Sonrası Kalp Kası Rejenerasyonu İçin Kardiyomiyosit Yüklü Polimerik Mikroküre lerin Hazırlanması ve *in vitro* Değerlendirilmesi

Kalp krizlerinin çoğu koroner arterlerde (kalp kasına kan ve oksijen taşıyan atardamarlar) oluşan pıhtılar (trombüs) sebebiyle meydana gelir. Pıhtılar genelde ateroskleroz sonucu meydana gelen değişiklikler yüzünden daralmış koroner arterlerde oluşur. Arter duvarının içindeki aterosklerotik plak bazen çatlar ve bu da pıhtı oluşumunu tetikler. Koroner arterin tıkanmasıyla oluşan kalp krizinde, o damarın beslediği kalp bölgesi, geri dönüşsüz olarak canlılığını kaybeder. Ayrıca, Miyokardiyal iskemi sonrası oluşan oksidatif stres, kalp kasına ciddi derecede hasar vermektedir. Kardiyak iskemi sonrası oluşan oksidatif stresin kalp kasına verdiği hasarı, hücre yüklü, ilaç conjugatı ile entegre edilmiş polimerik mikroküre tedavisi kullanılarak tersine çevrilebilir ve tedavi edilebilir. Hasarlı dokuyu yenileyebilmek için hücre yüklü, ilaç ve peptit ile bağlanmış polimerik mikroküreler geliştirdik. İn vitro deneylerini yaparak, peptidin ve ilacın iskemik rejenerasyon üzerindeki etkisini tartışmaktayız. Bu polimerik mikroküre lerin doğal ve biyouyumlu olması, oldukça biyomimetik bir skafold elde edileceğini gösterir, ve yine bu polimerlerin biyobozunur olması da, hücreler kendini yeniledikçe vücudu zararsız bir şekilde terk edeceğini garanti eder. Bu sayede, bu tedavi yönteminin geleceğin umut vadeden yeni nesil hücresel tedavisinde yer alması olasıdır.

Anahtar Sözcükler: Hücresel tedaviler,andioksidan ilaçlar,polimerik mikroküreler ilaç-polimer conjugatı,peptit sentezi

ABSTRACT

Cardiomyocyte Loaded Polymeric Microsphere Design For Treatment Of Cardiac Regeneration After Myocardial Ischemia And *in vitro* Evaluation

Most heart attacks are caused by clots (thrombi) in the coronary arteries (arteries that carry blood and oxygen to the heart muscle). Clots usually occur in coronary arteries that are narrowed due to changes caused by atherosclerosis. The atherosclerotic plaque in the artery wall sometimes cracks, which triggers the formation of a clot. During the heart attack caused by a blockage of a coronary artery, the area of the heart supplied by the coronary artery irreversibly loses its vitality.(1) In addition, oxidative stress after myocardial ischaemia severely damages the heart muscle. The damage caused by oxidative stress to the heart muscle after cardiac ischaemia can be reversed and treated using polymeric microsphere treatment integrated with the combination of cell and drug conjugate. Within the scope of this stıudy,we developed cell-loaded, drug- and peptide conjugate incorporated polymeric microspheres by using electrostatic spray drying technique to repair the damaged tissue. H9C2 rat cardiomyocytes were proliferated and cell viability inside the microspheres were investigated. We discussed the effect of peptide and drug on ischemic regeneration by performing in vitro experiments. Toxicity of the peptide and drug were investigated on day 4,8 and 12 incubation of the cell-loaded microspheres. Cell viability inside the microspheres were obtained on days 6 and 12 by applying live-dead assay. Since these polymeric microspheres are natural and biocompatible highly biomimetic scaffold will be obtained, and biodegradability of these polymers guarantess that they will leave from the body during cell regeneration. Thus, it is possible that this treatment method will be part of the promising next-generation cellular therapy in the future.

Keywords: Cellular therapies, antioxidant drugs,polymeric microspheres,drug-polymer conjugate,peptide synthesis

1. BACKGROUND AND AIM OF STUDY

The aim of this project is to develop Cardiomyocyte loaded and Lipoic acid integrated Polymeric Microsphere to provide Cardiac Regeneration after Myocardial Ischemia(MI), which occurs suddenly and cause serious damage.

Within the scope of this study, it is achieved to develop cell encapsulated bidegradable,biocompatible and injectible microspheres that may target to defected area in the cardiac muscle and provide cell regeneration and controlled release of the drug during the treatment period. The microspheres were prepared by combining natural polymers, cell-adhesion peptide and antioxidant drug molecule. In Tissue Engineering, the fixation of cells in the tissue which are transferred is also achieved by using peptide-based biomolecules. In addition, since there is no example of this Project in the literature in terms of peptide conjugated structure and cell-drug-peptide combination, the design can be evaluated as the novel design. The Novelty of this study is that the polymers forming the microsphere wall are loaded with anti-oxidant drugs has the potential to contribute to regeneration by reducing the oxidative stress of cardiomyocytes activated in hypoxia environment after MI. Whilst cells are encapsulated in the inner (core) parts of the microspheres, the physical cross-linking of the polymeric structure forming the microsphere wall surrounding them provides the formation of a porous wall and facilitates the cells inside to reach the surrounding medium and to remove the wastes which they produce.Furthermore, the peptide which expedites the cardiomyocytes can be produced and combined with the micropshere.Thus, peptide incorporated cell loaded microspheres can facilitate to adhere cardiac muscle tissue. The general design of the micropshere is shown in figure1.1.

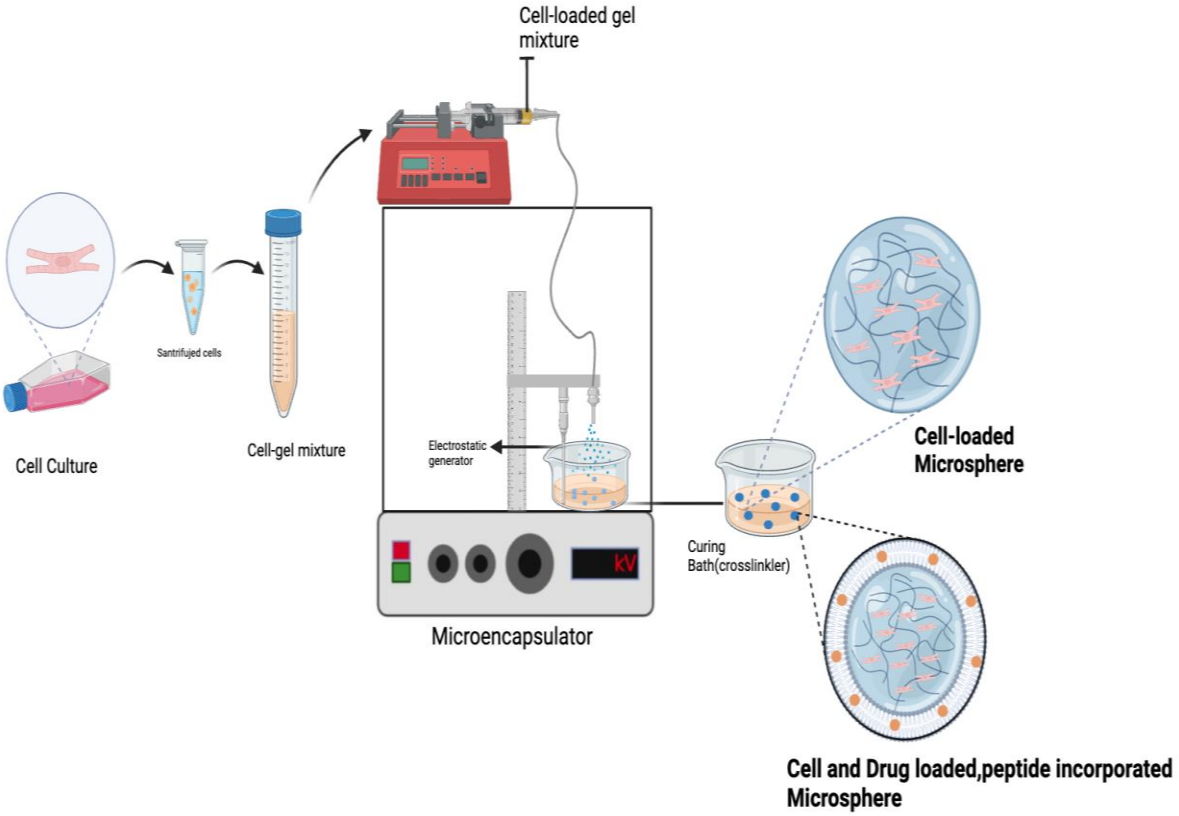

Figure 1.1.Shematic representation of the Cell loaded, and Cell loaded&Drug conjugated Polymeric Microsphere design

2 INTRODUCTION

2.1 Drug Delivery Systems (DDS) In Regenerative Medicine

Drug delivery systems (DDS) nowadays play crucial roles in medicine, enabling more effective therapies such as cancer and tissue regeneration than traditional medication products. Specifically, nanotechnology may fundamentally alter for finding a way to identify and treat cancer in the near future.(2). Nanoparticles (NPs) can selectively aggregate in tumors, cross biological barriers, and identify individual cancer cells, cardiac cells for treatment and detection because of their nanometrix size. Herein, nanocarriers enables target cancer treatment, regenerative therapy, and reduce their accumulation in healthy tissues. Furthermore, drug delivery technologies are one of the most important research fields in regenerative medicine. Recent tissue engineering studies with clinical success was done by Hoffma(3). In particular, the rapid degradation of protein and peptide biotherapeutics is a significant barrier that biomaterials can address in their formulation and delivery (4) .Herein, DDSs have been important role in preserving biomolecules from deterioration for biological settings and advancing their main purpose while decreasing deleterious side effects. Since increasing transplanted cell survival, differentiation, and engraftment has long been a key focus of this research, something similar occurs when DDS and stem cells are combined to enhance the transfer. Recent studies in the use of biomaterials for cell delivery demonstrated great ability to resolve these issues (5).Nowadays, it is possible to modify the host environment and affect the outcome of transplanted cells by using biomaterials with favorable properties. In the future, they will enable us to create entirely implanted organs that are customized to the requirements of the patient.

2.2 Cell Therapy and Encapsulation Technology

Sustained drug delivery systems have been made for over 40 years using a variety of materials, both synthetic and natural. Some of these are able to release growth factors, proteins, or medications over an extended period of time; more sophisticated ones can immobilize and protect live cells, keeping them isolated from their environment while releasing the targeted therapeutic molecules(6).Numerous illnesses with widely differing characteristics, including diabetes mellitus (DM), anemia, hemophilia B, and disorders of the central nervous system (CNS), have demonstrated great advantage for cell encapsulation Technologies. As a highly intriguing substitute, cell microencapsulation increases the transport of nutrients and oxygen inside the capsules while also significantly enhancing the surface/volume ratio. The immobilization of cells that generate therapeutically important chemicals into spherical particles with a diameter of roughly 100–1500 μm is the basis of the cell microencapsulation technique (7). Biocompatible natural polymers are used to fabricate the particles, which are then typically surrounded by a semi-permeable polymeric membrane that prevents high molecular weight molecules, antibodies, and other immune system components from passing through. This membrane shields the cells from the host's immune response as well as potential mechanical stressors that may arise from implant placement in the chosen tissue.

The Cell encapsulation technique involves immobilizing cells inside a semipermeable membrane (8). While the membrane permits the diffusion of nutrients, oxygen, and waste products in both directions, it protects the inner cells from host’s immune system and mechanical stress. This feature might be visualized as a significant benefit since it may lead to a decrease in the need for chronic immunosuppressant administration, which is something that should be taken into account in organ transplantation because of the serious side effects that these medications can cause in recipients. All things considered, only completely biocompatible materials that don't affect cell function should be used. Since the cells release the therapeutic products continually, the delivery of the product can last longer when cells are encapsulated

instead of therapeutic products(9). Additionally, non-human cells can be transplanted thanks to cell encapsulation, which offers a potential substitute for the finite supply of donor tissue. Furthermore, any desired protein can be expressed in vivo by immobilizing genetically modified cells without altering the host's genome(10,11). Due to their small size (ranging from 100 μm to 500 μm), the capsules can be implanted in close connection to the bloodstream. This could prove advantageous for the longtime encapsulated cell viability in certain applications, as the accelerated oxygen transfer within the capsules may contribute to their functionality.Moreover, microcapsules increase the permeability of nutrients and oxygen because they have a higher surface to volume ratio than conventional immobilization scaffolds(12,13).

Cell type is also important for the regeneration of the damaged tissue. For instance,Scientists have improved an emulsion-based technique to create engineered cardiac tissue (ECT) microspheres from mouse embryonic stem cells (mESCs) encapsulated in poly(ethylene glycol) (PEG)–fibrinogen(14). In order to repair damage and restore function, ECT presents a promising alternative therapeutic option for heart failure.Additionally, Gene expression alterations and pharmacological stimulus sensitivity were demonstrated in these ECT microspheres in a timely manner. As early as Day 10 of cardiac differentiation, microspheres began to develop into dense spheroidal ECTs and began to spontaneously contract. These findings show that encapsulating pluripotent stem cells via an emulsion method is feasible for application in microsphere-based cardiac differentiation(15,16).

While cell selection is significant in microsphere fabrication, the environment for the cell growth is also important. Cells are encapsulated using by polymeric networks. These polymeric networks are created via natural or synthetic polymers according to the goal.

2.3 Polymers Used in Encapsulation Technology

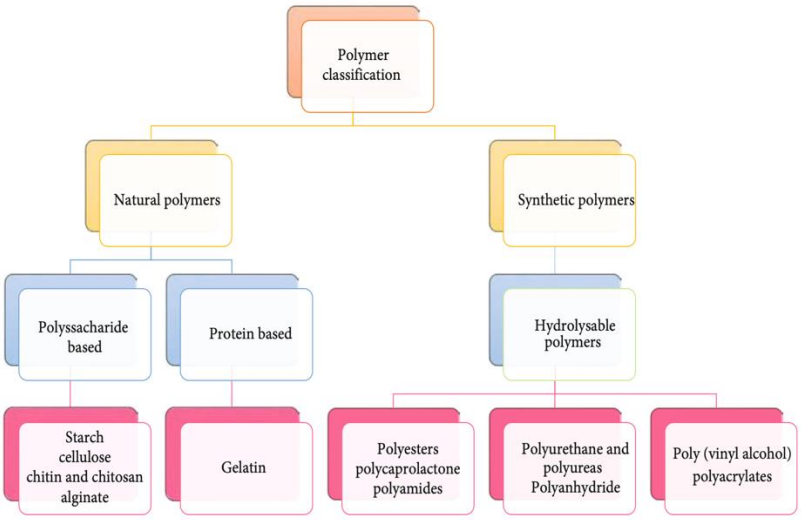

Figure 2. 1.Shematic classification of Polymers (16)

2.3.1 Synthetic Polymers

Recently, multitude of fields, including encapsulation technology, medication delivery, and tissue engineering research, have made use of synthetic polymers. Synthetic polymers are less expensive, less toxic to the body, and have greater mechanical properties than natural polymers. They can also be utilized for longer periods of time. It is possible to blend natural and synthetic polymers to give materials the required properties (17).

Esters, anhydrides, and amides are the most prevalent types of molecules. The primary factor contributing to the biodegradability of synthetic polymers is their weak hydrolyzable linkage. The blended creation of synthetic polymers, which are extremely hydrophobic, is frequently used in cell culture research to provide cell adhesion to material surfaces and assess the materials' in vitro biocompatibility. The most common used synthetic polymers are the aliphatic polyesters: Poly(ε-

caprolactone) (PCL), polylactide (PDLA, PLLA), Poly(lactide-co-glycolide) (PLGA) due to their Cytocompatibility Biodegradability Slow degradation rate, Thermal stability and degradation ratio and Poly Ethylene Glycol(PEG) as polyether(18).

2.3.1.1 Polyethers

In the chemical structure of polyether, there are several ether groups. It is a synthetic polymer due to the functionality of ether group in the main chain. One of the most common used polyether is Polyethylene glycol (PEG) in pharmaceutical technology. Chemical structure of Polyethylene glycol (PEG) is $H{-}(O{-}CH_2{-}CH_2)_n{-}OH$), providing it with an significant ability to encapsulate or covalently link hydrophobic drugs, providing them hydro-solubility. Additionally, PEG has demonstrated excellent stability during internal body circulation, which is aided by its low immunological clearance and non-toxic nature. PEG is a useful building block that has the favorable capacity to provide chemical functionalization with many different targeting groups or responsive regions, including acetal, ketal, imine, and ortho ester. It can release cargo medications into the tumor's microenvironment when necessary by becoming responsive to stimuli like pH, temperature, and redox reactions through wise functionalization. An increased permeability and retention (EPR) effect can be obtained by carefully adjusting the hydrogel nanoparticle size into an appropriate range when PEG is employed as a water-swelling hydrogel. These important features have provided PEG-based materials crucial role to improve for regeneration therapy and drug delivery(19).

2.3.1.2 Polyesthers

Polyesters are a class of polymers where each repeat unit of their main chain includes an ester functional group.Since the ester group in its primary chain serves a purpose, it is a form of synthetic polymer. Esters play a significant role in a broad range of therapeutic and drug delivery platforms PCL is a non-toxic, semi-crystalline

aliphatic polyester that degrades slowly over three to four years, making it suitable included in load-bearing applications. Its primary constraint is its hydrophobicity, which interferes with cell attachment and penetration (15).Other synthetic polymer included in regeneration therapies is the PLA. It is characterized by a few characteristics that are necessary for regeneration treatments, namely thermal stability, cytocompatibility, and the absence of harmful breakdown products. Likewise, PLA was investigated in conjunction with hydroxyapatite with a goal of enhancing its mechanical characteristics. For example, a PLA/hydroxyapatite scaffold produced using 3D bioprinting technology demonstrated good elastic behaviors and a microvascular-mimicking channel. Human mesenchymal cells were used in in vitro assays to compare higher levels of cell adhesion, proliferation, and osteo-differentiation to either the same scaffolds without the inclusion of hydroxyapatite or the scaffolds with larger micro-channels(20). PLGA is a linear copolymer made up of glycolic acid (PGA) and poly-l-lactic acid (PLLA). As it can be tuned to degrade at a rate of weeks to months by simply changing the ratio of the two monomers, polylactic acid (PLGA) is chosen in the field of tissue regeneration. Furthermore , A common biodegradable polymer for the administration of several therapeutic agents, including proteins, peptides, DNA, and other big and tiny molecular medicines, is poly(lactic-co-glycolic acid) (PLGA)(18). Especially, PLGA micro- and nanoparticles including bacterial and viral antigens have been used in pre-clinical research to test a variety of vaccine formulations (21). Moreover, PLGA particle-based formulation's ability to effectively immunize people has been linked to three mechanisms: dendritic cells and macrophages extending antigen presentation, regulated release of antigens at certain vaccine delivery sites, and particle phagocytosis. In one study, in order to create an oral vaccine, the scientists have integrated the high structural stability of the porins of Salmonella typhi with their microencapsulation into poly(lactic-co-glycolic acid) (PLGA)(22). After oral administration, encapsulated porins showed increased immunogenicity and were shielded from acidic breakdown. Strong S. typhi-specific B cell responses were specifically induced by the vaccination in mesenteric lymph nodes and Peyer's patches (23). PLGA microencapsulation, all things considered, significantly increased the effectiveness of oral vaccination against S. typhi (24).

2.4 Natural Polymers

Due to features of biocompatibility and biodegradability, It is possible to use natural polymers to increase efficacy of cellular treatments. Moreover, natural polymers are derived from biological sources, which include microorganisms, plants, and animals. The most useful and outstanding characteristics of a natural polymer are its biocompatibility, biodegradability, and simple integration with damaged tissue due to its structural resemblance to human tissue (25).

Microspheres in encapsulation technology are made from a variety of natural polymers. Naturally derived biomaterials in cell encapsulation technology mirror the structure of the extracellular matrix while also boosting the metabolic activity of the cells. Natural materials involving proteins (silk, collagen, gelatin, etc.), polysaccharides (cellulose, chitin/chitosan, glucose, etc.), and glycosaminoglycans (heparan sulfate, and keratan sulfate) are used to make artificial tissue architectures. After being cellularized, artificial tissue is frequently utilized in cell therapy to restore damaged tissue. When the closed fibril structure is constructed and loaded with components taken from the extracellular matrix, cell transplants are preserved from the immune response (26).

A more hydrophilic environment and a greater capacity to store water in a three-dimensional build could be offered via encapsulation technology. The longtime viability and functionality of cells within polymeric microbeads may be enhanced (27). Microbeads made of natural polymers are a widely utilized approach in cell treatment. Its various attributes include its easy injection side contact, high biocompatibility inside the patient's body, good inflammatory response, and great potential for tissue regeneration within damaged areas. Two primary types were identified for natural polymers. Polysaccharides are the first class of natural polymers.

2.4.1 Polysaccharides

Polysaccharides are polymeric sugar macromolecules made up of at least ten monosaccharides and sugar chains connected by glycosidic linkages. Polymers consisting of the same monosaccharides are termed homopolysaccharides; these include starch, cellulose, and glycogen. Polymers consisting of the same monosaccharides are called heteropolysaccharides; examples of these include gum arabic, which is formed of pentose and galactose. Polysaccharides are mixtures of molecules with varying degrees of polymerization rather than pure compounds. Due to their various chemical structures,they provide various physicochemical qualities, including solubility, emulsion stability, digestibility, and water-holding capacity. (28). Polysaccharides may only be modified chemically or biologically due to the presence of surface groups such as amino, carboxyl, and hydroxyl groups which allow to produce several functional derivatives. These derivative groups typically have an impact on bioadhesion by non-covalent interaction with biological tissues, containing mucous membranes and epithelium microorganisms. They have a positive charge at their ammonium group (NH3+) and negatively charged at their carboxylic groups (COO-) and sulfate group (SO-3). Natural polymers can be categorized positively and negatively charged such as alginate and chitosan.

Alginate is negatively charged natural polysaccharide that is a derivative of brown algae. It is an anionic polymer chain that is linear and unbranched, composed of β-D-mannuronic acid and α-L-guluronic acid connected by β-(1 → 4) glycosidic linkages(29). Moreover, The special gel qualities of alginate include its crosslinking structure, stability both chemically and physically, biocompatibility, low toxicity, ease of sterilization, affordability, and soft gelatinizing process. Alginate microcapsules and a highly elastic and strong gel are produced when it comes into contact with divalent cations or polycations, which causes ion transfer. Because alginate is non-biotoxic, it can be used to prepare microcapsules for bacteriological agents and has several advantages over other materials which involve biodegradability, high surface

porosity of the final capsules, compatibility with microbial organisms, ability to embed at room temperature, and controllable pore size through alginate concentration (30).

The structure of alginates was specified related to partial hydrolysis and insoluble fraction (resistant to hydrolysis). A soluble (hydrolysable) fraction is produced during fractionation, while an insoluble fraction primarily consists of L- or D-mannuronic acid (G) residues. Glycosidic bond cleavage causes alginate degradation through processes mediated by acids or alkalis, heat depolymerization, or polymer modification. The alginates that are rich in mannuronic acid (M-rich) are more vulnerable to degrade by acids and heat than the alginates that are rich in guluronic acid (G-rich) (31). The presence of alginates' enzymes renders them vulnerable to biodegradation. These neutral pH-functioning enzymes are found in seaweed, marine mollusks, and microbes. Alginate also has the property of being a biomaterial that is used to carry other medications, proteins and bioactive compounds. One of the most important aspects of drug delivery is alginate's biocompatibility. Contaminants in alginates have the potential to cause the body to react toxically to immune responses.

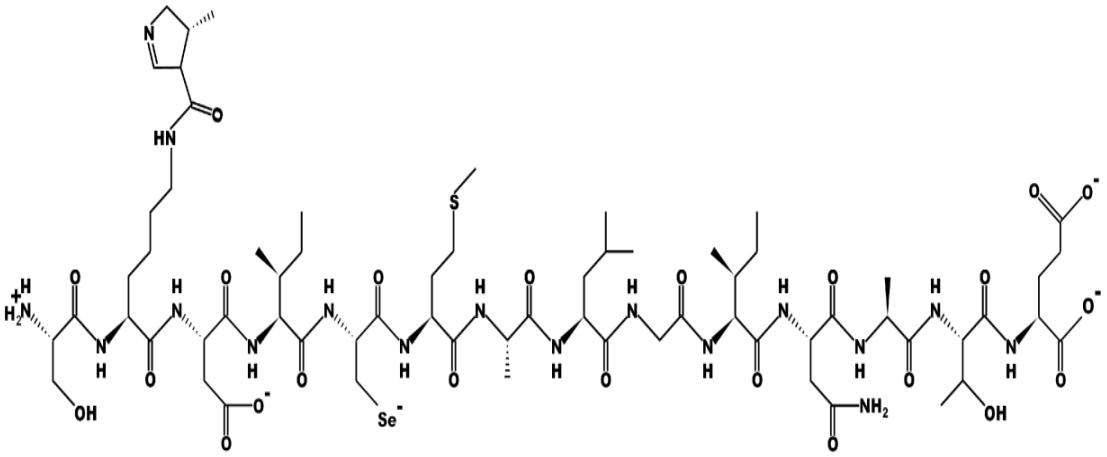

Figure 2. 2.Chemical structure of sodium alginate

Alginate has reached popularity as a biomaterial for microencapsulation due to its low cytotoxicity and great biocompatibility. In one study, the Scientists created mechanically resistant alginate microcapsules to encapsulate and shield microorganisms (Lactobacillus plantarum ST-III) from lactic acid (LA). Different mechanical strengths and particle sizes of microcapsules were achieved by adjusting the gelation capacity and viscosity of the alginate solution. Optical microscopy was used to characterize these microcapsules which demonstrated that the projected microcapsules have a spherical sform and very little aggregation (32).

2.4.2 Proteins

Proteins are kinds of polymers that comprise amino acid components. One element of the extracellular matrix is protein. Several methods, including the usage of glycosaminoglycans, fibrous proteins, sticky proteins, and proteoglycans, were employed to simulate extracellular materials. Since proteins are typically neither soluble or bound without degradation, they are used in the environment in their native state. Since proteins are copolymers made up of many amino acid configurations, the production of proteins is a complicated process involving a variety of enzymes. Proteases are used in the enzymatic protein degradation, which is an amide hydrolysis reaction (33).

The collagen (tropocollagen) is a triple helix consist of three parallel α-chains. Collagen is essential for providing tissues with their physical support and for maintaining the structural integrity of the extracellular matrix (ECM). Collagen is derived from a variety of sources, including bone, cartilage, tendon, ligament, blood vessel, nerve, and skin. Collagen is the main structural protein of most hard and soft tissues. Moreover, it has low immunogenicity, permeability, good biocompatibility, and biodegradability. Due to its porous structure, collagen provides adhesion, migration, and differentiation of the cells. The most prevalent collagens are known as classical fibril-forming collagens and are kinds I, II, and III. In most tissues of higher

order animals, collagen type I predominates. In its original tissues, it has a consistent diameter of 50 nm and is composed of two α1 chains and one α2 chain. Three identical α1(II) chains make up collagen type II, which forms fibrils with a diameter of less than 80 nm (25). Collagen type III is thought to be a little impurity in collagen type I that has been manufactured from skin because it is only discovered in small amounts (10%) in conjunction with collagen type I. It is made up of three α1(III) chains that combine to create fibrils that can vary in diameter from 30 to 130 nm. Collagen has also low immunogenicity (33).

Gelatin is most abundant protrein derived from pig skin, bovine hide, cattle and pig bones.Also it is generated by hydrolysis of collagen. For in vitro cellular development, gelatin has the ability to operate similarly to collagen in biomaterials due to it’s derivative of collagen.(33)Gelatin is also a hydrophilic biodegradable and biocompatible polypeptide that is obtained from the partial hydrolysis of collagen, a fibrous protein mostly found in the body's connective tissues. Functional groups found in gelatin include a range of ionizable groups, such as the -NH2 group of lysine, the imidazolium group of histidine, the guanidinium group of arginine, terminal -NH2 and -COOH groups, and aspartic acid -COOH groups and also carboxyl and phenolic groups can act as sites for different types of chemical modifications and conjugation opportunities. (34)Unlike synthetic polymers, which are generally nonionic, gelatin is a polyelectrolyte polymer that includes ionizable carboxylic and amino groups. Also, gelatin can chemically alter or conjugate with a variety of synthetic or natural polymers, biopolymers, and other compounds thanks to their functional groups (35).

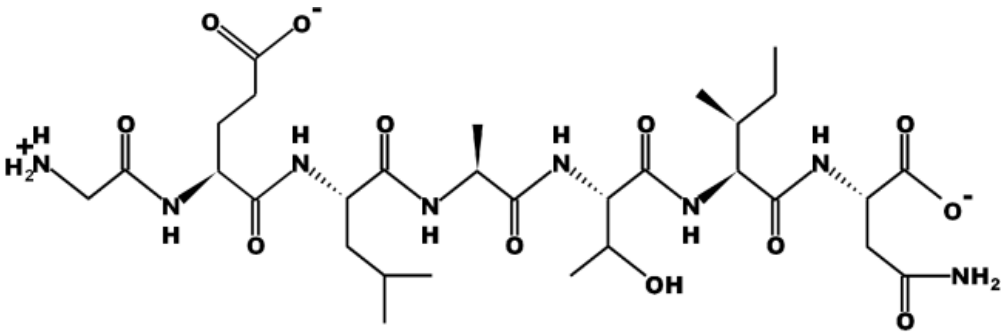

Figure 2. 3.Chemical structure of gelatin

Whereas extreacellular matrix (ECM) proteins are more expensive, gelatin is more widely available. Gelatin is easier to use in biomedical applications because it is more soluble than other ECM proteins. It may not be necessary to employ whole proteins because only a small portion of the entire sequence of ECM proteins is significant for cell attachment and inducing a cell response (36,37).

The backbone of gelatin, like that of collagen, contains arginine-glutamine-aspartic acid (RGD) peptide sequences, which are bioactive and can interact with cells by being recognized by cell receptors (38). As it contains the RGD sequence of GelMA hydrogel and the amino acids glycine, proline, and hydroxyproline, which offer an extracellular matrix (ECM)-like environment for cell adhesion, proliferation, and assistance with nutritional exchanges and metabolic waste disposal, gelatin is a great choice for tissue engineering and drug delivery (35).Furthermore, gelatin can be made even more flexible by adding functional groups that have the ability to be photocrosslinked or substantially polymerizable.With the development of the first photocrosslinkable gelatin derivative, GelMA, gelatin stabilizing techniques can be used to process materials in an easy way.Methacrylamides are created when methacrylic anhydride and the main -NH2 groups on the side chains of ornithine and hydroxylysine in gelatin to functionalize the polymer (39).

2.5 Microencapsulation Techniques

There are several techniques of microencapsulation.One of the techniques is Spray-drying tecnhique. Spray-drying, a fast, continuous, one-step process that combines drying and encapsulation, is a viable way to create microspheres in volume. Firstly, the medication must be dissolved, emulsified, or dispersed in an alginate solution. Secondly, the liquid feed must be atomized into droplets using a nozzle. Lastly, the droplets must be dried in the drying gas to produce dry microspheres. Both aqueous drug -and micronized drug particles can be encapsulated using this method (13).

The emulsification/gelation method is cheap very simple to set up experimentally, and it also allows for particle sizes of less than 100 mm, so it is applicable to create microspheres at the microscale. Peptides, proteins, vaccines, and water soluble drugs are the greatest candidates for this method of microsphere creation, which causes creating multiple emulsions or double emulsions of type w/o/w. With both polymers, this approach is applicable (40). A continuous layer of lipophilic organic matter disperses the aqueous protein solution. It is possible that the active ingredients are in the protein mixture. Consisting of a polymer solution that finally encapsulates the protein in a dispersed aqueous phase, the continuous phase is typically encountered. Before adding the primary emulsion to the poly vinyl alcohol aqueous solution, it is homogenized or sonicated (41). This concludes in the double emulsion formation. After that, the emulsion is removed using a solvent extraction technique or solvent evaporation. The double emulsion solvent evaporation/extraction process can be used to successfully incorporate a variety of hydrophilic drugs, including vaccines, proteins, leutinizing hormone releasing hormone (LH-RH) agonists, and conventional compounds into the microspheres.Furhermore, Natural polymers including proteins and carbohydrates are microparticulate carriers made by the single emulsion method (42).

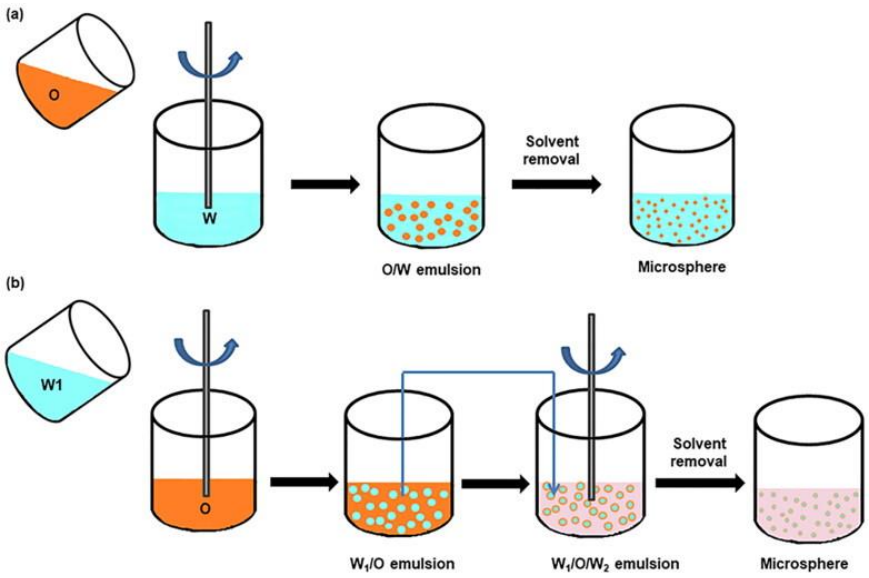

Figure 2.4.Shemas of the (a)single emulsion technique and (b)double emulsion technique (21)

Extrusion technique is the simple and widely used method in Microencapsulation by ionic gelation of solution which involves a simple diffusion and cross-linking reaction. Accordingly, extrusion technique is also called as ionic gelation technique. For instance, to create alginate microspheres alginate solution is dropped using a basic dripping equipment into a gelling bath containing calcium chloride (CaCl2). In the alginate structure, the Ca2+ ions take the place of the Na+ ions and stiffen the membrane to form particles. With this method, setting up an experiment is simple and can be done with a simple dripping tool (like a syringe or pipette) or a complicated tool (like stainless steel needles) (43). Due to the ability of Ca2+ ions to attach to the carboxylic group of alginate and form a thermostable gel, the gelation of alginate in the extrusion technique is greatly reliant on the concentration of CaCl2. By modifying the CaCl2 concentrations, stirring rate and duration of the operation, and needle size, Lin et al. were able to effectively encapsulate 90% of astaxanthin into alginate beads. Over 90% of the initial astaxanthin concentration was still present after 21 days of storage at 25 °C, according to their study, which focused on the effect of Ca2+ ions in improving drug stability in alginate matrix (44).

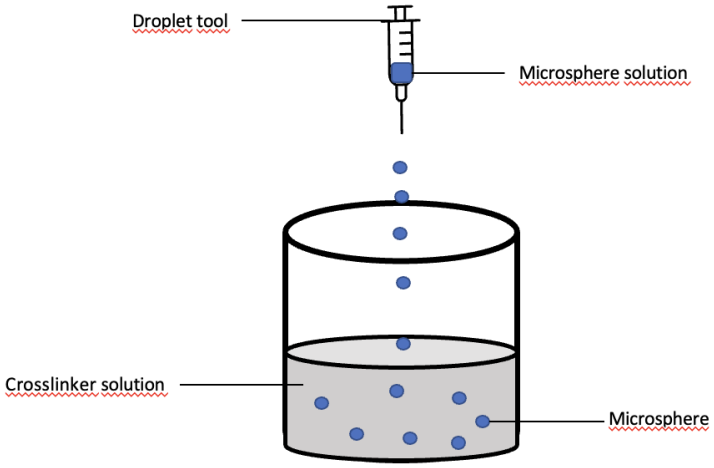

Figure 2. 5.Schema of extrusion microencapsulation technique

2.5.1 Types of Microencapsulation

2.5.1.1 Bio-adhesive Microencapsulation

Adhesion may be described as the drug's ability to attach to a membrane using the water-soluble polymer's sticky characteristic. Bio adhesion is the phrase used to describe the adherence of a drug discovery instrument to a mucosal membrane, like the nasal, ophthalmic, or buccal. These microspheres produce superior therapeutic activity because they stay longer at the application site, form close contact with the absorption site, and have a longer residence time (45).

2.5.1.2 Polymeric Miroencapsulation

There are two categories of the polymeric microsphere encapsulation which are Synthetic and biodegradable polymeric micropsheres.

2.5.1.2.1 Synthetic Polymeric Microencapsulation

In addition to being utilized as bulking agents, fillers, embolic particles, drug delivery vehicles, and other applications, synthetic polymeric microspheres have shown great promise in clinical settings and have been shown to be both safe and biocompatible. However, the primary drawback of these microspheres is their propensity to disperse from the injection site, which increases the risk of embolism and further organ injury. As radioactivity acts from various emitters such as a emitters, ß emitters and within the normal radioisotope distance rather than being emitted from microspheres (45).

In one study, PLGA micropheres(MSs) with the purpose of cathcing small peptides were developed. The produced MSs were intended to be taken orally as an

adjuvant to HIV vaccine. To create tiny PLGA microspheres with excellent encapsulation efficiencies, several formulations were created and investigated in addition to the MSs, which were made using the W1/O/W2 solvent evaporation method (46). Furthermore, the peptide was not released into the stomach media in less than four hours. Then, the 7-amino acid peptide pBC 264—a strong CCK-B receptor agonist with promise for treating neurological conditions such as Parkinson's disease (PD)—was ensnared in the previously created PLGA MSs (24). The brain rapidly eliminates this peptide,so trapping it in biodegradable MSs should make it more stable and enable them to investigate its long-term biological and pharmacological consequences. In order to achieve a high encapsulation efficiency and sustained release when the particles were given into the brain, the formulation had to be adjusted from their first analysis, which involved encapsulating pBC 264 under the same conditions as V3 BRU.The sluggish transport of neuropeptides in the brain was demonstrated by the near-identical in vivo release kinetics of MSs that were locally implanted in the rat brain using stereotaxic surgery. Lastly, spray-drying was used to encapsulate vapreotide, an equivalent of the somatostatin octapeptide, within PLGA MSs (22). This peptide has potential applications in the management of neuroendocrine tumors and acromegaly. The study's major goal was to keep rat’s plasma levels stable for two to four weeks following a single injection. An injectable delivery method for vapreotide was created twice a month using the best and most optimal MS formulation (47).

2.5.1.2.2 Biodegredable Polymeric Microencapsulation

Biodegradable, biocompatible, and bioadhesive natural polymers, including starch, are used in various applications. Biodegradable polymers have a high degree of swelling property with aqueous medium, which occur gel formation and prolongs the residence period in contact with mucosal membranes. The polymer concentration and the sustained release pattern regulate the magnitude and speed of medication release. It is difficult to control medication release when using biodegradable microspheres because of their complex drug loading efficiency in clinical settings(48).

2.6 Bioactive Microspheres for Regeneration Therapies

Microcapsule performances are mostly determined by the material's physicochemical characteristics and the methods of preparaton used. To physically isolate an implanted cell mass from the outside world within the confines of a semipermeable membrane barrier is the ultimate aim of cell encapsulation. This technique was initially motivated by the need to overcome graft rejection, a significant barrier that arises when nonautologous cells or tissue are transplanted (49).

Allograft utilization has expanded thanks to the ability to combine cells with polymer scaffolds utilizing microencapsulation techniques to develop "living cell medicines" that deliver drugs over an extended period of time. For instance, therapeutic proteins can be frequently delivered by encapsulated cells for extended duration of days, weeks, or even months. These characteristics made this method especially appealing for the transplantation of islets to treat diabetes. Cell encapsulation makes it possible to use a range of cell types, including primary, stem, and genetically altered cells that might be changed to represent necessary protein *in vivo* without changing host genome (50).

Cells loaded microspheres were studied *in vitro* for potential applications on different diseases including cancer, diabetes, skin, liver, and cardiovascular, bone,neurodegenerative and cartilage disorders.

Healing capacity is limited for articular cartilage.Cartilage repair with autologous chondrocyte implantation demonstrated great achievement,but gigh rates of graft failure, delamination, and tissue hypertrophy were observed when chondrocytes were implanted without a carrier (51).Thus, it is necessary to create next-generation methods for cartilage recovery that combine matrix growth and alternate cell sources. Mesencymal stem cells(MSC) are important in cartilage repair. MSC are distinguished with several features which contain numerous potentially differentiation, regeneration, and hypoimmunogenic property, accessible. They might be extracted from autologous

resources including adipose tissue and bone marrow (52). In order to inhibit graft delamination and mistake matrix augmentation for cell-based cartilage regeneration seeks to locate and give anchorage support to the cells upon implantation at the defect site. (53)Structurally, mechanically stable, chondroconductive, biocompatible, and biodegradable scaffolds are ideal for cartilage repair matrix augmentation. Cartilage tissue engineering has developed use of a variety of scaffolds, including collagen, alginate, and hyaluronic acid gel.

The microspheres allow the encapsulated MSC to migrate and locate themselves at the implantation as well as perfect integration. Significantly, when the encapsulated MSC are sensitive to chondrogenic induction signals, the collagen microspheres are perfect chondroconductive scaffolds, as the template type I collagen matrix is altered and mimicked with a cartilage-like matrix (9).

Unexpected disease may arise from a brain damage as a result of disability or death. Trauma, illnesses, and tumors can all harm the central nervous system (CNS). When combined, neurological conditions rank as the world's second most common cause of mortality (54). Damaged brain tissue cannot be repaired by the brain. Growth inhibitory environments, glial scar development, restricted chemical transport into or within brain tissue, and the CNS's limited ability for regeneration are only a few of the many obstacles that impede any therapeutic intervention for the brain.Furthermore, in cases where medications or growth factors are larger in size or non-lipid soluble, the protective blood-brain barrier (BBB) restricts their ability to get into the brain. It may be possible to restore brain tissue brain by stem cell transplantation with microencapsulation with combination of natural and synthetic polymers to deliver drugs across the blood-brain barrier. Collagen microspheres containing embedded progenitors of oligodendrocytes facilitated their development into mature oligodendrocytes and enhanced the myelination of co-cultured neurons by acting as cell carriers (55). Additionally, fibrin is used to create hydrogel and microsphere materials for the delivery of cells and drugs. When employed as local NGF reservoirs in vivo, fibrin-based hollow microspheres with great loading efficiency offered a

continuous release over a period of 14 days. The release of GDNF and the survival of entrapped cells were not adversely affected by the loading of fibrin gels with MSC overexpressing GDNF. Furthermore, upon transplantation into the rat striatum, these fibrin gels, as compared to transplanted cells alone, reduced activation of surrounding tissue's astrocytes and microglia.

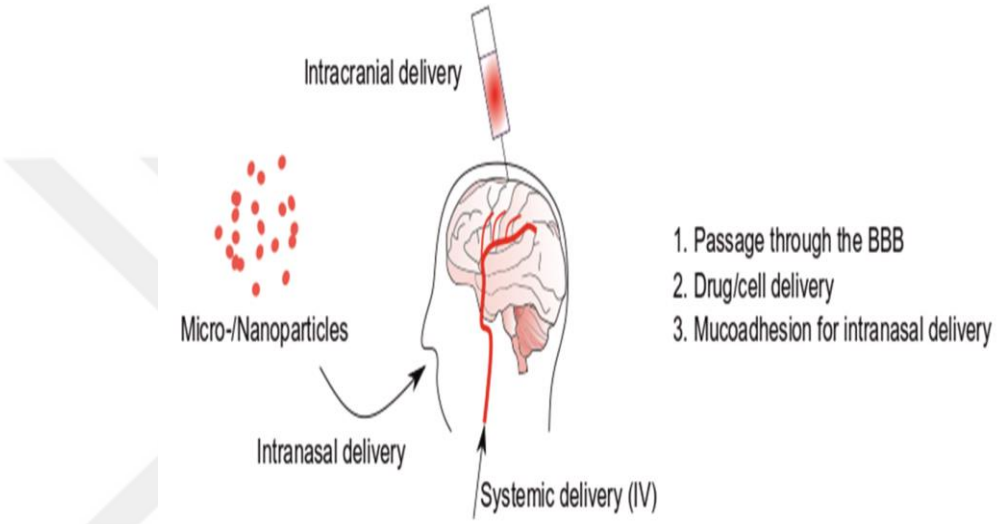

Figure 2. 6.Schema of Micro-/nanoparticles possibilities to bypass the BBB by intranasal, intracranial or systemic delivery for therapeutics

One of the major issues facing researchers is the restoration of bone abnormalities. Degenerative illnesses, including those affecting the bones, undoubtedly arise from the loss of stem cells. The likelihood of restoration of injured tissue or stopping more tissue deterioration increases when active stem cells are present.Stem Cell therapy might be recover damaged tissue. Among the most effective stem cell types, mesenchymal stem cells have a great capacity for differentiating into different types of connective tissue. In addition, MSCs play a role in a variety of biological processes, such as bone regeneration, hypoxic-ischemic encephalopathy, and wound healing (10). Even though MSCs show a lot of promise for tissue engineering and medical regeneration, their distribution, safety, stability, fate, and function need to be assessed prior to transplantation.Encaspsulated stem cells is one method to maintain them safe

and stable.Furthermore, The encapsulation of stem cells in alginate hydrogel has been widely employed in tissue engineering applications because of its simple fabrication and tenable biophysical characteristics, including matrix mechanics, biocompatibility, disintegration, and non-antigenicity (12)(13).Treatment of neurodegenerative diseases with primary cells is now being researched as well. Scientists developed an alginate-based encapsulating device that immobilized the choroid plexus in order to achieve the best possible distribution of neurotrophic substances to the brain in a monkey model of Huntington's disease (HD). Transplants of choroid plexus showed a strong protective effect on striatal neurons, indicating that encapsulated choroid plexus may be helpful in inhibiting neuronal degeneration in HD patients (56).

Another study field for cell encapsulation is diabetes. Approximately 3-5% of people worldwide suffer from diabetes, making it a global health concern.There are 150 million people worldwide who have diabetes mellitus based on current WHO statistics, and that number is expected to double by 2025 (13). Microencapsulated human islets were implanted to 10 patients with type 1 diabetes who were not immunosuppressed The results of two patients have been released, and while the use of exogenous insulin was not completely stopped, the assessment of several indicators showed that the pancreatic islets were still metabolically active (57).

Tissue-engineered dermis (TED), which consists of a collagen- Chitosan scaffold, is a component of skin tissue constructs used in regeneration research (58). The primary drawback of this architecture is that it lacks any vascular structure. It is known that when VEGF gene-modified hUCMSC-derived fibroblast cells are microencapsulated and transplanted into pig skin scars, vascularization structure of the collagen-chitosan laser-drilled acellular dermal matrix composite scaffold is increased (59).

2.6.1 Gelatin-Alginate (GA) Microencapsulation for Cardiac Regeneration

Cardiovascular disease is one of the serious issue worldwide. Insufficiency is caused by disturbances in the cardiovascular system's operation.Globally, Myocardial ischemia (MI) is one of the main causes of death. This disorder is brought on by both coronary artery stenosis and an imbalance in the supply and demand of oxygen, which results in a shortage of oxygen in the heart muscles (60). Numerous cardiomyocytes beat rhythmically, and these rhythmic beatings of cardiomyocytes contribute to the rhythmic contractions of heart tissue. Acute myocardial infarction and cardiomyocyte mortality can result from coronary artery stenosis, which prevents oxygen and nutrients from reaching the heart tissue.Generally, cell-based therapy encounters a more severe set of obstacles, involving the quick time to administer the medication, the hostile microenvironment, inadequate cell engraftment, and rapid removal from the ischemic tissue (61). The pace of regeneration may be accelerated by the application of unique cell and tissue engineering techniques. Until now, a number of techniques have been used to administer and transplant cells into target tissues. Given the constraints imposed by direct cell administration using traditional techniques, it makes sense to develop new strategies for cell delivery. For example, the physical link between cells is eliminated, and a significant fraction of cells perish during administration due to mechanical stress with a direct cell administration system (40) The studies demonstrated that a significant proportion of cells underwent programmed cell death when being administered to myocardial that had been damaged. The majority of transplanted cells notably lose their ability to transdifferentiate into cardiac lineage. Furthermore, polymers are significant to create extracelluar microenvironment for the cell migration, adhesion and proliferation (62).

Alginate(alg) is widely used natural polymer for the microencapsulation due to its biocompatibility, biodegradability properties. In the presence of cations such as Ca^{2+}, Mg^{2+}, Ba^{2+}, Fe^{2+}, and Al^{3+}, alginate can function as a 3D backbone both independently and in conjunction with other substrates to ensnare biological macromolecules and cells within a restricted size. The proliferation and differentiation

of stem cells are stimulated when sodium alginate is cross-linked with calcium, as demonstrated by numerous studies. Although the results are encouraging, there are certain drawbacks and difficulties with using Alg in regenerative medicine. This material consists of high charge density polyanionic polysaccharides that have a restricted rate of cell attachment. Alg can undergo chemical modification through the binding of natural proteins, including gelatin(gel) which shares a structure with the extracellular matrix (ECM) (63). Several motifs that are necessary for various cellular bioactivities are produced by the modification of the Alginate backbone with gelatin. The scientists confirmed that encasing rat cardiomyoblasts in GA preserved their stemness potential and reduced oxidative stress.It has been determined from earlier research that a portion of heart cell damage is brought on by mitochondrial malfunction in pathological circumstances (64).

In tissue engineering, peptide-based biomolecules also stabilise the cells in the tissue to which they are transferred. Many studies demonstrated that cell or tissue specific peptides trigger cell adhesion and adhesion. These peptides are short amino acid sequences that support the cell- cell attachment. For instance, RGD (R: arginine; G: glycine; D: aspartic acid) are used for many cells such as fibroblasts and cancer cells, while YIGSR (Tyr-Ile-Gly-Ser-Arg) and IKVAV (Ile-Lys-Val-Ala-Val) can be used for neural cells. CMP (WLSEAGPVVTVRALRGTGSW) is 20-mer peptide and significantly selective ligand produced by phage display biopanning. WLSEAGPVVTVRALRGTGSW is a cardiomyocyte-specific peptide. Exosomes expressing WLSEAGPVVTVRALRGTGSW may improve cardiomyocyte selective uptake, reduce cardiomyocyte death, and increase cardiac retention after intramyocardial injection in vivo. CMP has the ability to specifically target primary cardiomyocytes (65). An isolated CMP phage has been shown to bind primary cardiomyocytes 180 times more than control phages. Additionally, when compared to a panel of other cell types, phages exhibiting this peptide favorably adhere to cardiomyocytes. In order to therapy of cardiovascular disease, CMP has been utilized to change a bioreducible polymer carrier involving Fas siRNA. One study discovered that the polymers modified with CMP may specifically bind cardiomyocytes,

increasing the efficacy of transfection (66). Chemical structure of CMP peptide was demonstrated in figure 2.7.

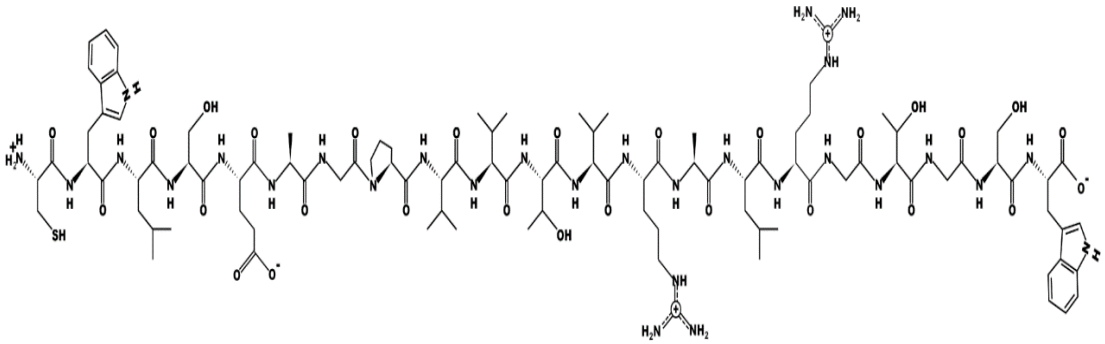

Figure 2. 7.Chemical structure of CMP peptide

α-Lipoic acid(LA) is a natural short-chain fatty acid with both hydrophilic and lipophilic properties, containing a carboxyl group and a disulfide bond, produced by plants, animals and humans. Recently, it has been recognized that it has one of the most significant cellular oxidation regulators with exceptional antioxidant features. Both surface-seeded and microsphere-encapsulated cells are available, depending on the application. The term "cell-on" systems refers to the usage of cells on the surface of microspheres for in vitro cell growth (67). Cell adhesion and proliferation with varying surface modifications are supported by this system, which can be cultivated in vitro prior to distribution to the target site. An improved means of promoting cell attachment and proliferation is through a microsphere's three-dimensional structure. In a variety of MSCs, osteogenesis has been shown to be promoted by a straightforward switch from plate to microsphere cell culture (68,69).

3 MATERIALS AND METHODS

3.1 Materials Preparation

Sodium Alginate(NaAg) (low viscosity, A1112, Sigma-Aldrich) solution was dissolved with 4%(w/v)concentration in saline (Polypharma) solution and stirred on magnetic stirrer at 37 ℃.Gelatin (Gelatin from bovine skin, Type B, Sigma Aldrich) solutions were prepared with 1%, 0.1% and 0.05%, concentration (by weight) in saline .Gelatin solutions (5, 10 ratio by volume) were added into total alginate solution and gelatin/alginate mixtures (GA) were prepared.

Calcium chloride (CaCl2) (75 mM and 100 mM) were used as a crosslinking agent. Incubation of needle tips (0.35 mm) inside sodium citrate solution (85 mM) or double distilled water to inhibit blockage of the system. Sterilization of encapsulation systems performed with ethanol (70%). Thereafter, the device sterilized with UV (2 hour) in the laminar flow hood before the cell culture experiment.

3.2 Alginate Microsphere Fabrication

Alginate polymer, a natural and biocompatible polysaccharide, was studied to obtain micron-sized particles by physical cross-linking with divalent Ca+2 ions.(86) For this purpose, a 4% w/v polymer solution of low viscosity alginate polymers (50% mannuronate units, Sigma-Aldrich A1112) and 75mM Calcium Chloride (CaCl2) prepared in distilled water were used for curing.

The prepared microspheres were obtained between the targeted 150-200 μm. This value is suitable for the encapsulation of large amounts of cardiomyocytes (cell diameter: 17-25 μm) into the polymeric microspheres to be obtained.4 % w/v prepared Alginate mixture was filled to a 5mL injector and connected to the syringe pump of

the Microencapsulator to be used for microsphere formation (NiscoEncapsulator VAR V1 LIN-0043, NiscoEngineering AG, Zurich, Switzerland).(70)

On the other hand, 75 mM CaCI2 solution was transferred into the curing bath. A magnetic stirrer was placed in the solution bath to prevent the accumulation of microspheres in the solution bath.

The microsphere size might be varied with various parameters. After adjusting the parameters of the device (solution flow rate, voltage, distance between the nozzle and the curing agent beaker), microsphere formation was performed. The polymer solution flow rate (5 mL/h), inner diameter of the nozzle (0.35 mm) and voltage values (6 kV electrostatic potential) were optimized to form the diameter of microspheres as 150-250 μm . After dripping the polymer solution into the curing solution at a certain speed and voltage value, these microspheres (Alg-MS) obtained by stirring in the curing bath for at least 1 hour were washed twice with saline to remove excess calcium salts in the medium.

3.3 GA Microsphere Fabrication in Different Concentrations

It is predicted that these hybrid microspheres, which are aimed to be prepared by adding gelatin polymer (Gelatin from bovine skin, gel strength ~225 g Bloom, Type B) at different ratios into the alginate polymer, can enhance cell adhesion.

To obtain alginate-gelatin microspheres (GA-MS), 0.05 % and 0.1% (by weight) of gelatin was dissolved in saline(0.9%). Different volumes (5 % and 10 %, v/v) of gelatin solution prepared by 0.05% and 0.1% (by weight) of the gelatin was added to previously prepared 4 % (w/v) alginate solution. The final mixture was used in the cell encapsulation experiment using the Microencapsulator device.

The prepared GA solution was filled to a 5mL syringe and connected to the syringe pump of the Microencapsulator to be used for microsphere formation (NiscoEncapsulator VAR V1 LIN-0043, NiscoEngineering AG, Zurich, Switzerland). On the other hand, 100 mM CaCI2 solution was transferred into the curing bath. A magnetic stirrer was placed in the solution bath to prevent the accumulation of microspheres in the solution bath. After adjusting the parameters of the device (solution flow rate, voltage, distance between the nozzle and the solution beaker), the desired GA microsphere was obtained. The polymer solution flow rate (5 mL/h), inner diameter of nozzle (0.35 mm) and voltage values (6 kV electrostatic potential) were optimized to form microspheres of 150-200 μm in size. A total of 5 mL of the solution was dripped into the curing solution with the help of a syringe pump.

3.4 A-Lipoic Acid -Gelatin (Gel-LA) Conjugation

A procedure in accordance with the literature was used for the synthesis of gelatin polymer conjugated with α-lipoic acid, a powerful natural anti-oxidant.(Hoch, et al.; 2013) Gelatin polymer was dissolved in 10 % (w/v) sodium bicarbonate solution in the round bottom flask. Afterwards, 50 mg of lipoic acid , 51.1 mg of (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide)EDC-HCl and 27.9 mg of activating molecule (N-Hydroxysuccinimide) NHS were dissolved in 500 μL DMSO in order to activate the carboxylic acid group in the structure of these drug molecules and reaction left for 30 mins on the magnetic stirrer for the 1,4-Michael addition reaction.As *a*-lipoic acid is light sensitive, the beaker full of the mixture was covered alumium foil to protect from the light. After 30 mins, the carboxylic acid group NHS-activated lipoic acid solution was transferred onto the gelatin solution with a syringe by dropwise and reaction left for 24 hours at room temperature in nitrogen gas.

After the reaction completed, dialysis of the gel-LA conjugate was carried out using RC (regenerated cellulose) bags with a MWCO value of 10 kDa was used.Deionized water was used as the receptor solution and was continued for 2 days,

Finally, the purified conjugate solution was lyophilized by freezing to store the modified gelatin polymer in a solid form. Briefly, The conjugation was performed with the chemicals listed in Table 3. 1.

Table 3. 1.The amount of chemicals for a-lipoic acid-Gelatin Conjugation

Name of the Chemical	**Equivalent**	**Molecular Weight (Da)**	**n (mole)**	**Mass (mg)**	**Volume**
Gelatin	10			500	
A-lipoic Acid	1	206.33	0.2423	50	
EDC•HCl (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide)	1.1	191.7	0.2666	51.1	
NHS (N-Hydroxysuccinimide)	1	115.09	0.2423	27.9	
Dimethyl Sulfoxide (DMSO)	1				500 µL
Sodium Bicarbonate($NaHCO_3$)					5 ml

3.4.1 Gel-LA Conjugated GA Micropsphere Fabrication

2 methods were used to obtain Gel-LA conjugated GA MS. First method is that 2 mg of Gel-LA conjugate was dissolved in 8 ml pure ethanol for 30 minutes. Afterwards, the solution was diluted with a range of 1:8 which is 56 ml dH_2O and stirred on magnetic stirrer at 3 hours.

Different volumes (5 % and 10 %, v/v) of Gel- LA solution prepared was added to previously prepared 4 % (w/v) alginate solution and mixed for 30 minutes. The prepared Gel-LA-Alg solution was filled inside to a 10mL syringe and connected to the syringe pump of the Microencapsulator to be used for microsphere formation (NiscoEncapsulator VAR V1 LIN-0043, NiscoEngineering AG, Zurich, Switzerland). In parallel, 100 mM CaCI2 solution was transferred into the curing bath. A magnetic stirrer was placed in the solution bath to prevent the accumulation of microspheres in the solution bath.

In the second method 0.1w/v % (15 mg) of the Gel-LA was dissolved in 15 ml 0.1 M of Carbonate buffer approximately 4 hours and then, 5(v/v)% of the solution was added to 4(w/v) % Alginate solution and stirred for 30 mins. Thereafter, it was filled to 10 ml syringe and proceeded with the microsphere fabrication with above mentioned set-ups. For 10 mL syrringe, encapsulator set up parameters was demonstrated in table 3.2.

Table 3. 2.Encapsulator parameters

Flow Rate	Diameter	Volume	Voltage	Pump	Agiator
5 ml/hr	14.5	off	6.00	20	30

3.5 Peptide Synthesis and Purification Analysis

3.5.1 WLSEAGPVVTVRALRGTGSW (CMP) Peptide Synthesis

In order to covalently bind the WLSEAGPVVTVRALRGTGSW (CMP) peptide to the side branches of the gelatin polymer, a sequence with C (cysteine) was synthesized. The free thiol group from the Cystein amino acid at one end was

used for conjugation. Peptide synthesis was carried out by SPPS (solid phase peptide synthesis) method using by the Peptide Synthesis Device CEM, Liberty^{TM} Blue and CEM Discover^{TM} Microwave) available in the University infrastructure. General overview amino acid names and their specific derivative was demonstrated n in table 3.3.

Table 3. 3.Overview of aminoacids and codes

Amino Acid	**Three Letter Code**	**One letter code**	**Specific Derivative**
Alanine	Ala	A	Fmoc-Ala-OH
Arginine	Arg	R	Fmoc-Arg(Pbf)-OH
Glutamate	Glu	E	Fmoc-Glu(OtBu)-OH
Glycine	Gly	G	Fmoc-Gly-OH
Leucine	Leu	L	Fmoc-Leu-OH
Proline	Pro	P	Fmoc-Pro-OH
Serine	Ser	S	Fmoc-Ser(tBu)-OH
Threonine	Thr	T	Fmoc-Thr(tBu)-OH
Tryptophan	Trp	W	Fmoc-Trp(Boc)-OH
Valine	Val	V	Fmoc-Val-OH
Asparagine	Asn	N	Fmoc-Asn-OH
Aspartate	Asp	D	Fmoc-Asp(OtBu)-OH
Glutamine	Gln	Q	Fmoc-Glu-OH
Histidine	His	H	Fmoc-His-OH
Isoleucine	Ile	I	Fmoc-Ile-OH
Lysine	Lys	K	Fmoc-Lys(Boc)-OH
Methionine	Met	M	Fmoc-Met-OH
Phenylalanine	Phe	F	Fmoc-Phe-OH

Tyrosine	Try	Y	Fmoc-Tyr-OH

The peptide synthesis process involved two main steps: the peptide synthesis itself, which was done with a DMF solution, and the Rink Amide ProTide resin cutting process, which was done with a DCM solution. First, the computer and device were turned on, and the liberty Blue program was used to enter the peptide sequence that was to be synthesized. After this, the program's required data was filled in to specify the necessary amounts which shown in Table 3.4. The necessary amounts were weighed in accordance with the information which was mandatory. The resin was inflated in DMF for 30 minutes before the synthesis initiated. To ensure that any potential residues were removed, DMF was used to clean the entire equipment containing amino acid falcons to ensure that any potential residues removed.

The prepared solutions of oxyma, DIC, DMF and piperadine which given in Table 3.4 and 3.5 were filled into the instrument, and the amino acids were transferred to the falcon and fastened to the equipment. The resin was transferred immediately to the synthesis device after calibration was performed by the Liberty Blue program. Before applying the synthesis, the falcons containing the amino acids were labeled for control purposes. Finally, The synthesis was carried out for approximately 2 hours and 30 minutes.

In the second step of the synthesis, resin cutting was initiated.The chamber was opened and arranged 37℃, 15 minutes before the reaction ended . A cleaning filter was attached to column 11, where the experiment was conducted. An empty waste flask was placed beneath, and DCM was added and washed. After three times washes, the cleavage cocktail was prepared (Table 3.6). At the end of the reaction, the resin was carefully removed and transferred to filters. After washing with DCM to remove DMF, the cleavage cocktail was added after placing the filter on top of the 11^{th} flask. The lid was then closed, and the solution was allowed to rest for 30 minutes. At the end of this period, all of the solution was removed by vacuum. Three washes were

performed with cold ether in a sterile flask. The centrifuge was set at 5000 rpm for 3 minutes. After the last wash, the cold ether was discarded, and the flask was covered with aluminum foil, wrapped in parafilm, and perforated. After being stored in a desiccator overnight, the flask was sealed and stored at +4 ℃ The reagent table which were used for CMP peptide synthesis was listed in table3.4. For detection of the peptide purification,1 mg of synthesized CMP peptide was dissolved in 0.5µL acetonitrile and placed an HPLC vial and HPLC analysis conducted.

Table 3. 4.The reagent list for CMP synthesis

Reagent	**DMF Volume (mL)**	**Mass(g)**
Alanine	6	0.38
Arginine	12	1.56
Glutamate	3	0.26
Glycine	9	0.54
Leucine	6	0.43
Proline	3	0.21
Serine	6	0.47
Threonine	6	0.48
Tryptophan	6	0.64
Valine	9	0.62
Cystein	3	0.36
Main Wash (DMF)	542	
Resin mass required:	7.5	0.143

Table 3. 5.The amount of the chemicals for activator preparation

Chemical	Amount	DMF Volume(ml)
Diisopropylcarboimide (DIC)	2.2 ml	25.8
Piperidine	22 ml	88
Oxyma	1.989	14

Table 3. 6.The amounts of cleavage cocktail

Chemical	Molecular Weight (g/mol)	Volume
TFA	114.02	4.75 ml
Dd H2O	18.02	125µL
TIS	158.36	125µL

Finally, obtained molecular weight of the synthesized peptide is 67 mg.

3.5.2 Gelatin-Metacrylamide (Gel-Mal) Synthesis

The CMP peptide-Gelatin conjugation was performed using by the 1,4 Michael Addition reaction. This stage comprises of two main steps. Firstly, 100 mg of gelatin was dissolved in 50 mL of 1% Phosphate Buffered Saline (PBS) on a magnetic stirrer at 300 rpm without heat. Thereafter, 7.65 µL of Triethylamine (Et_3N) was added to the the gelatin solution and dissolved on the magnetic stirrer for 15 mins. Meanwhile, 1 mL of anhydrous dimethyl sulfoxide (DMSO) which given in Table 3.7 was placed

in a different round-bottom flask dissolved. In parallel,12 ,95 mg of NHS, 21,5 mg of EDCI and 15,85 mg of the 6-maleimidohexanoic acid (Mal) were added to DMSO and round bottom placed on the magnetic stirrer and the reaction left for 2 days.The reaction was performed at a room temperature in nitrogen gas.

The Gel-Mal was purified by initiating a dialysis process. 10kDa membrane was used to proceed with the dialysis . The Gel-Mal solution was poured into one knotted end. The other end was then knotted and submerged in 500 ml of ddH_2O that had been earlier heated in a beaker. The ddH_2O was replaced every 2 hours in less than 8 hours. Consequently, purified Gel-Mal solution was put into lypholizator to obtain dried polymer. The lypholized Gel-Mal polymer was weighed as 384 mg. FT-IR analysis of the polymer was performed to obtain functional groups of the conjugate. Overall, The reaction table for Gel-Mal syhtesis was listed in table3.7.

Table 3. 7.The reaction table for Gel-Mal syhtesis

Name of Chemical	**Equivalent**	**Mass(mg)**	**Volume**
Gelatin	1	500	
6-Maleimidohexanoic acid (KRSRGYC)	20	15.85	
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide(EDCI)	30	21.5	
Triethylamine(Et_3N)	20		7.65 µL
N-Hydroxysuccinimide (NHS)	30	12.95	
1X Phosphate Buffered Saline			50 ml
Anhydrous Dimethyl sulfoxide (DMSO)			1ml
Total Product		384	

3.5.3 (Gel-Mal)- CMP Peptide Conjugation

Gel-Mal-CMP peptide conjugation consist of 2 stages. Firstly,350 mg of Gel-Mal was dissolved in 21 ml 1% PBS and 43,5 mg of the CMP peptide was dissolved in 2 ml DMSO respectively. Afterwards,7.1 μL of Triethylamine(Et_3N) was added to the CMP peptide solution and reaction left approximately 1 hour on a magnetic stirrer for the 1,4-Michael addition reaction. After Gel-Mal was dissolved, the CMP peptide solution was added dropwise with a syringe and and reaction left for 2 days on the magnetic stirrer. The second step is that the (Gel-Mal)-CMP solution was purified with a dialysis process. 10kDa membrane was used to proceed with the dialysis. The (Gel-Mal)- CMP solution was poured into one knotted end. The other end was then knotted and submerged in 500 ml of ddH_2O that had been earlier heated in a beaker. The ddH2O was replaced every 2 hours in less than 8 hours. Finally, purified (Gel-Mal)- CMP solution was put into lypholizator to obtain dried polymer. The reaction rable for the (Gel-Mal)-CMP pep conjugation was summarized in table 3.8.

Table 3. 8.The reaction table for (Gel-Mal)- CMP conjugation

Name of Chemical	**Equivalent**	**Mass(mg)**	**Volume**
Gel-Mal	1	350	
Triethylamine(Et_3N)	2	5.2	7.1 μL
DMSO			2ml
WLSEAGPVVTVRALRGTGSWC	15	43.75	
PBS			21 ml

3.6 Characterization of CMP Peptide and Conjugates

3.6.1 FT-IR Analysis

FT-IR spectroscopy was used to assess the functional groups of obtained CMP peptide, Gel-LA conjugate, and GelMal-CMP conjugate in the 3500-500 cm−1 wavelength range.

3.6.2 ^{1}H NMR Analysis

The chemical structure of the CMP peptide and conjugates was determined using a 400MHz Bruker NMR spectrometer in DMSO-d6.

3.7 Characterization of the Microspheres

3.7.1 FT-IR Analysis of the Fabricated Microspheres

FT-IR spectroscopy (Thermo^{TM}Nicolet iS10) meausred the properties of functional groups of the chemical structures in microsphere samples with ranges of the 3500-500 cm−1.

3.7.2 Scanning Electron Microscopy (SEM)

Pore sizes and Porous structure of the Gel-CMP conjugates integrated Microspheres and Gel-LA conjugated microspheres were carried out by using SEM (Themorscientific Quatro S). Also, Semi -quantitative elemental observations were investigated with energy dispersive x-ray spectroscopy(EDS) detector. Dried

microspheres were covered with 40 nm gold under vacuum. Thereafter, DS of the microspheres were investigated.

3.7.3 Rheological Analyzes

Rheological analyzes of obtained GA MS in the concentrations of 0.05(w/v) % and 0.1% gelatin(w/v), 0.1(w/v) % Gel-CMP Conjugated MS, 0.1(w/v) % Gel-LA conjugated -MS were carried out using by Malvern Ultra+ rheometer instrument under shear stress.

3.8 (Gel)- CMP Conjugated GA Microsphere Fabrication

0.1(w/v) % (15 mg) of the (Gel-Mal)- CMP conjugate was dissolved in 15 ml ddH_2O and 1 M of Carbonate buffer approximately 4 hours and then, filtered with 0.45 μm membrane. 5%(v/v) of the the (Gel-Mal)- CMP conjugate solution was added to 4%(w/v) Alginate solution and stirred for 30 mins. Afterwards, it was filled to 10 ml syringe and connected to microencapsulator for microsphere formation (NiscoEncapsulator VAR V1 LIN-0043, NiscoEngineering AG, Zurich, Switzerland). On the Contrary, 75 mM CaCI2 solution was transferred into the curing bath. A magnetic stirrer was placed in the solution bath to inhibit the accumulation of microspheres in the solution bath. To obtain microspheres with targeted diameter, set up of the encapsulator and syringe pump was arranged as given in table 3.9.

Table 3. 9.Encapsulator parameters

Flow Rate	Diameter	Volume	Voltage	Pump	Agiator	Syringe ml
5 ml/hr	14.5	off	6.00	20	30	10

3.9 Cell Culture Experiment

Rat cardiomyocyte H9c2(2-1)-CRL-1446 were proliferated in DMEM (Dulbecco's Modified Eagle's Medium) 10% Fetal Bovine Serum (FBS) advanced , %1 Penicillin Streptomycin (PSA). Cells were thawed according to the manufacturing procedure. Cells were cultured at 37 ℃ and 5% CO2. Medium exchange were performed each 48 hours. Cells were trypsinized at 90-92% confluency and centrifuged at 300 RCF for 6.30 mins. After centrifuging, the cells mixed with 3 ml of complete medium. The Cell numbers were calculated with a hemocytometer with dying 1:1 trypan blue.

Cells (cell / mL) = Mean of cells x Dilution factor (DF) x Volume of Thoma (10^4) (eq.1)

3.10 Cell-Loaded Microsphere Fabrication in Different Formulations

Different concentrations of Cell loaded microspheres were named as follows:

- Microspheres with the concentrations of 0.1(w/v) % Gel-CMP and 0.1%(w/v) Gel-LA conjugates = MS1
- Microspheres with the concentrations of 0.1(w/v) % Gel-LA conjugate and %4(w/v) Alginate = MS2

- Microspheres with the concentrations of 0.1(w/v) % Gel-CMP conjugate and 4(w/v) % Alginate = MS3
- Microspheres with the concentrations of 0.1(w/v) % Gel and 4(w/v) % Alginate = MS4

To obtain MS1, firstly, 4(w/v) % alginate was dissolved in 0.9 % izotonic sodium chloride, saline(polifarma), 0.1(w/v)% Gel-CMP pep in double distilled water and Gel-LA conjugate in 0.1M carbonate buffer respectively. PH of the Gel-CMP pep was balanced to approximately 8.0 by adding 450µL 1M of NaOH. For 5 mL of syringe, 500 µL of Gel-CMP pep solution and 500 µL of Gel-LA solution were added to 2.5mL of alginate solution. Afterwards, 500 µL of complete medium (10% Fetal Bovine Serum advanced(FBS advanced),1% penstrep(PSA)) was added to final solution to protect cell viability during the MS fabrication,gently mixed on the magnetic stirrer at 37℃ for 1-2 hours. Additionally, 100mM of CaCI2 solution was prepared to use as curing agent and filtered by using 0.45 mm filter. 0.35 mm of nozzles were used for the MS fabrication.

All tools of the encapsulator were sterilized with autoclave. The encapsuılator device were cleaned with 70% ethanol and placed to laminar flow hood and UV-sterilized for 2 -3 hours. Final gel solution and filtered $CaCI_2$ solution and 0.9%saline were UV-sterilized in laminar flow hood and UV-sterilized for 2 -3 hours.

In parallel, The cells were passaged by trypsinization. The trypsinized cells were centrifuged at 300 rcf for 06:30 mins.Then, supernatant were removed from the cells and 2 ml of fresh complete medium was added to cell pellet. The cell number was calculated as $5.430.10^6$. After calculation, the cells were re-centrifuged and supernatant was removed from the cells. The prepared gel solution was added to the cell pellets and gently dissolved. The cell loaded gel solution was filled to 5mL of sryinge and put into the syringe pump. The incubation batch was placed to the encapsulator device.

The encapsulator device and syringe pump set ups were arranged as per table 10 and MS1 were fabricated. The device were closed after 30-35 mins. The excess $CaCI_2$ solution were removed from the MS1 and washed twice with saline. The MS1 were put into T75 flask and 15 mL of the complete medium was added to MS1 and incubated at 37℃and 5% CO_2 incubator. The diameter and stability of the MS1 were analyzed by using optical microscope. The medium of the MS were refreshed each 2 days. The same procedure was carried out for MS2, MS3 and MS4 fabrications respectively. The $5.505.10^6$ of the cells were encapsulated for MS2. The $5.201.10^6$ cells were encapsulated for MS3 and the $4.310.10^6$ cells were encapsulated for MS4. Number of the cells and cell loaded microspheres were investigated according to eq.2 and eq.3.

Table 3. 10.Encapsulator parameters for MS1, MS2, MS3 and MS4 fabrications

Flow Rate	**Diameter**	**Volume**	**Voltage**	**Pump**	**Agiator**
5 ml/hr	12.07	off	5.61	20	30

Total number of the microspheres = Number of the microspheres/mL x total volume of the crosslinking agent which was used. (eq.2)

Cell in the MS/ mL = Number of the encapsulated cells / Number of microspheres in total solution (eq.3)

3.11 Cytotoxicity Assay

Rat cardiomyocyte H9c2(2-1)-CRL-1446 were proliferated in DMEM (Dulbecco's Modified Eagle's Medium) 10% Fetal Bovine Serum (FBS) advanced , %1 Penicillin Streptomycin (PSA). Cells were thawed according to the manufacturing procedure. Cells were cultured at 37 ℃ and 5% CO2. Medium exchange were performed each 48 hours. Cells were trypsinized at 90-92% confluency and centrifuged at 300 RCF for 6.30 mins. After centrifuging, the cells mixed with 3 ml of complete medium. The Cell numbers were calculated with a hemocytometer with dying 1:1 trypan blue.

Cells (cell / mL) = Mean of cells x Dilution factor (DF) x Volume of Thoma (10^4) (eq.4)

After counting the cells, $8 \text{ X } 10^4$ cells were placed into the 8 well of the 96 well plate. In parallel, cell loaded Gel-LA conjugate integrated MS gel-pep&gel-LA conjugates integrated MS were placed to 16 well of the 96 well plate and 150 µL of fresh medium was added to 8 well and incubated for 24 hours at 37 ℃ and 5% CO2 humidified incubator. After 24 hours 10 µL of the CCK8 solution was added to each well of the plate. The well plate was incubated for 3 hours in the incubator. After 3 hours, the well plate was covered with aluminium foil to protect from the light and shaked in the orbital shaker at 100 °C for 1 minute. Thereafter, the well plate was placed to microplate reader and measured the cytotoxicity and cell viability at 450 nm. The same protocol was carried out for day 4 ,8 and 12.

0.1(w/v) % MS1, MS2, MS3 and MS4 were fabricated according to above mentioned fabrication step. The obtained MSs were incubated in T75 flasks with in complete medium at 37 ℃ and 5% CO2 humidified incubator on 12 days period. After counting the cells, 21 x10 cells/well in 100 µL were placed in 4 flat-bottom 96-well plates for 4 different MSs and incubated in 5% CO2 at 37°C and for 24 hours to attach the cells. Afterwards, the medium on the cells was discarded. 200 µL Mediums of the

MSs were added to the cell seeded well plates by preparing serial dilution in DMEM with a total volume of 100 µL and incubated in 5% CO2 at 37°C for 24 hours. After 24 hours , 10 µL of the CCK8 kit was added to each well of flat-bottom 96-well plates. The well plates were incubated for 2 hours in the humidified incubator at 5% CO2 at 37°C. After 2 hours, the well plates were covered with aluminium foil to protect from the light and shaked in the orbital shaker at 100 °C for 1 minute. Thereafter, the well plates were placed to microplate reader and measured the cytotoxicity and cell viability at 450 nm on day 4. The same protocol was carried out for days 8 and 12.

3.12 Live/Dead Assay

The Cell viability in the microspheres was carried out with Live/dead assay kit solution (ab115347, Abcam). The cell loaded MS1, MS2, MS3 and MS4 were placed to 4 wells of the flat bottom 24 well plate on day 6.The waste medium was removed from the microspheres which are in the wells. 2 mL of 1X DPBS was added to 15 mL falcon tube. Then, 4 µL of the live/dead assay kit solution was added to the DPBS and gently mixed. Afterwards, 500 mL of the DPBS&live/dead mixture was added to micropsheres which are in the 24 well plates and incubated for 10 mins in the dark. After 10 mins, the cell viability was visualized with Florescence microscope (EVOS™ M5000 Cell Imaging System, ThermoFisher, Bothell, WA, USA) The same protocol was carried out on day 12.

3.13 Reactive Oxygen Species (ROS) Assay

To evaluate reactive oxygen species of the MS1 and MS2, DCFDA / H2DCFDA Cellular ROS Assay Kit was performed by following ROS protocol recommended by the manufacturer (ab113851, abcam). Firstly, 25.000 cells/well (H9C2 rat cardiomoycte) were seeded in a flat bottom 96 well plate and incubated for 24 h at 37 ℃, 5% CO_2 humidified incubator . After 24 h incubation, 100 ng/mL LPS was applied to each well and incubated for 3 h at at 37 ℃, 5% CO_2 humidified incubator. After LPS

treatment,the medium was discarded from each well. Thereafter, 100 µL of the DCFDA was added to the LPS treated wells and incubated for 45 mins at 37 ℃, 5% CO_2 humidified incubator. After 45 mins incubation, the wells were washed with 1X buffer and then, the mediums of MS1 and MS2 were carried out to the wells with different concentrations for 4 hours.Finally, the datas were obtained by varioscan plate reader at 485/535 nm.

4 RESULTS

4.1 MS1 Microsphere Fabrication

0,1 (w/v) % MS1 microspheres were fabricated and cultured in 100mM $CaCI_2$ for 15 days incubation. 35.000 microspheres in 50 ml $CaCI_2$ were obtanined in approximately 30 minutes. Diameter changed between 150 μm-260 μm were observed with optical microscope. Diameter results were graphed using Microsoft excel.The diameter of the microspheres is increased around from 150 to 260 μm day by day among the groups of the experiment (Figure 4.1).

The number of microspheres was counted at day 0 and day 15.250 microspheres were counted in 1 ml of the microspheres at day 0 and day 15.

The cell loaded MS1 were successfully encapsulated and obtained by applying the procedure given in the methods section.

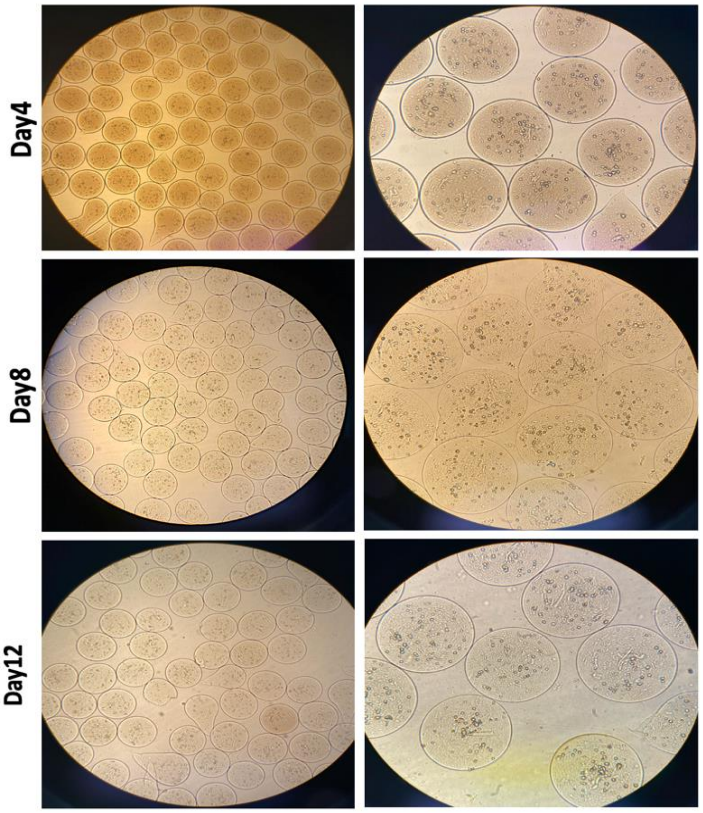

Figure 4. 1.Cell loaded MS1 on days 4,12 and 8 under optical microscope (10x, scale bar: 300 μm)

4.2 MS2 Microsphere Fabrication

0.1 (w/v) % (Gel-LA)-incorporated microspheres were fabricated and incubated in 100mM $CaCI_2$ for the 15 days period. 30.000 microspheres were obtanined in approximately 30 minutes. Diameter changed between 150 μm-250 μm were visualized with microscope. Diameter results were graphed using graphpad. The diameter of the microspheres is increased around from 150 to 250 μm day by day among the groups of the experiment (Figure 4.2A and 4.2B).

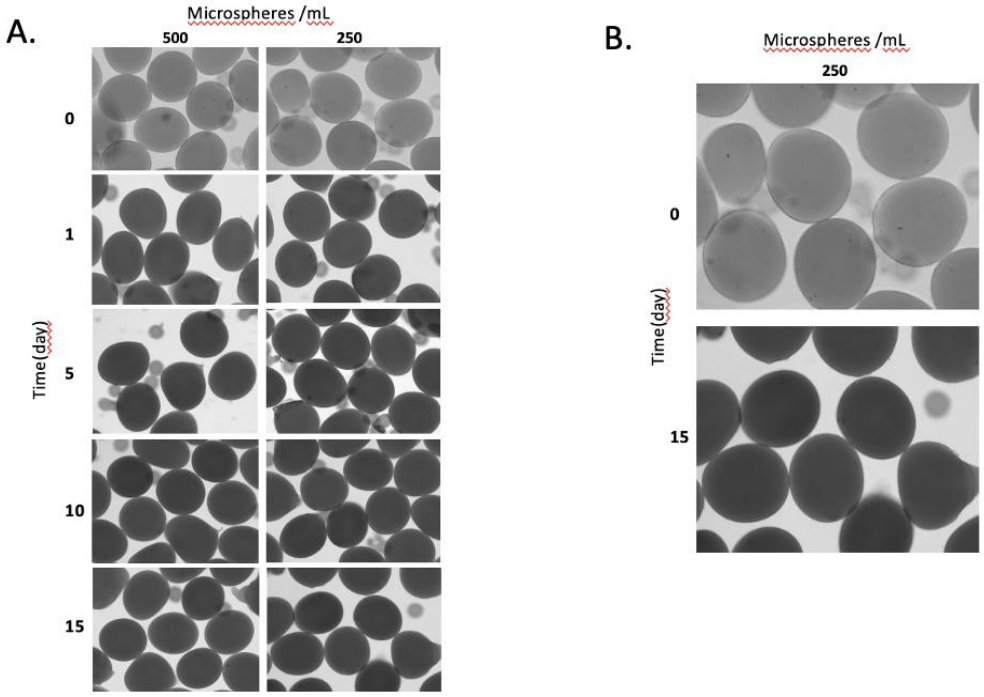

Figure 4. 2. Diameter changes of the 0.1(w/v) % Gel-LA conjugate integrated MS in CaCI_2 for 15 days period under optical microscope (10x, scale bar: 200 μm)

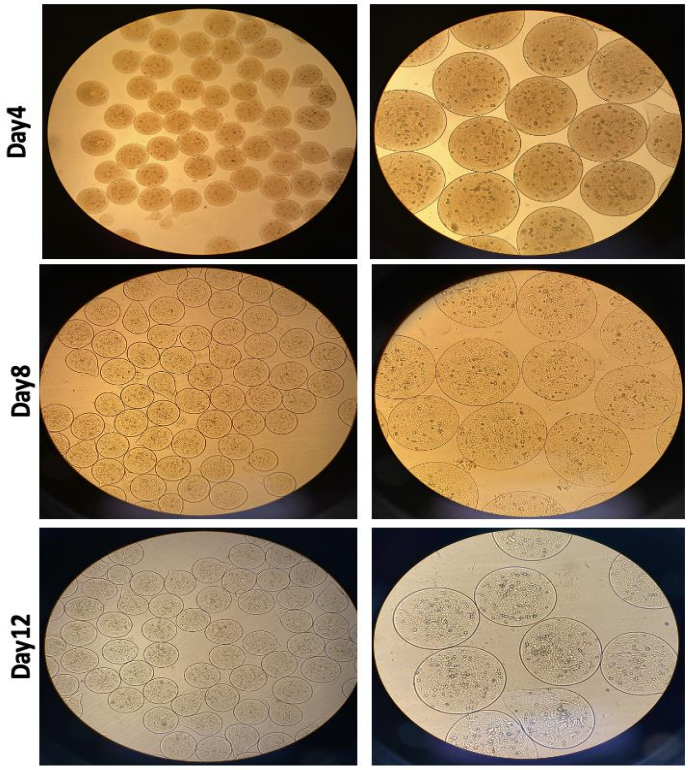

Figure 4. 3.Cell loaded MS2 on days 4,12 and 8 under optical microscope (10x, scale bar: 300 μm) MS3 Microsphere Fabrication

0,1(w/v) % Gel- CMP microsphere was fabricated and cultured in 100mM $CaCI_2$ for 15 days incubation. 30.000 microspheres in 50 ml $CaCI_2$ were obtanined in approximately 30 minutes. Diameter changed between 150 μm-260 μm were observed with optical microscope. Diameter results were graphed using Microsoft excel.The diameter of the microspheres is increased around from 150 to 260 μm day by day among the groups of the experiment (Figure 4.3)

The number of microspheres was counted at day 0 and day 15. 300 microspheres were counted in 1 ml of the microspheres at day 0 and day 15

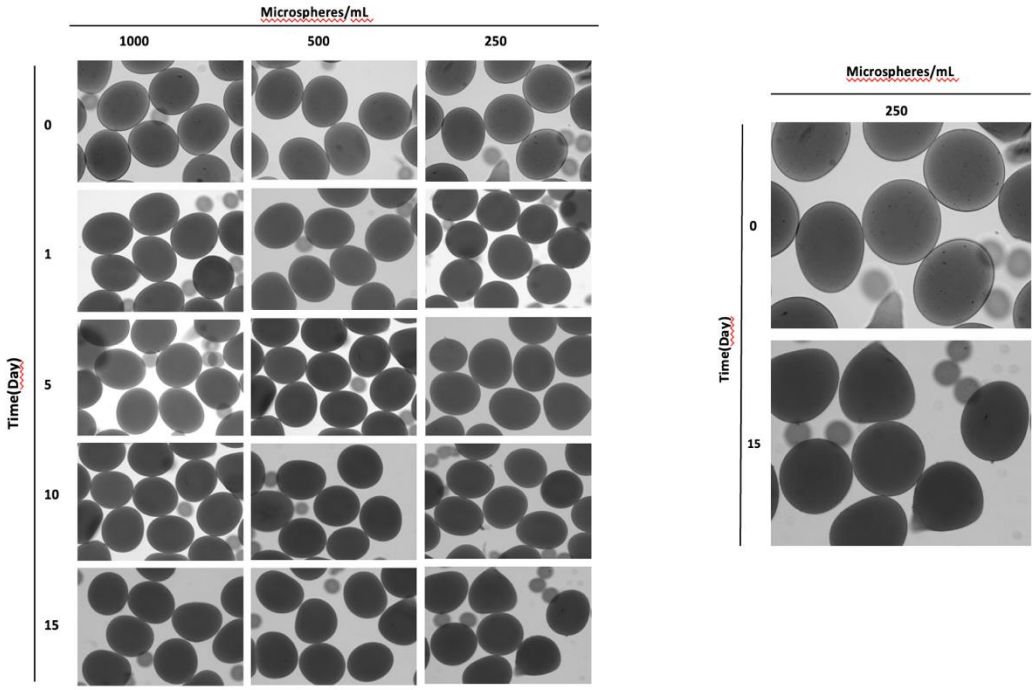

Figure 4. 4.Diameter changes of the 0.1(w/v) % Gel-CMP peptide conjugate incorporated MS during the incubation of 15 days period under optical microscope (10x, scale bar: 200 μm)

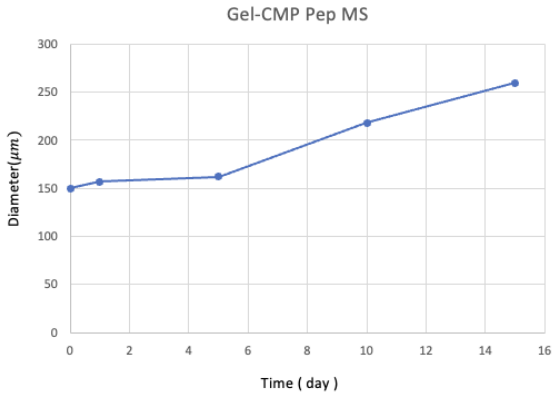

Figure 4. 5.Diameter changes of the 0.05(w/v) % MS3 in the CaCI2 for 15 days

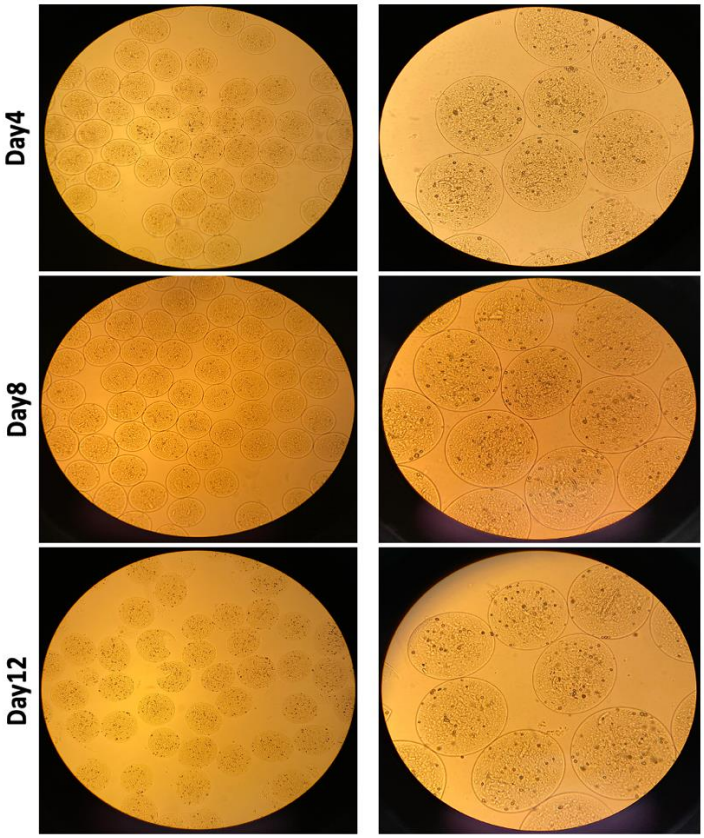

Figure 4. 6.Cell loaded MS3 on days 4,12 and 8 under optical microscope (10x, scale bar: 300 μm)

4.3 MS4 Microsphere Fabrication

Gelatin-Alginate microspheres were fabricated a in the concentrations of 0.1% by weight and 0.05% w/v obtained microspheres were cultured in 75mM $CaCI_2$ during 15 days incubation.

In this stage, 40.000 microspheres were obtanined in 30 minutes. Diameter changed between 180 μm-230 μm were recorded with microscope. Diameter results were graphed using graphpad. The diameter of the microsphered is increased around from 180 to 230 μm day by day among the groups of the experiment (Figure 4.7)

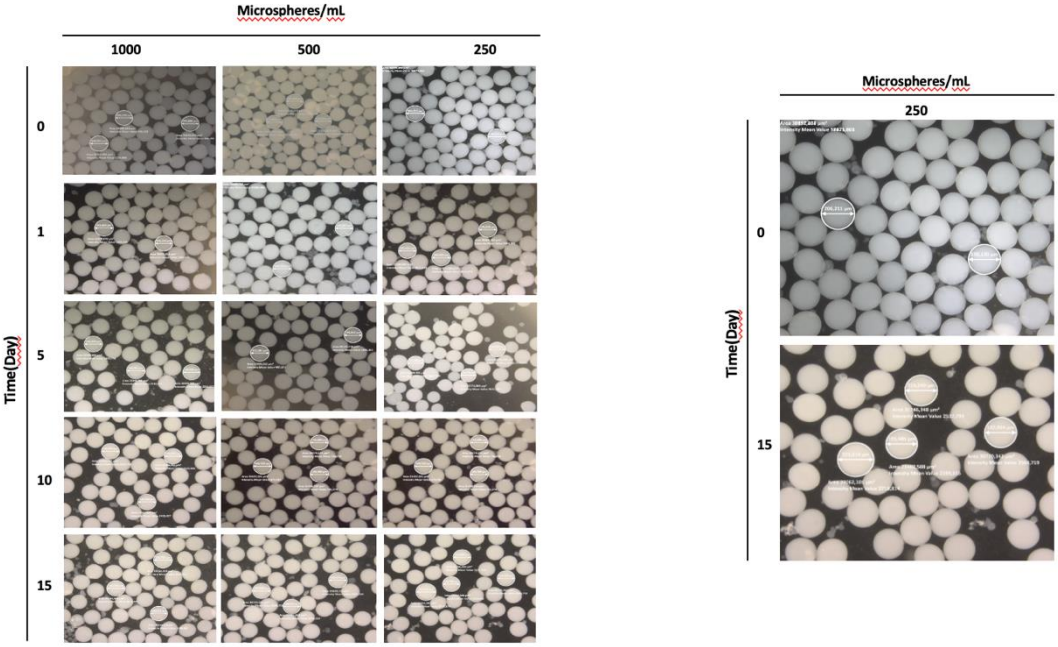

Figure 4. 7.Diameter changes of the 0.05(w/v) % GA MS during the incubation of 15 days period under optical microscope (5x, scale bar: 200 µm)

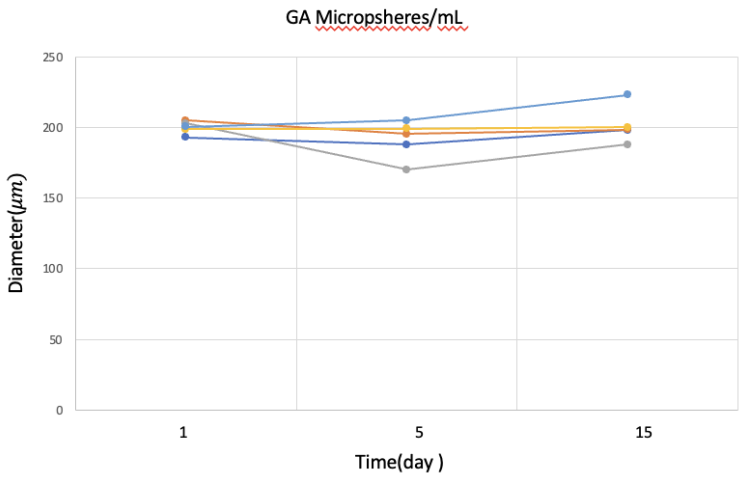

Figure 4. 8.Diameter changes of the 0.05(w/v) % GA MS in the CaCI_2 for 15 days

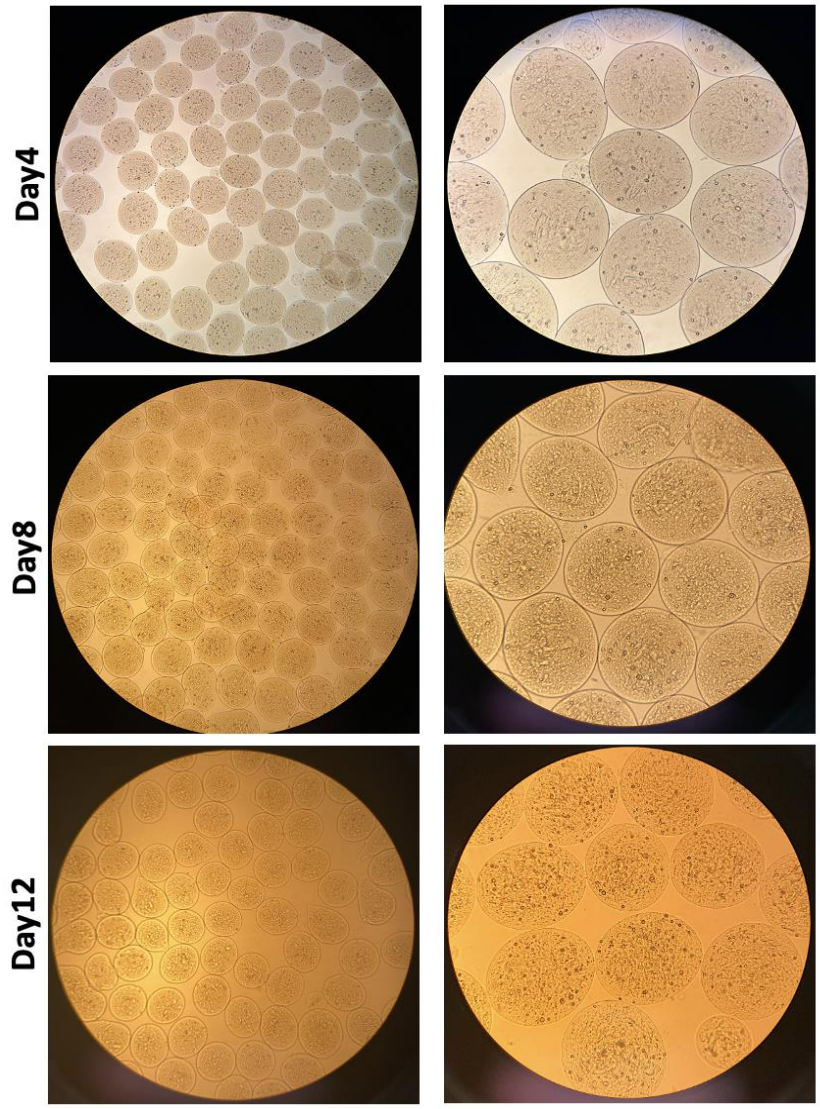

Figure 4. 9.Cell loaded MS4 on days 4,12 and 8 under optical microscope (10x, scale bar: 300 μm)

4.4 Characterization of the Microspheres

4.4.1 Rheological analyzes of The Microspheres

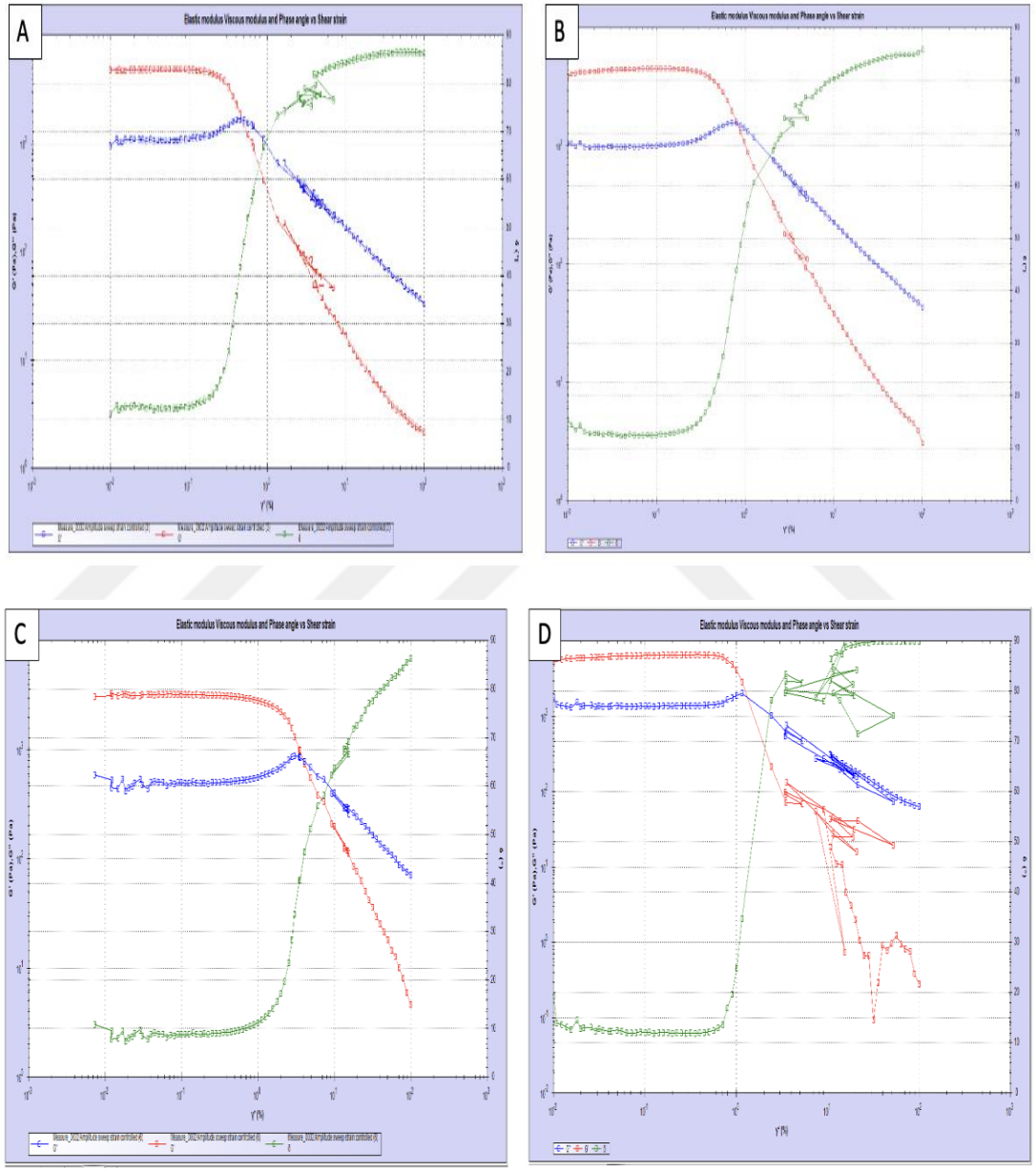

Figure 4. 10.Rheological analyzes GA Microspheres in different concentrations of the gelatin and conjugates; a) 0.05(w/v) gelatin %, b) 0.1(w/v) % gelatin, c) 0.1(w/v) % MS3 and d) 0.1(w/v) %MS2

To obtain mechanical properties , different concentrations of the GA microspheres which demonstrated in figure4.10A 4.10B ,4.10C and 4.10D were measured by using Malvern Kinexus ultra^{+} Rheometer instrument.The mandatory arrangements were applied at the device. The mechanical prpperties were performed at 25℃, with a geometry of 10 mm ,oscillatory stress sweep f=1.000 Hz and γ=0.01 by following the procedure.Figure A and B represents the comparison of mechanical characteristics of the microspheres between different concentrations of gel solutions..The incorporation of different concentrations of gelatin solution was changed both hardness and flexibility of the polymeric microspheres. Likewise, the effect of gel-CMP and gel-LA conjugates combined with alginate solution might be analyzed for stiffness and biodegradation evaluations in figure 4.10D and 4.10 C.

4.4.2 Morphological Analysis

The expected porous structure, pore size and elemental analyzes of the polymeric microspheres were visualized in figure 4.11 after being exposed to high energy electrons in a high vacuum chamber of Scanning Electron Microscopy (SEM) device. In addition, The figure 4.11,4.12 4.13 and 4.14 SEM images represents morphological difference of the GA microspheres in different concentrations.

On the contrary, figure 4.12A and 4.12B provides data of the Alginate microspheres incorporated with Gel-LA and gel-CMP conjugates. Likewise, SEM images can also be compared with respect to the concentrations of Gel-CMP and Gel-LA conjugates within the polymeric microspheres. EDS detector allowed to investigate about the weight percentages and elemental analysis of the fabricated microspheres. Figure 4.13 and figure 4.14 indicated the scanning outcomes of the specified fields for the Gel-CMP and Gel-LA conjugated microspheres respectively. The percentages of atomic and weight of the elements were obtained by EDS-SEM semi-quantitative analysis explained in table 4.1 and 4.2

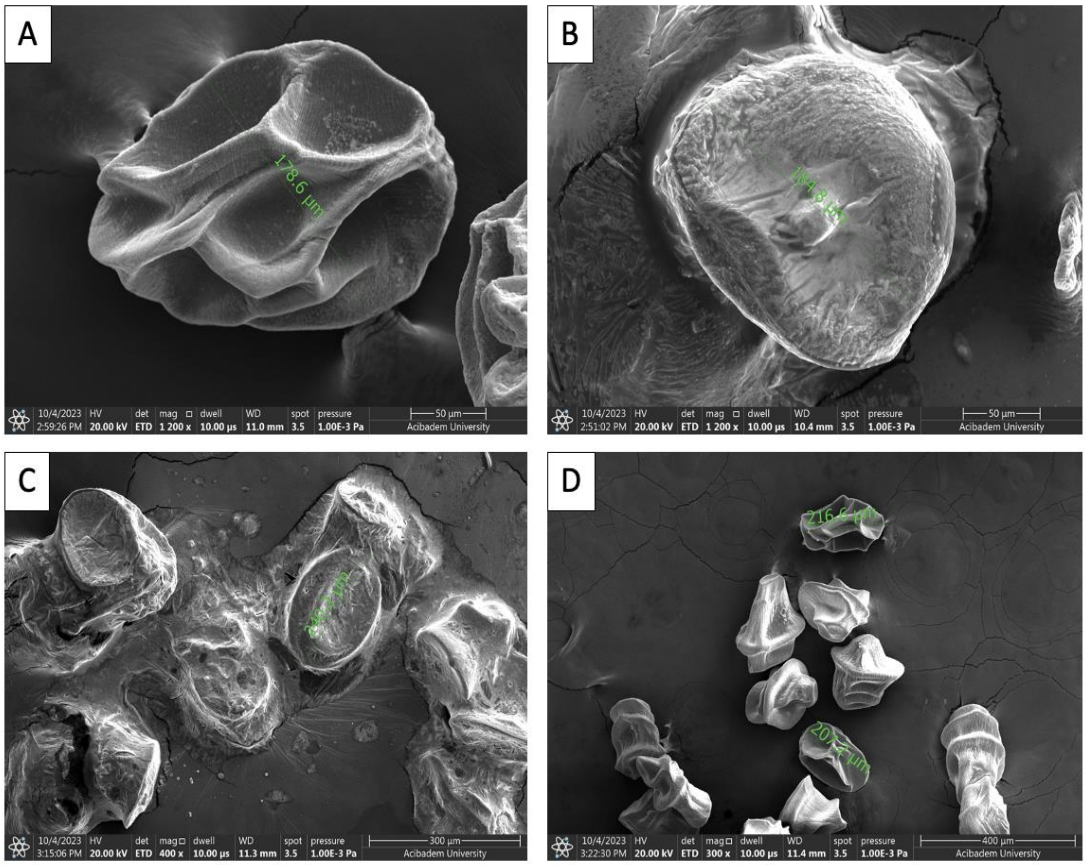

Figure 4. 11.SEM images of the GA MS in different concentrations of the gelatin; a) 0.05(w/v)% , b) 0.1(w/v)% , c) 10(v/v)% , d) 5(v/v)%

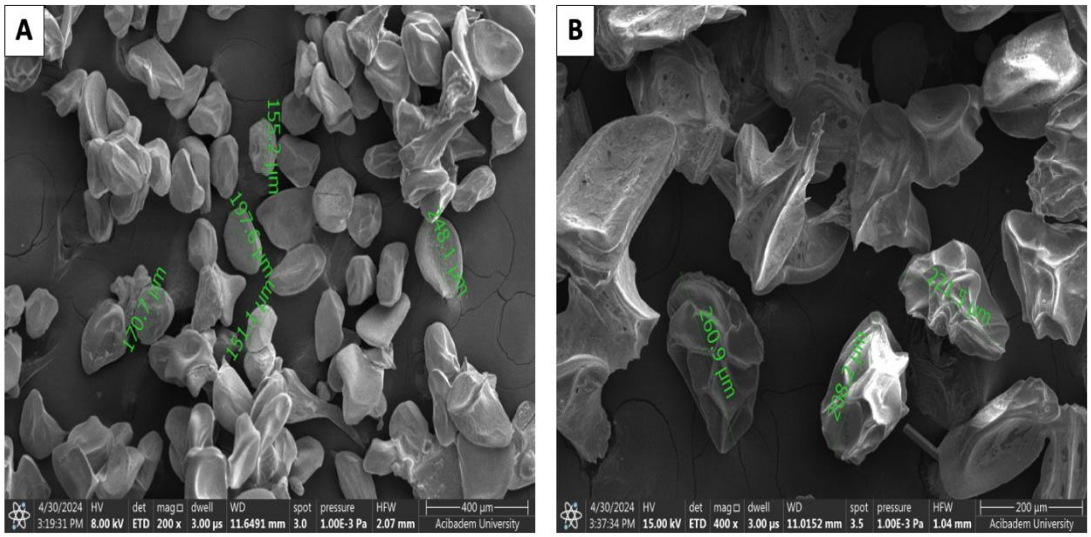

Figure 4. 12.SEM images of the alginate microspheres including a) 0.1(w/v) %Gel-LA and b)0.1(w/v) % Gel-CMP conjugates

Table4. 1.Atomic and weight percentages of the elements in the 0.1(w/v) %Gel-LA conjugate integrated alginate microspheres

Element	Weight%	Atomic %
C	15.9	26.3
N	7.9	11.3
O	29.6	36.8
Na	1.6	1.3
S	5.6	3.5
CI	20.1	11.3

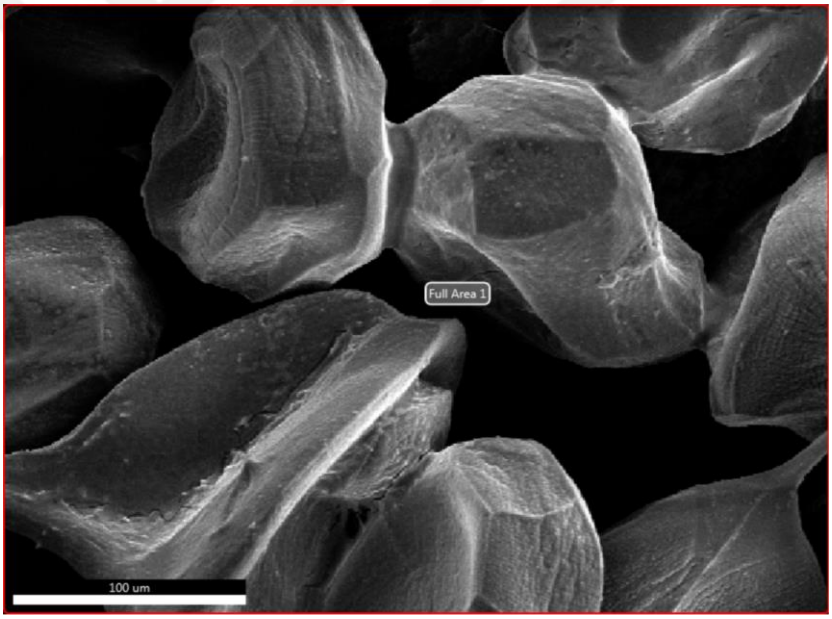

Figure 4. 13.EDS-SEM data: Scanning image of the Gel-LA incorporated microsphere

Table4. 2.Atomic and weight percentages of the elements in the alginate microspheres incorporated with 0.1(w/v) %Gel-CMP conjugate

Element	Weight %	Atomic%
C	23.9	38.1
N	6.7	9.2
O	24.4	29.2
Na	1.7	1.4
CI	24.1	13

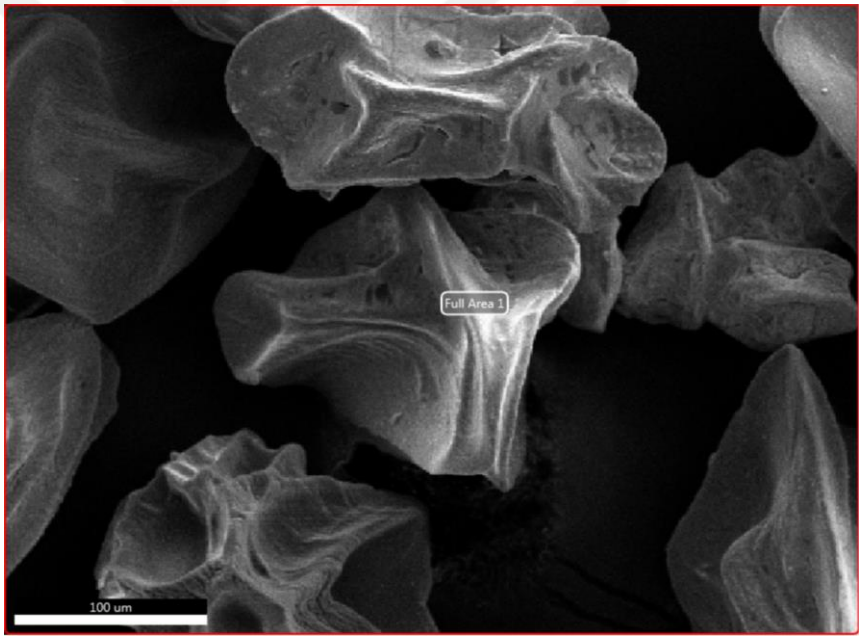

Figure 4. 14.EDS-SEM data: Scanning image of the Gel-CMP incorporated microsphere

4.4.3 Functional Group Analysis (FT-IR)

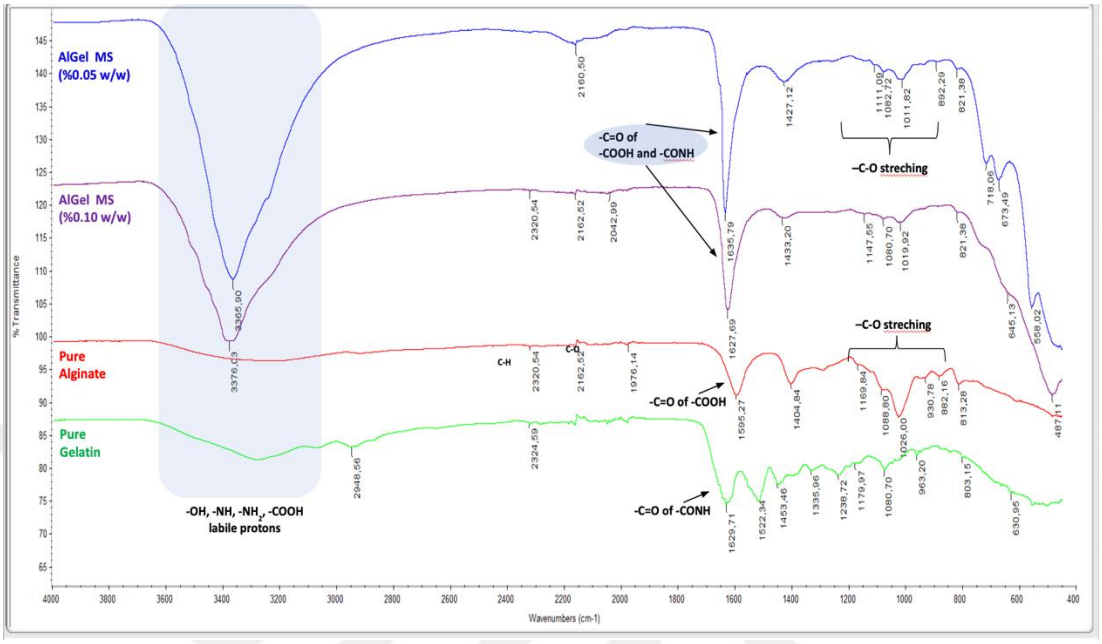

Figure 4. 15.FT-IR analyzes of GA MS in different concentrations

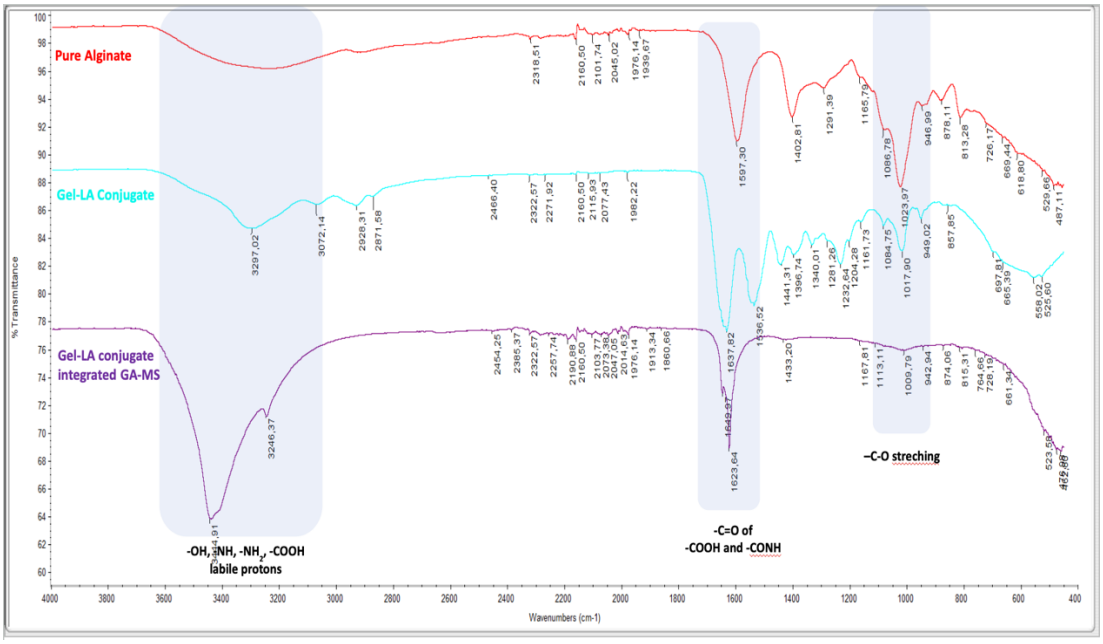

Figure 4. 16.FT-IR spectras of Pure Alginate, Gel-LA conjugate, and Gel-LA conjugate integrated MS

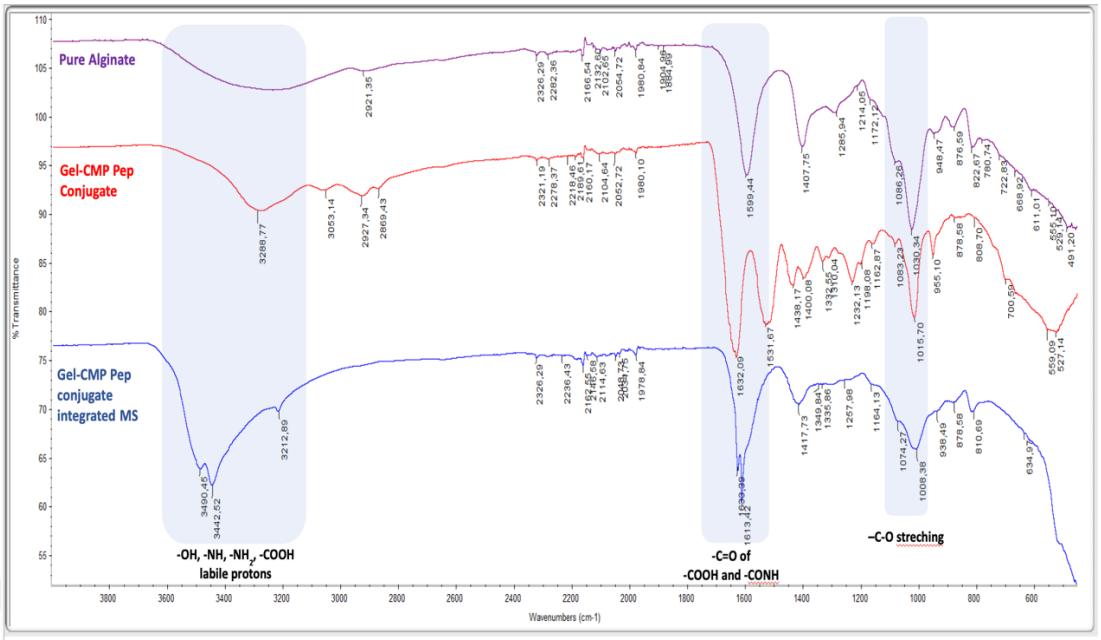

Figure 4. 17.FT-IR spectras of Pure Alginate, Gel-CMP Peptide Conjugate, and Gel-CMP Pep conjugate integrated MS

4.5 Characterization And Analysis of CMP Peptide and Conjugates

4.5.1 HPLC Analysis

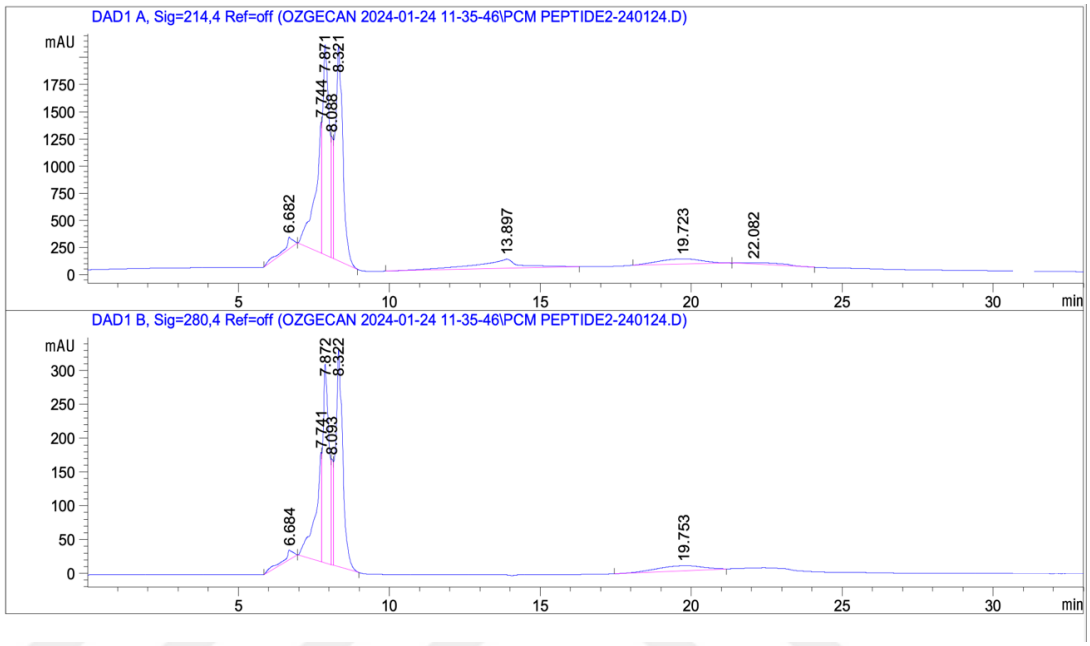

Figure 4. 18.HPLC chromatograms of CMP peptide

4.5.2 FT-IR Analysis

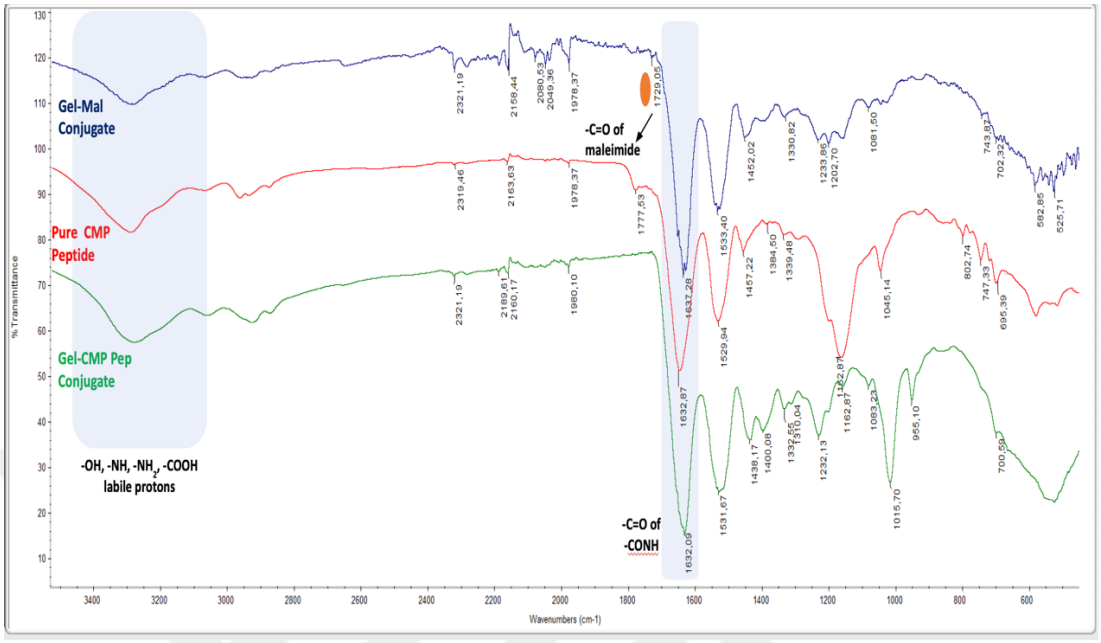

Figure 4. 19.FT-IR analyzes of Gel-Mal Conjugate,Pure CMP peptide and Gel-CMP-Pep conjugate

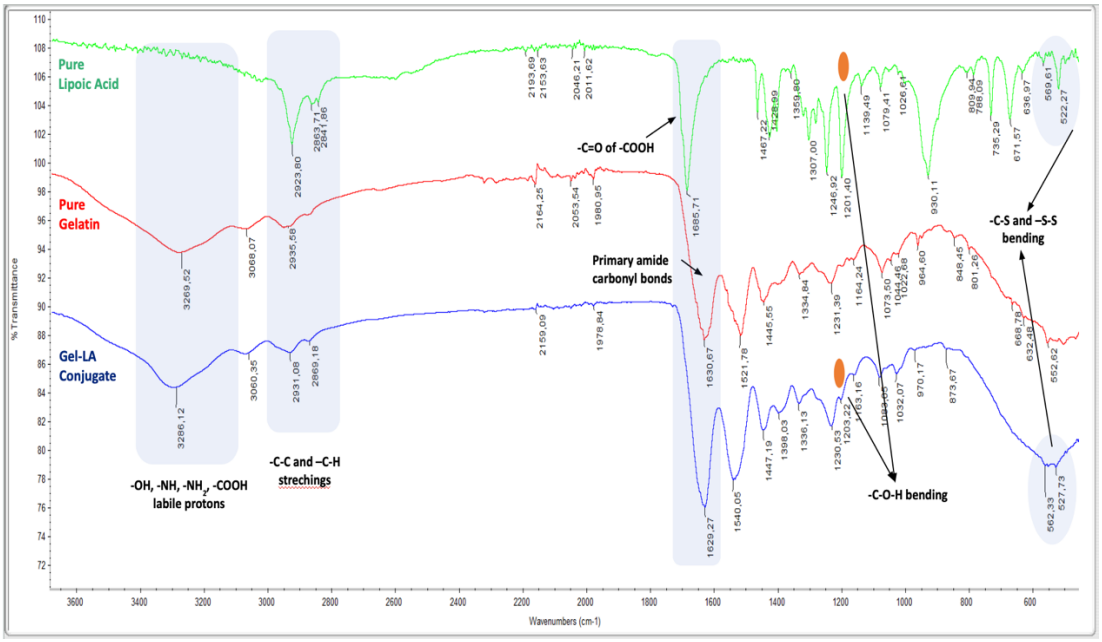

Figure 4. 20.FT-IR analyzes of Pure Lipoic Acid,Pure Gelatin and Gel-LA conjugate

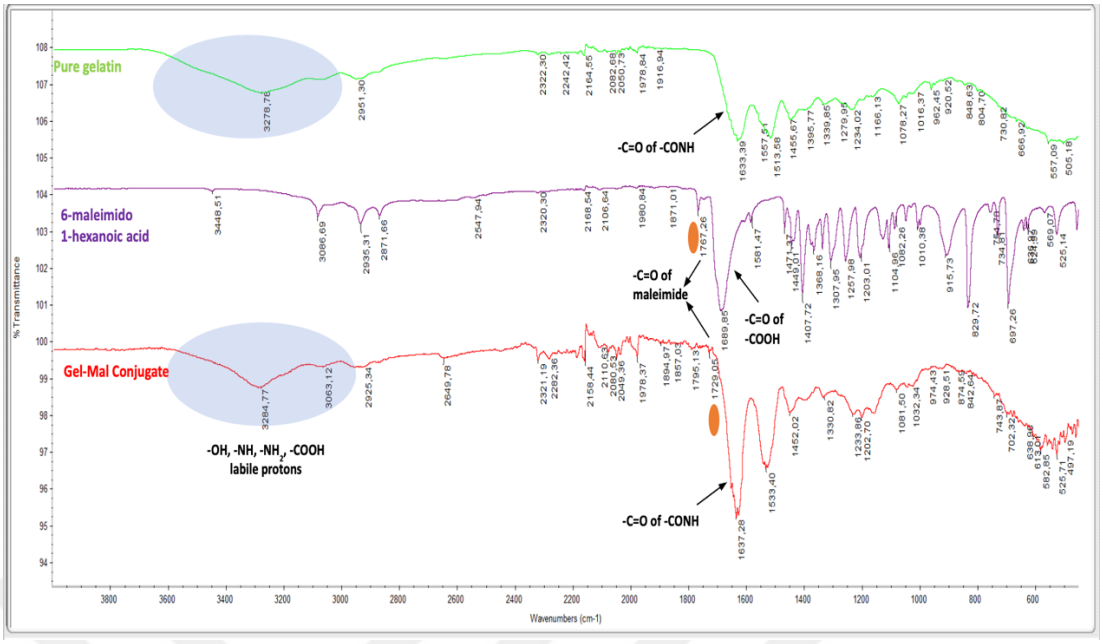

Figure 4. 21.FT-IR spectras of Pure Gelatin ,6-maleimido 1-hexanoic acid and Gel-Mal conjugate

FT-IR measurements were carried out by Thermo Scientific Nicolet iS10 device.The Wavelenghts of the measurements were performed by Omnic software. These analyses were performed to observe functional groups of the conjugates, peptides and polymers.Figure 4.15,16,17,19,20,21 demonstrate the chemical groups of the microspheres and conjugates. The chemical variety and different peaks of the Gel-Mal conjugate, Gel-LA conjugate,Gel-CMP pep conjugate and GA MSs indicated that The reactions were succesfully performed. Each conjugates and microspheres were analyzed in dry form which were lypholized to remove the liquids from the samples.

4.5.3 ^{1}H NMR of Gel-LA and Gel-CMP Peptide Conjugates

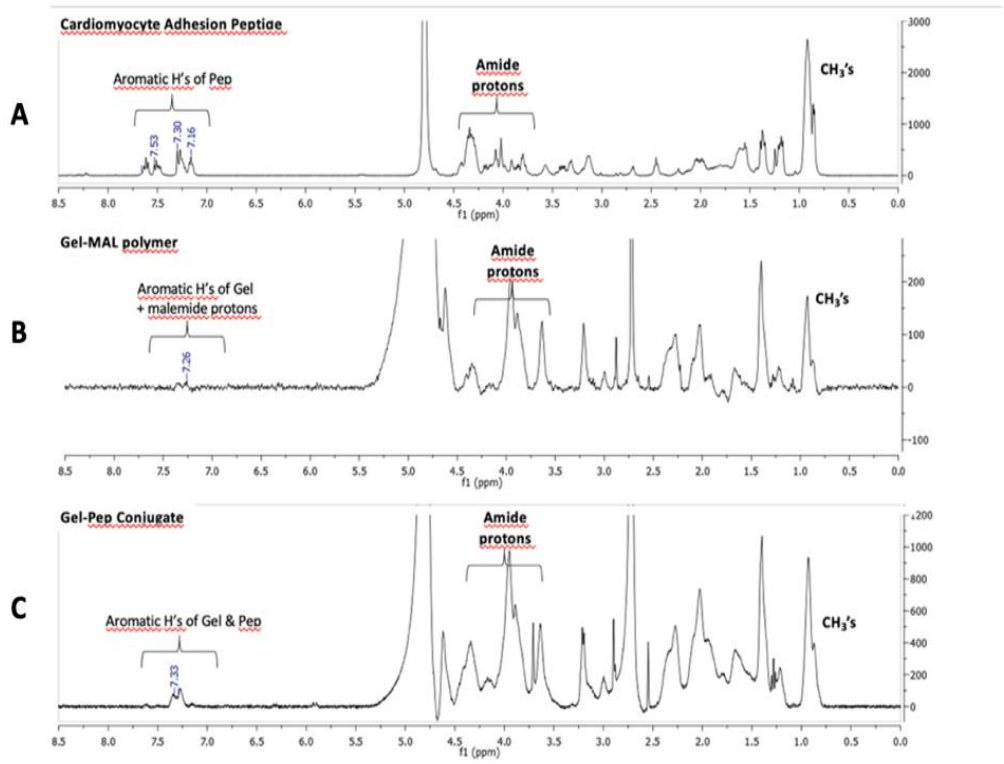

Figure 4. 22.^{1}H NMR analyzes of CMP peptide, Gel-MAL polymer and Gel-Pep conjugate

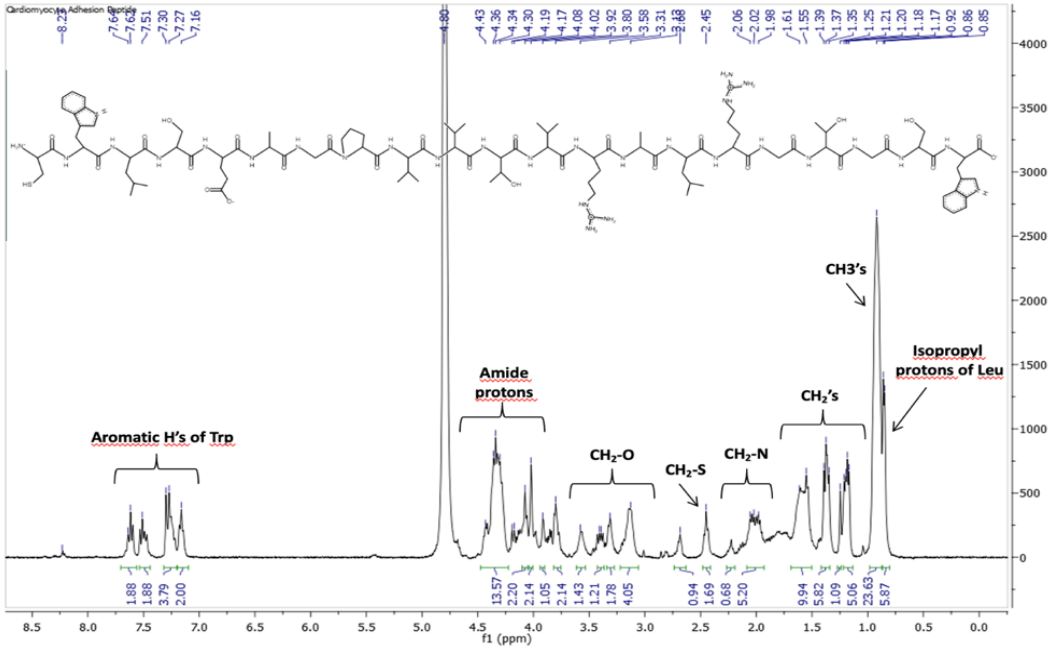

Figure 4. 23.[1]H NMR analysis of CMP peptide

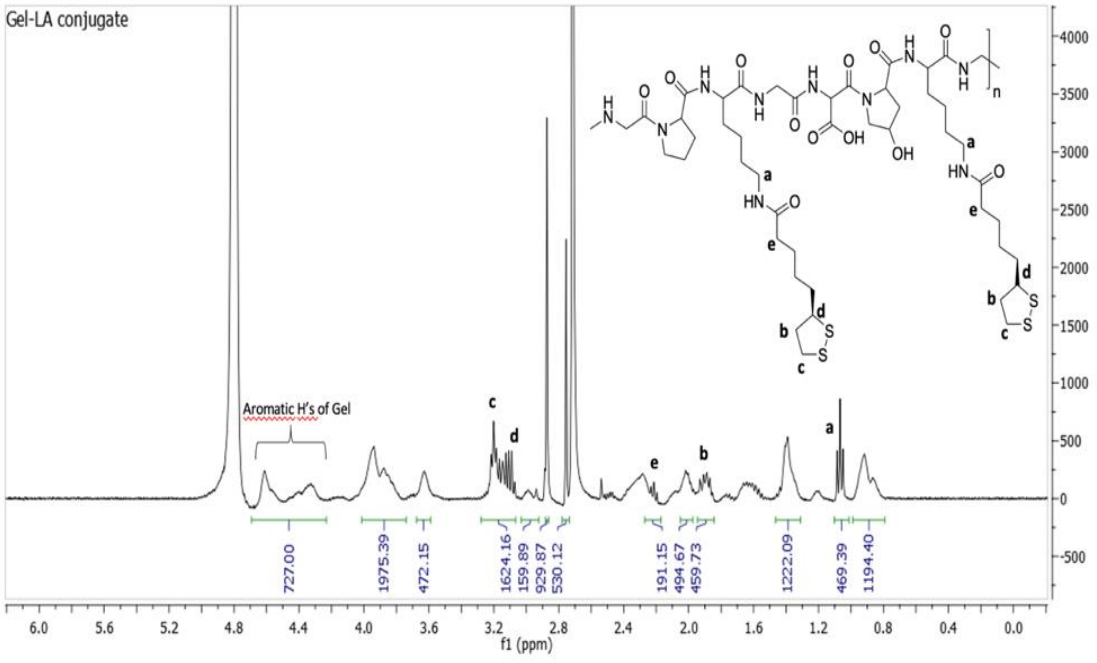

Figure 4. 24.[1]H NMR analysis of Gel-LA conjugate(71,72)

Proton NMR spectroscopy (Fig 4.22, fig.4.23 and fig.4.24) performed in d_6-DMSO, was carried out invetigate the chemical structure of the peptide and conjugates, indicating the existence of peptide via its specific protons, as well as other protons in LA and the CMP peptide (74,75).

4.5.4 LC/MSMS

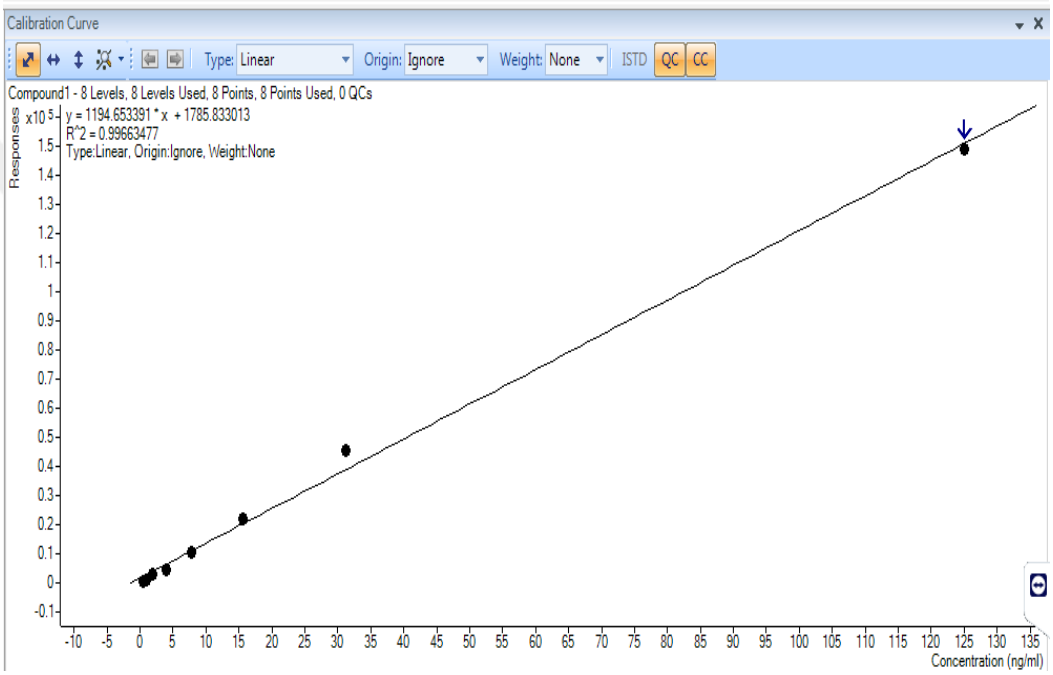

Figure 4. 25.Calibration curve of Gel-LA MS

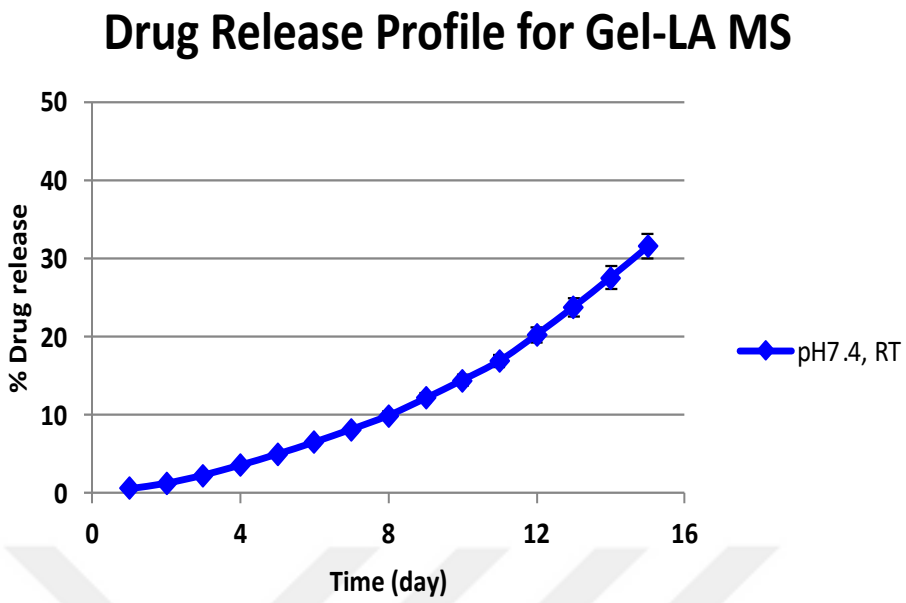

Figure 4. 26.Drug release of MS2 during the 15 days period.

4.6 Cytotoxicity Assay of the Cell loaded MSs

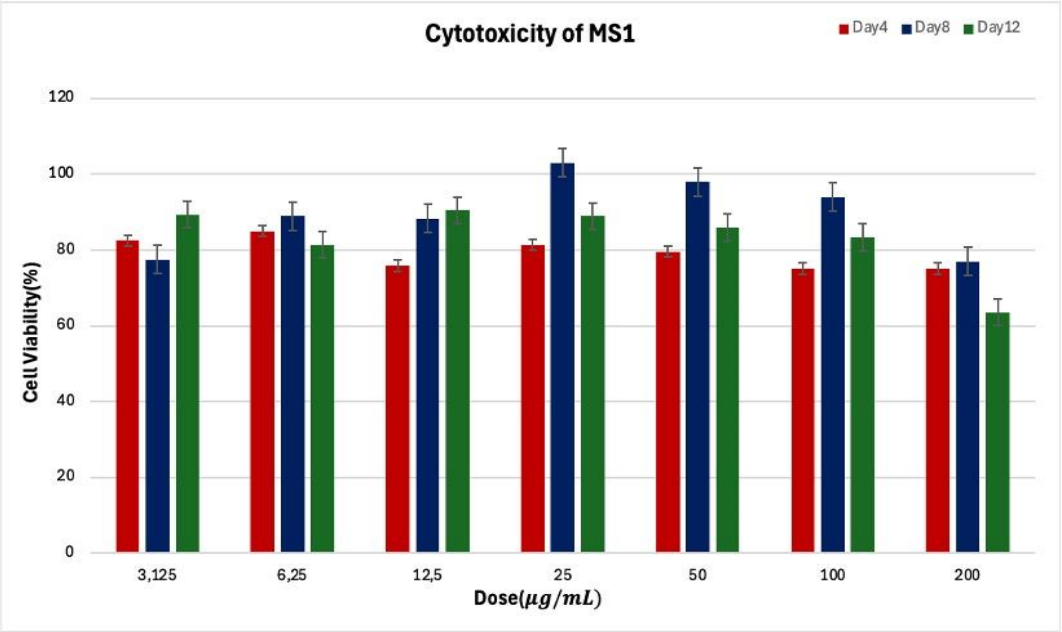

Figure 4. 27.Dose dependent cytotoxicity of the MS1on period of day4, day8 and day12

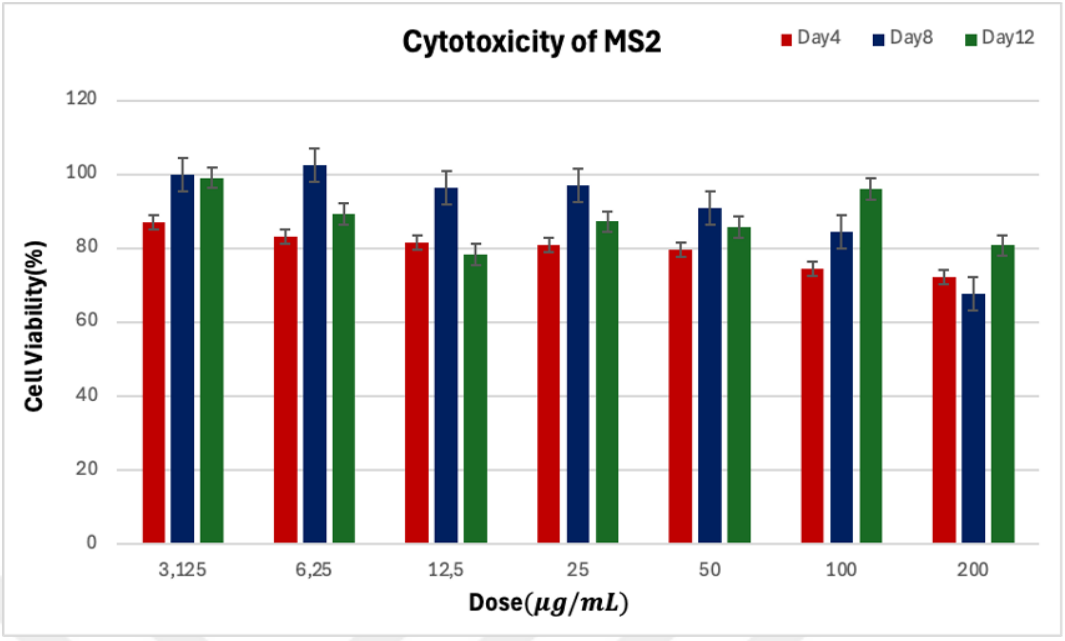

Figure 4. 28.Dose dependent cytotoxicity of the MS1on period of day4, day8 and day12

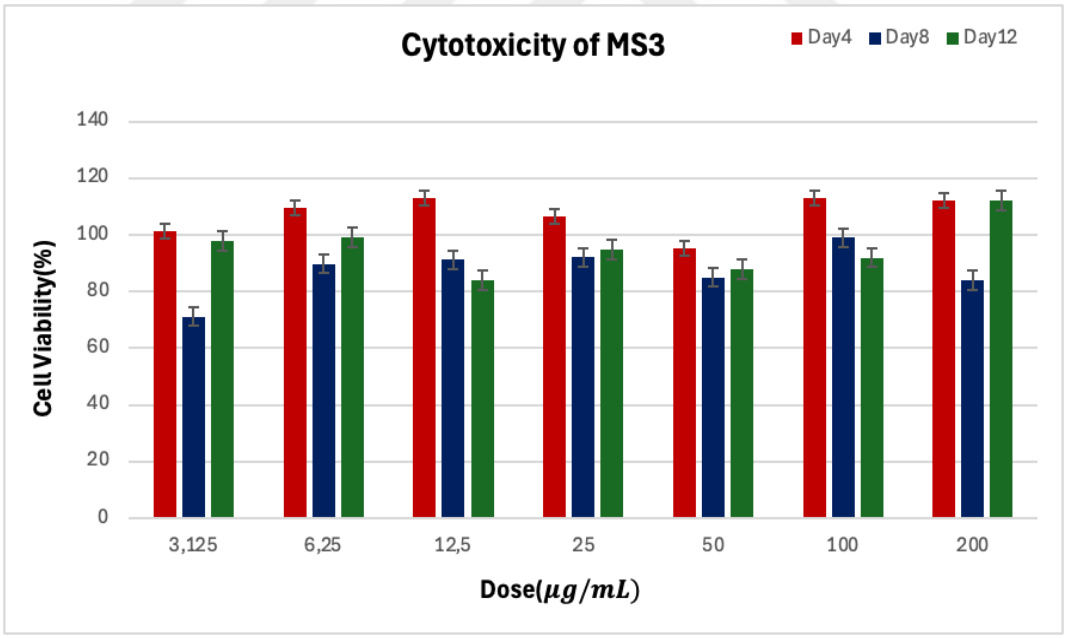

Figure 4. 29.Dose dependent cytotoxicity of the MS1on period of day4, day8 and day12

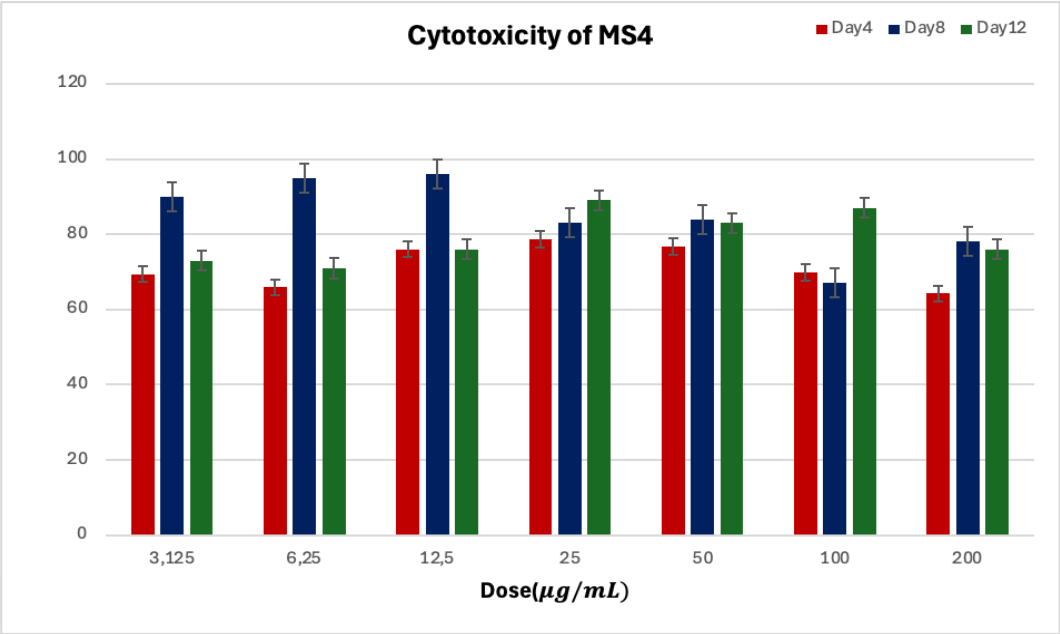

Figure 4. 30.Dose dependent cytotoxicity of the MS4 on period of day4, day8 and day12

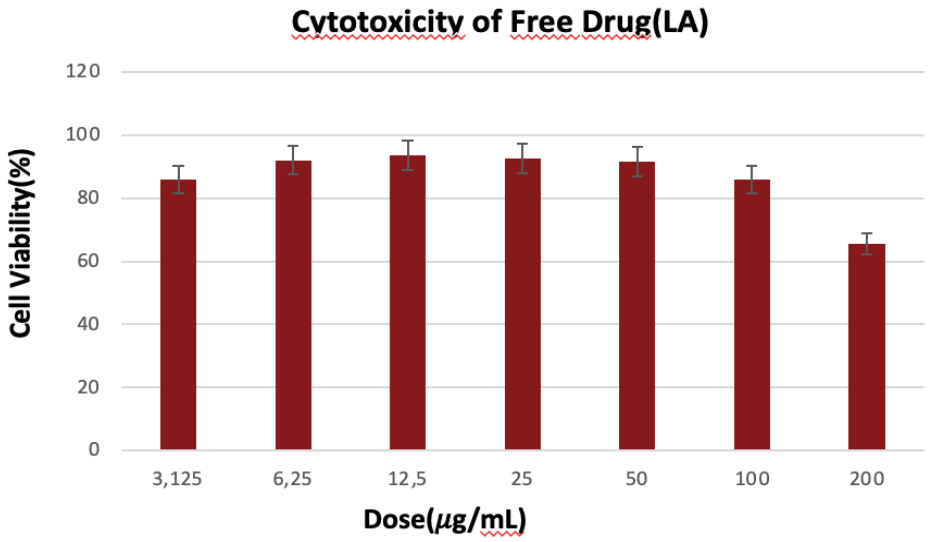

Figure 4. 31.Dose dependent cytotoxicity of pure lipoic acid

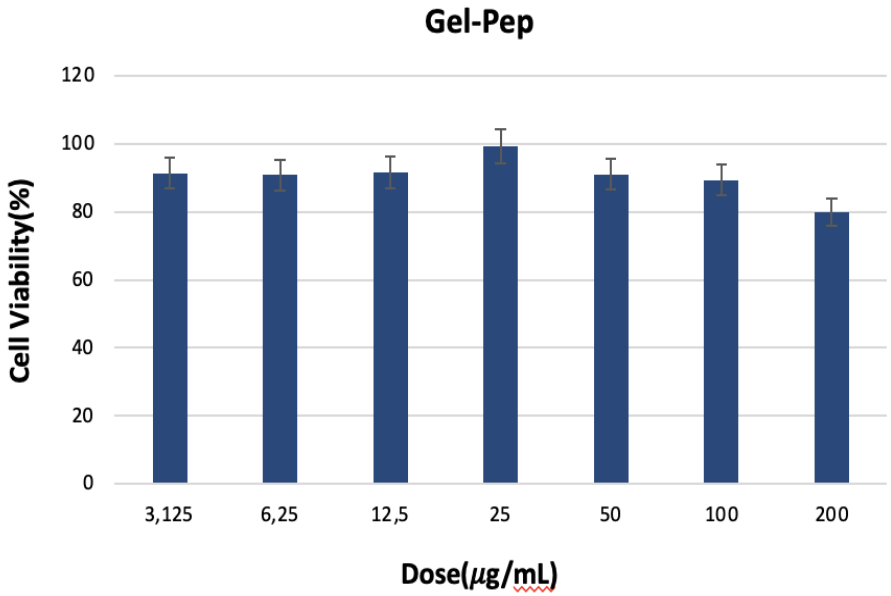

Figure 4. 32.Dose dependent cytotoxicity of Gel-Pep conjugate

4.7 Live/Dead Assay

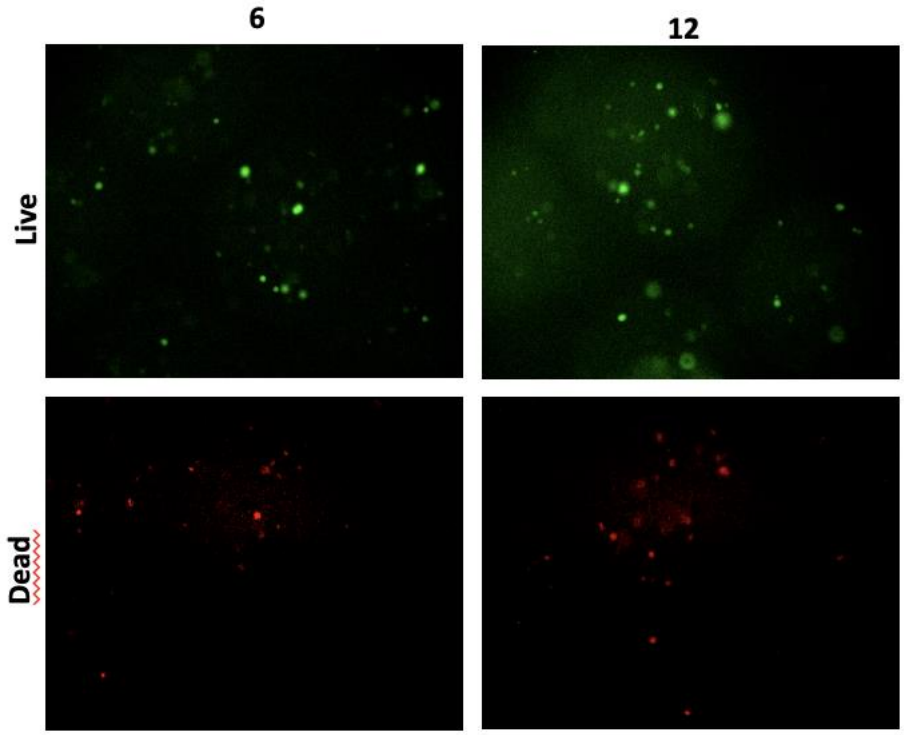

Figure 4. 33.Fluorescence images of live/dead staining for H9C2 rat cardiomyocytes in the MS1 on days 6 and 12 (scale bar;300 mμ,10X)

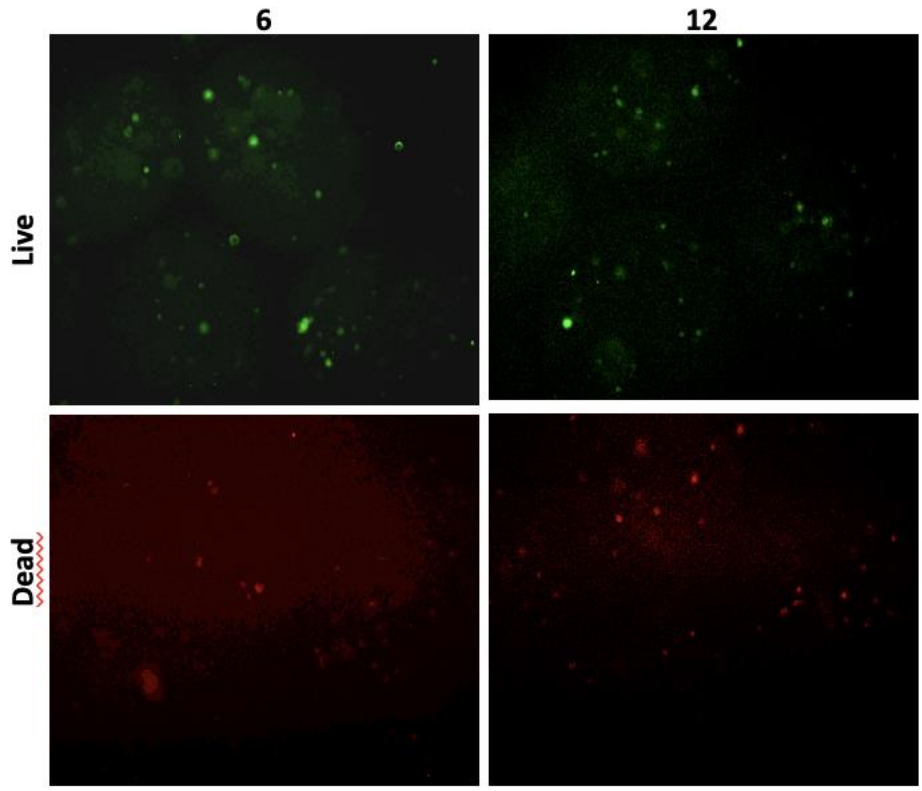

Figure 4. 34.Fluorescence images of live/dead staining for H9C2 rat cardiomyocytes in the MS2 on days 6 and 12 (scale bar;300 mμ,10X)

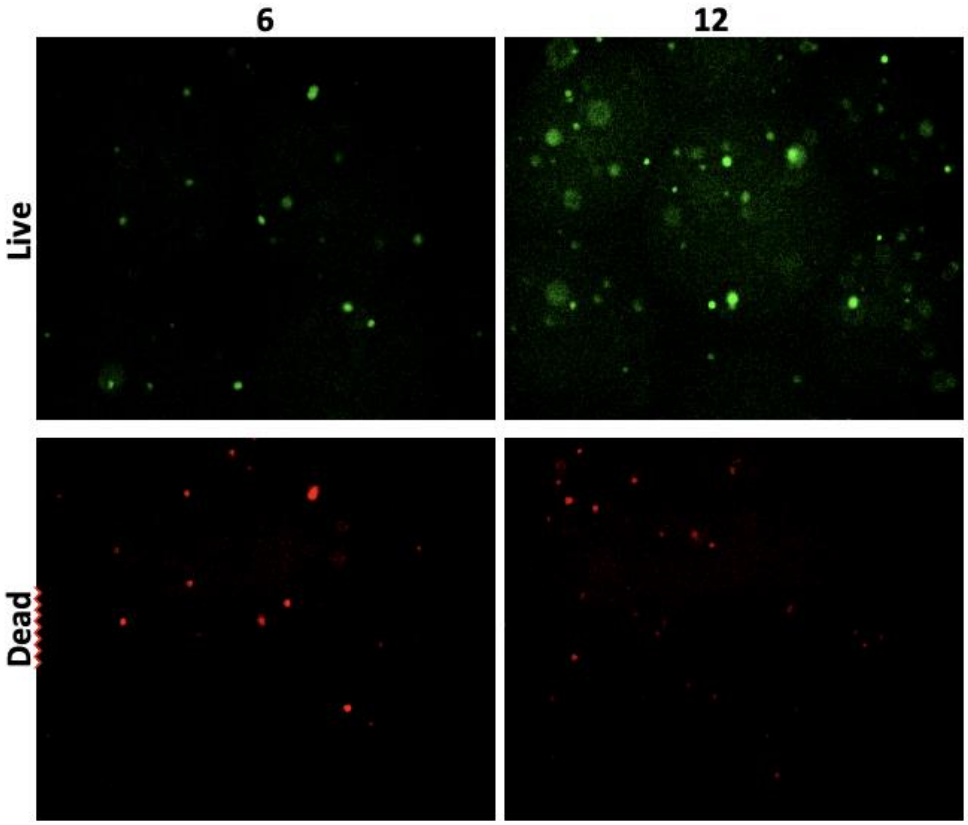

Figure 4. 35.Fluorescence images of live/dead staining for H9C2 rat cardiomyocytes in the MS3 on days 6 and 12 (scale bar;300 mμ,10X)

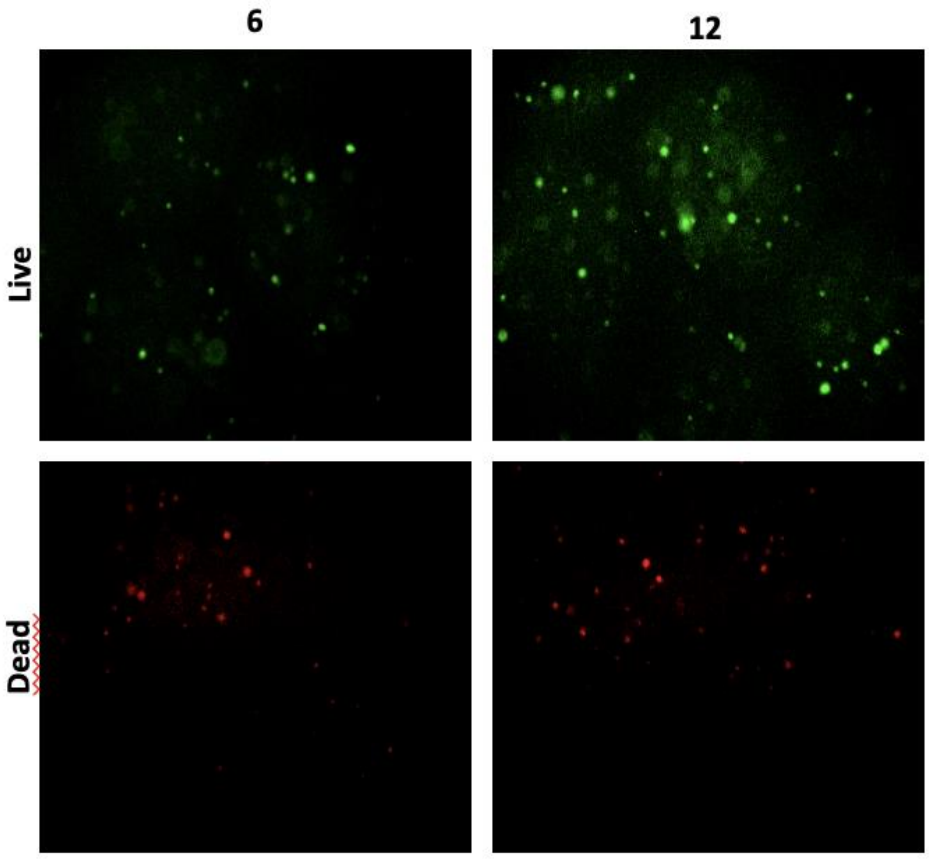

Figure 4. 36.Fluorescence images of live/dead staining for H9C2 rat cardiomyocytes in the MS4 on days 6 and 12 (scale bar;300 mμ,10X)

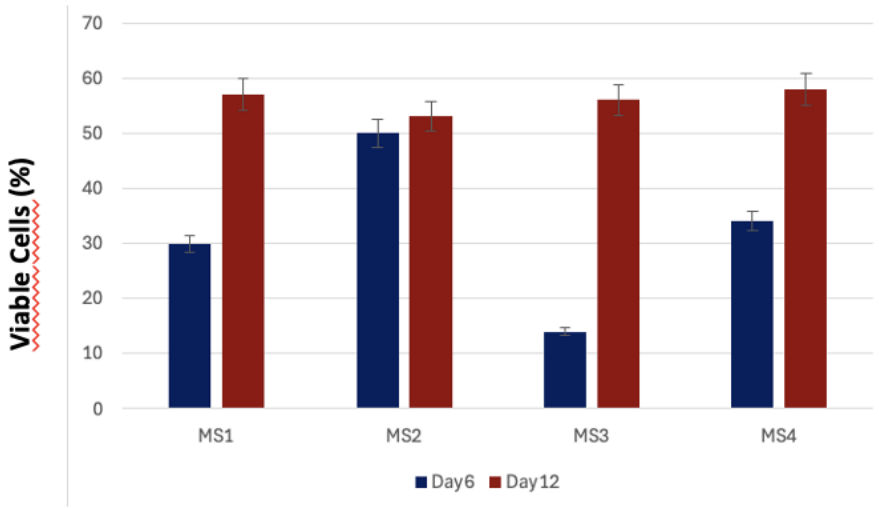

Figure 4. 37.Percentages of viable cells after live/dead staining on days 6 and 12

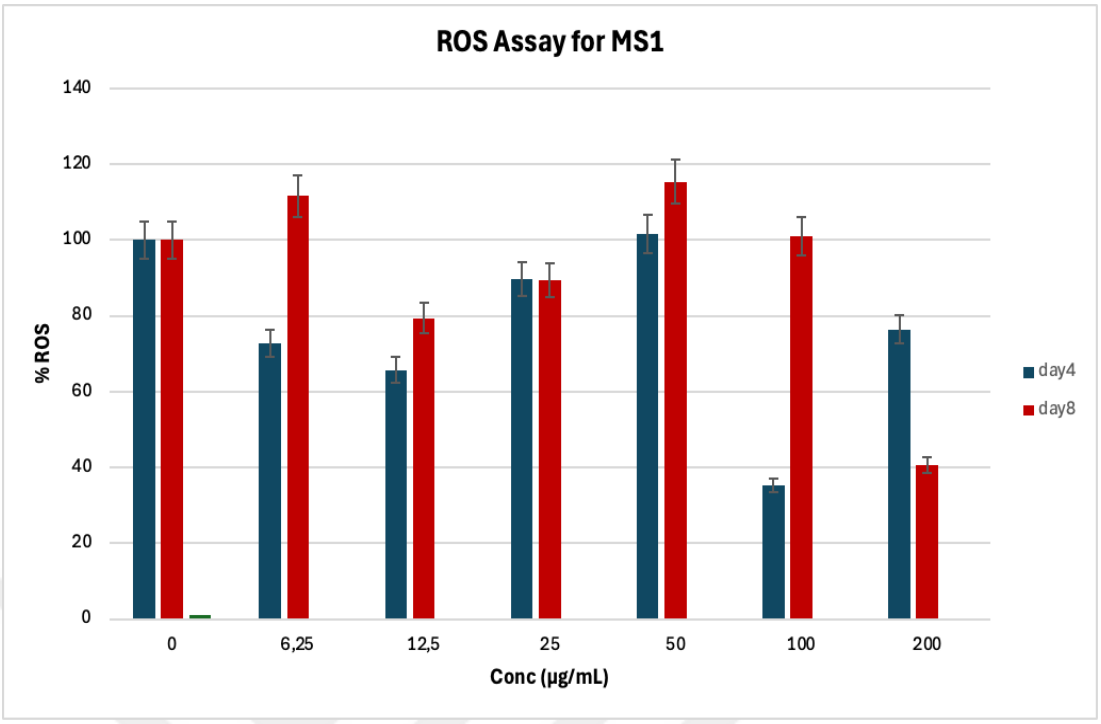

Figure 4. 38.ROS Analysis of MS1 on day4 and day8

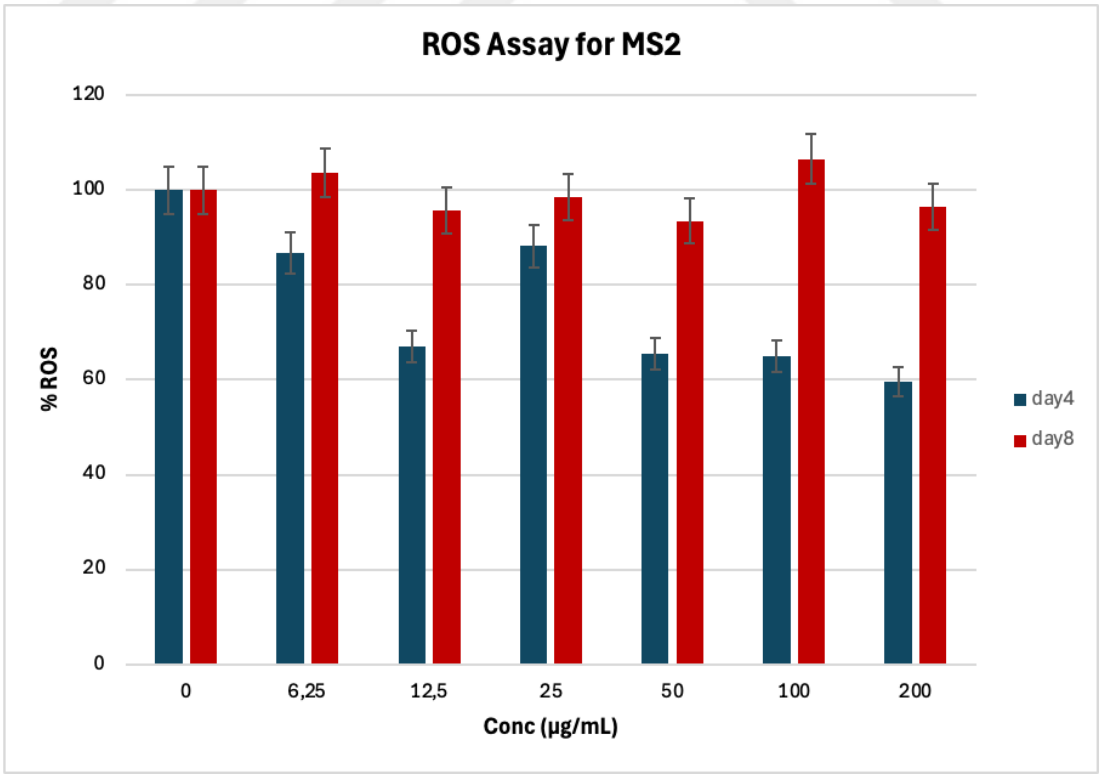

Figure 4. 39.ROS Analysis of MS2 on day4 and day8

DISCUSSION

Cardiovascular disease is one of the serious global problem. Deficiency is caused by disturbances in the cardiovascular system's operation. In humans, ischemic-reperfusion injury can result from head trauma, cardiac arrest, stroke, or subarachnoid hemorrhage (73).The aim of this study is to fabricate drug and peptide incorporated cardiomyocyte loaded microspheres which can present cellular therapy to provide cardiac regeneration after MI which delevops suddenly and cause serious damage. After production of cell loaded MS1 and MS2, they were analyzed with optical and fluorescence microscope.The literature showed that the alginate MS were protect the stability during 12 days period *in vitro* conditions (42,81). In our study, we observed the stability of GA microspheres ,MS1 and MS2 in *in vitro* conditions during 15 days period. Anionic polymers have a net negatively charged group in their structure. The effect of the alginate determined that the microspheres swell during the 15 days period due to the existence of non-crosslinking divalent cations , chelators, monovalent ions. When the CaCl concentration increased, MS was well cross-linked due to calcium release. It was proved that the microspheres swell day by day and concentrations of $CaCI_2$ keep the swelling rate a little stable.

The gel solution of cell loaded MS was first prepared with PBS to provide physiological pH conditions. Due to the salt amounts ofthe PBS, the microspheres were not crosslinked. To increase crosslinking of the micropsheres, 0.9%saline was used in gel solution fabrication. It was demonstrated that the 0.9% saline protected the stability of the microspheres. Cardiomyocytes were encapsulated to target cardiac regeneration. The Cardiomyocytes loaded MS1 and MS2 were successfully fabricated. The diameter of the microspheres reached between 150 and 250mμ as targeted. When We increased the amount of complete medium, the microspheres burst due to the contents of medium (high glucose, L-glutamine).Accordingly, the amount of the medium was decreased from 1000 μL to 500 μL.The viability of the cells was analyzed with Live and Dead assay. As the cells encapsulated in the gel solution and 3D culture

constucted, the viable cells observed layer by layer. While research on lipoic acid's potential impact on maintaining and/or enhancing central nervous system function is still in its early stages, the findings are encouraging. The possibility of lipoic acid having a protective impact on nerve cells was initially noted in a thorough analysis of oxygen toxicity that was published in 1968 (71). Following a period of more or less intensified hypoxia, abrupt reoxygenation of tissues (reperfusion) causes brain damage from stroke, cardiac arrest, hemorrhage, or head injury. When lipoic acid was given to animals undergoing ischemia-reperfusion (such as when a cerebral artery was blocked), the effects of the reperfusion were lessened. As a result, the amount of reactive oxygen species (ROS) in the brain cells decreased, the degree of the damage was lessened, and the animals' survival times were noticeably longer than those of the control group. In addition, Humans can sustain ischemia-reperfusion damage from stroke, cardiac arrest, subarachnoid hemorrhage, or head trauma. The effects of lipoic acid are dose-dependent, according to literature evidence as well.An increase which demonstrated by the sicentists the rate of cell proliferation at low doses of lipoate (1 mmol/l); lipoate functions as a growth factor. Lipoic acid demonstrated a clear antiproliferative action at high concentrations (100 mmol/l). It is possible that lipoic acid has two distinct nature, and that this complexity extends to its mode of action (72).

Comperative representation of the live/dead staining indicated that while viable cells of MS1 is approximately 30% on day 6, the viability increased to 58% on day12 in figure 4.33 and figure 4.34. Moreover, MS2 has 50% viability on day6, and the viability increased to 55%. The literature suggested that engineered cardiosphere derived cells (CDCs) exosomes to express CMP(WLSEAGPVVTVRALRGTGSW) and targeted delivery to cardiomyocytes and decreased cardiomyocyte apoptosis. Statistically, it was observed that there is an essential increase on the viable cells of the MS3 between from day6 to day12. We can prove that the CMP peptide maintained positive effect for the cell viability. Additionally, it was indicated that the cell viability gradually increased in MS1 by comparing MS1. The literature reported that LA induced apoptosis and supported the wound healing thanks to it’s andioxidant

property. The lipoic acid provided smooth cell proliferation by supporting the literaure. In addition to the potent characteristics of the lipoic acid, The incorporation of the CMP peptide with the LA expedited to cell proliferation and induced the apoptosis. These combination may demonstrate positive effect on regeneration ischemic area in the heart after myocardial infarction (71).

The mechanical strength of the MS were investigated by Rheometer instrument. G' Specifies the elastic modulus(elasticity) and G'' represents viscous modulus(viscosity) of the microsphere. In addition, Overlap of the $G'$and G'' indicates the elasticity of the micropshere. Deformation of the microsphere started after the elastic and viscous modulus diverged. The deformation increases as the stress increases in figure4.10b, on the contrary, the deformation in figure 4.10a increased slowly. The amount of the gelatin impacted the deformation rate.Furthermore, the deformation increased sharply under shear stress in figure 4.10c and 4.10d The amount of the gel-CMP pep and gel-LA conjugates affected the degradation rate.

Functional groups and chemical structures of the MS and conjugates were analyzed by FT-IR instrument. It is indicated that carbonyl group of the alginate represents at between 1595 cm^{-1} and 1627 cm^{-1}(C=O and -COOH) in figure Amine groups of the gelatin (NH_2) shows by peaks at 3365 cm^{-1}(GA MS) It is clearly seen that overlaps of the peaks shows the different amide and carbonyl peaks and these peaks indicate that the amide groups of the were bound to the gelatin(-NH_2) at 3284 cm^{-1} in figure16 and 4.17. Additionally, the disappearance of COOH in the linker indicates that the reaction was successfully performed and the maleimide ester carbonyl remained in the conjugate. Moreover, figure 4.15 indicates that the dissapearency of maliemide ester peaks in the the Gel-Mal conjugate demonstrates that the CMP peptide successfully bound to gelatin from maliemide (70,74).

The ^{1}H NMR data of Gel-LA conjugate demonstrates that when appending LA to gelatin, the resulting spectrum is impotantly a composite of the two starting materials in figure 4.24.The main contribution from the gelatin moiety seems as broad multiplet at 4.4 ppm. Compared to the pure gelatin, c and d adjacents of sulfur atoms were shifted and broadened at 3.2 ppm-2.8 ppm which corresponded to thiol groups ($S\text{-}CH_2$). These results indicated that the LA was successfully grafted with gelatin (75,76).

A comparative representation of the spectra for CMP peptide,Gel-Mal and Gel-Pep conjugate indicated the presence of labile amide protons that appear as a broad multiplet between 4.5 ppm and 3.5 ppm in figure 4.22(a,b,c) and figure 4.23. The figure 4.18 demonstrates the proton spectra of peptide and conjugates. As the gelatin is the fundamendal polymer of the conjugates, spectras of amide protons were identical.Additionally, CMP peptide's tryptophan amino acid is the source of the aromatic group of peptide protons. While DMSO protons appear at 2,5 ppm, the peaks at 1.0 ppm-0.5 ppm in figure 4.18 belong to the proton of CH_3 methyl group of the CMP peptide. Moreover,The aromatic group of protons of the CMP peptide as a broad multiplet at 7.5–7.00 ppm and isopropyl group protons of leucine (Leu) as a doublet at 1.00–0.50 ppm; these may have originated from the structure of the CMP peptide in figure 4.22 (77,78).

The morphological evaluations of the microspheres were investigated in dry form by SEM. Figure 4.11 a,b,c and d show the pore sizes of the GA microspheres in different concentration of the gelatin by weight and volume. The diameters of the microspheres were successfully observed as desired .Figure 4.13 shows the SEM image of the 0.1(w/v) Gel-LA conjugate incorporated alginate microsphere and the image was measured by sending X rays with the EDS detector which integrated to the SEM instrument. Atomic and elemental analyzes of the micropshere were evaluated quantitatively. The atomic diversity of the microsphere was analyzed and the peaks are sulfur(S) atom Nitrogen(N) were observed as 3.5 % and 11.3 % and these are the spectrum of the Gel-LA conjugate (table 4.1). The atomic diversity indicates that the

gelatin was successfully bound to lipoic acid. Additionally, It is clearly seen that N and Na atoms come from gelatin and sodium alginate which are the fundamental structure of the microsphere.

Similarly, the atomic evaluation of the gel-CMP pep integrated microsphere was visualised with the EDS detector by SEM device. C and N atoms in table 4.2 demonstrate that the gel-CMP pep initially bound to sodim alginate during gel preparation.

Comparison of the Molecular Weight Distribution of Gelatin Fractions by Size-exclusion Chromatography and Light Scattering in figure 4.25 demonstrates that Paper for Gelatin molecular weight (MW) is approximately 80kDa and molecular weight of 1 amino acid is 110 kDa, so gelatin has almost 727 monomers. Correspondingly, lipoic acid CH_2 peak's integration is 468, so we have 234 LA in 1 gelatin chain. The Calculation of LA on Gelatin polymer by weight is 37,6%.

Additionally, figure 4.26 indicates the drug release of MS2 during the 15 days period. The MS2 was incubated in 10 mL of release medium (100 mM $CaCI_2$, pH=7.4) at 37 ℃ with thermoshaker during the 15 days period. According to the literature, there are several factors which impact the drug release kinetics of the microparticles. These factors contains polymer molecular weight (MW),particle porosity and size. For instance, Drug release from PLGA microparticles can include variable molecular mechanisms containing drug diffusion throughout the polymer matrix, drug diffusion through the aqueous pores, polymer degradation and dissolution into the aqueous phase and drug dissolution into the bulk aqueous phase. The diffusion process through the polymer matrix is only related to small hydrophobic drugs (79). Hydrophobic drugs do not show an induction time since they just rely on Fickian diffusion via the polymer matrix. A straightforward comparison of the drug's effective diffusion coefficients in aqueous fluids is insufficient to comprehend the variations in their overall release behaviors because of the distinct drug diffusion paths between the hydrophilic and

hydrophobic situations.the lipoic acid is hydrophobic property, so delayed biphasic drug release performed. A delayed biphasic drug release profile (= L + P) starts with an initial lag period followed by a rapid release of drug, giving rise to a sigmoidal shaped curve. As the Lipoic acid binded to amide bonds, the drug release started based on the polymer backbone.

CCK-8 assay was performed to determine cytotoxicity of the different microsphere formulations on H9C2 rat cardiomyocytes by applying dose-dependent concentrations. Cytotoxicity data were shown as mean ± SD and analysis with two-way ANOVA by performing 3 replicates(n=3) with graphpad prism (80). P values of each graph were evaluated statistically significant ($p < 0.0001$). The figure 4.27,4.28,4.29 and 4.30 demonstrate the cytotoxicity of the MS1, M2, M3 and MS4 on days 4,8 and 12 respectivelty.

It was determined that the cell viability of MS2 is over 72% at highest concentration on day4 while cell viability of MS1 is over 75 % which is statistically significant (81). As the MS2 includes CMP peptide which is cell adhesive peptide, it maintains to increase the cell proliferation. Even the concentration was decreased,the cell viability increased .Meanwhile,there were no any toxicity associated with the gel-LA conjugate and gel-pep conjugate at 7 levels of concentration.

Likewise, the cell viability of MS3 at highest concentration(200μg/mL) was 106% on day4 while it was 112% on day12. It was found that the viability increased by time dependent as the CMP peptide is cell adhesive peptide , decrease cell death and cell proliferation. While cell viability increased sharplyduring the incubation days on MS3, there were gradual increase on MS2.Lipoic acid provided increasing of the cell viability as it is a strong lipophilic antioxidant which performs several tasks, including cell apoptosis induction ,glucose absorption,improvement and scavenging reactive oxygen species (ROS) (82,83). Moreover, it was proved that LA and Gel-pep aren’t toxic on cell viability in figures 4.31 and 4.32.

Furthermore, Figure 4.30 demonstrated that MS4 is non-toxic to H9C9 cells on days 4,8 and 12. MS4 inludes gelatin and alginate. Gelatin is the perfect natural bioactive substance for the purpose of creating the microsphere growth factor release system. Cell surface adhesion molecule recognition is facilitated by its ability to bind with cell surface receptors, include short peptides with biological activity related to amino acids, and respond to different surface modifications, so it has positive effect for the cell adhesion.

5 CONCLUSION

In conclusion, controlled drug release and cellular therapy platform were developed with the integration of gel-CMP pep conjugate to treat the ischemic heart muscle. Polymeric microspheres were successfully constructed with the incorporation of peptide and drug and loaded with the Cardiomyocytes. The microspheres were developed by using the sodium alginate and gelatin as they are natural polymers, on-toxic, biodegradable and biocompatible. The microspheres were functionalized with the gel-drug and gel-pep conjugates to expedite the healing process. The cell adhesion peptide was used to provide better-adhesion and induce apoptosis for the cardiac regeneration. Since the lipoic acid has antioxidant properties, it is accepted one of the strongest oxidation regulators. To sum up, the combination of peptide and drug with the microspheres can be evaluated as promising candidates to contribute and expedite regenerate damaged heart muscle after the Myocardial Ischemia.

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7 APPENDIX

Appendix 1 Calibration Curve of Lipoic Acid

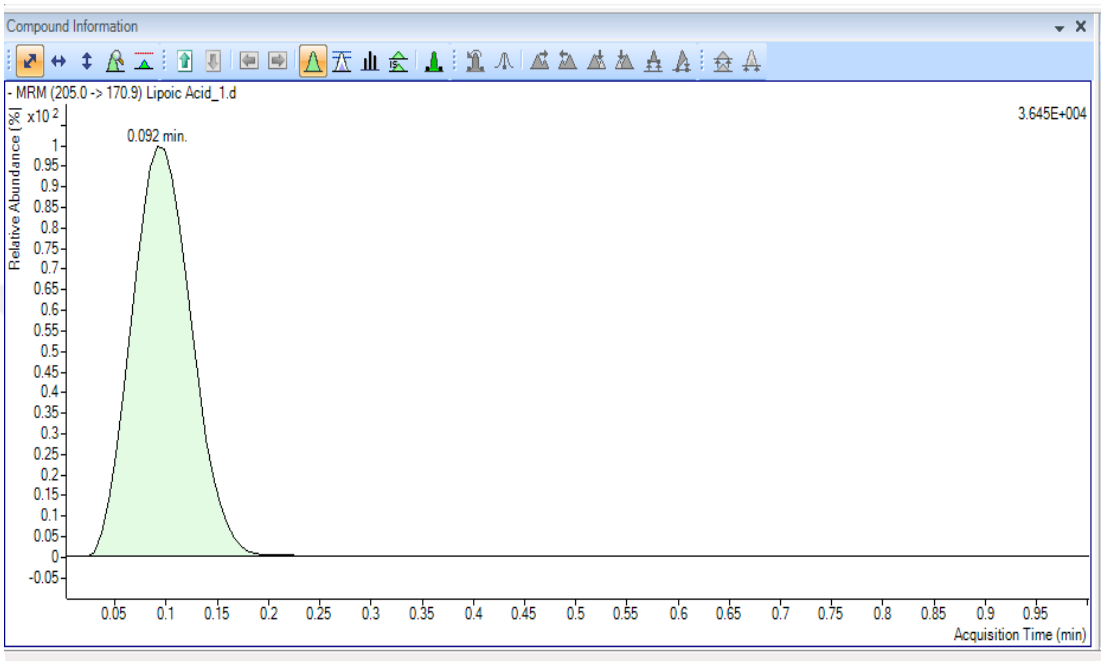

Figure 4. 40.Calibration curve of lipoic acid

8 CURRICULUM VITAE

Personal Information

Name		**Surname**	
Place of Birth		**Date of Birth**	
Nationality		**Phone**	
E-mail			

Education

	Institution Name	**Graduation Year**
Doctor of Philosophy		
Master of Science		
Undergraduate		
High School		

Work Experience

	Position	**Corporation**	**Duration**
1.			-
2.			-
3.			-

Language	**Reading***	**Speaking***	**Writing***

***** Evaluated as advenced, good, intermediate, beginner

	Foreign Language Exam Results #								
KPDS	ÜDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE	OTHER

All Successful exams should be enrolled.

KPDS: Kamu Personeli Yabancı Dil Sınavı; ÜDS: Üniversitelerarası Kurul Yabancı Dil Sınavı; IELTS: International English Language Testing System; TOEFL IBT: Test of English as a Foreign Language-Internet-Based Test TOEFL PBT: Test of English as a Foreign Language-Paper-Based Test; TOEFL CBT: Test of English as a Foreign Language-Computer-Based Test; FCE: First Certificate in English; CAE: Certificate in Advanced English; CPE: Certificate of Proficiency in English

	Quantitative	**Equally Weighted**	**Verbal**

Computer Skills

Program	**Ability to Use**

***** Evaluated as advenced, good, intermediate, beginner

National and International Publications/Posters/Certificates/Prizes/Other