

**T.C.
REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES**

**DEVELOPMENT OF NANO-FORMULATIONS CONTAINING
NEUROPROTECTIVE ACTIVE INGREDIENT AND
EVALUATION OF EFFICIENCY IN PERIPHERAL NERVE
INJURY MODEL**

MSc. Pharmacist. Mohammad Karim HAIDAR

Program of Biopharmaceutics and Pharmacokinetics

DOCTOR OF PHILOSOPHY THESIS

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Mohammad Karim HAIDAR (Pharmacist, M.Sc.)

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ADVISOR OF THE THESIS

Prof. Dr. HAKAN EROĞLU

ANKARA

2018

**Development of nano-formulations containing neuroprotective active ingredient
and evaluation of efficiency in peripheral nerve injury model**

Mohammad Karim Haidar

Supervisor: Prof. Dr. Hakan EROĞLU

This thesis study has been approved and accepted as a Ph.D. dissertation in “Biopharmaceutics and Pharmacokinetics Program” by the assessment committee, whose members are listed below, on 02.11.2018

Chairman of the Committee: *Prof. Levent ÖNER*

Hacettepe University Faculty of Pharmacy

Member :

Prof. Kezban ULUBAYRAM

Hacettepe University Faculty of Pharmacy

Member :

Prof. Selma ŞAHİN

Hacettepe University Faculty of Pharmacy

Member :

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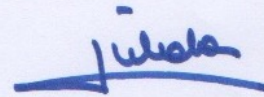
Gazi University Faculty of Pharmacy

Member :

Assoc. Prof. Atilla KAZANCI

Yıldırım Beyazıt University Faculty of Medicine

This dissertation has been approved by the above committee in conformity to the related issues of Hacettepe University Graduate Education and Examination Regulation.



Prof. Diclehan ORHAN, MD, PhD

Institute Manager

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Yükseköğretim Kurulu tarafından yayınlanan "*Lisansüstü Tezlerin Elektronik Ortamda Toplanması, Düzenlenmesi ve Erişime Açılmasına İlişkin Yönerge*" kapsamında tezim aşağıda belirtilen koşullar haricince YÖK Ulusal Tez Merkezi / H.Ü. Kütüphaneleri Açık Erişim Sisteminde erişime açılır.

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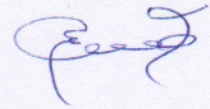
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ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with advisor (Prof. Dr. Hakan EROĞLU) and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.

Mohammad Karim HAİDAR



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My heartfelt thanks to my lab friends and the entire family of **Pharmaceutical Technology** for their endless and enduring help.

ABSTRACT

HAIDAR Mohammad Karim, Development of Nano-formulations Containing Neuroprotective Active Ingredient and Evaluation of Efficiency in Peripheral Nerve Injury Model, Hacettepe University Graduate School of Health Sciences, Ph.D. Thesis in Department of Biopharmaceutics and Pharmacokinetics, Ankara, 2018. Peripheral nerve injury (PNI) is one of the common traumas. Causes of acquired peripheral neuropathy include physical injury, diseases or disorders, exposure to toxins etc. Depending on nature and severity of injury, several therapeutic methods have been proposed. Crush injury is the most common type (80% of PNI) of PNI and decompression is the only way for treatment. However, the outcome of this treatment method is not satisfactory. Therefore, accelerating the curative process by additional treatment is strongly required. The aim of this thesis is to develop a novel polymeric composite nanofiber which containing nanoparticles for dual and localized delivery of neuroprotective drugs for accelerating regeneration process of peripheral nerve injury. For this purpose, alpha lipoic acid (ALA) and atorvastatin calcium (ATR) were used as neuroprotective agents. At first step ATR loaded chitosan nanoparticles were prepared by Nano Spray drying method. At the second step, Nano Spray dried ATR loaded chitosan nanoparticles were suspended into solution of poly lactic-co-glycolic acid (PLGA) and ALA. The resulting mixture was exposed to electrospinning and electrospun nanocomposites were collected for further study. After characterization of these formulations, regrading to composition of formulation, selected formulation was used subsequently and implanted into animal model of sciatic nerve trauma. Trauma was formed with the compression method in Sprague Dawley rats. The motor function and sensory tests in the post-surgery stage revealed significant improvement in the regeneration process of nerve injury in the treated animals. The ultrastructural examination of sciatic nerve tissues samples at different timepoints also implies that the number of normal myelinated axons were increased in the treatment groups. The determination of pro-inflammatory cytokines also supported the effectiveness of the developed formulation. As the conclusion of this study, we might state that ATR loaded chitosan nanoparticles that are embedded in ALA containing nanofibers could be promising drug delivery systems for neuroprotection after peripheral sciatic nerve injury, especially depending on the faster recovery within the first 15 days period after trauma.

Key Words: Atorvastatin Calcium, Alpha Lipoic Acid, Nanoparticle, Nanofiber, Peripheral Nerve Injury, Cell Culture

Acknowledgement: This study was supported by TÜBİTAK-1001 Scientific Project with the number 115 S 202.

ÖZET

HAIDAR Mohammad Karim, Nöroprotektif etkin madde içeren nanoformülasyonların geliştirilmesi ve periferik sinir hasarı modelinde etkinliklerinin değerlendirilmesi, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Biyofarmasötik ve Farmakokinetik Programı Doktora Tezi, Ankara, 2018. Periferik Sinir Hasarı (PSH), en sık görülen travma türleri arasında yer almaktadır. Oluşum sebepleri arasında fiziksel yaralanma, hastalıklar ve toksinlere maruziyet gibi çok sayıda sebep bulunmaktadır. Yaralanmanın türü ve şiddetine bağlı olarak farklı tedavi yaklaşımları bulunmaktadır. Çarpma hasarı, PSH türleri arasında en sık görülen türü (%80) olmakla birlikte tedavi için tek yok uygulanan basının kaldırılmasıdır. Buna karşın uygulanan bu tedavinin çıktıları çoğu zaman yeterli olmamaktadır. Dolayısı ile ek tedavi yöntemleri ile tedavi sürecinin hızlandırılması ihtiyacı vardır. Bu çalışmanın amacı, periferik sinir hasarında rejenerasyon sürecini hızlandırmak için nöroprotektif etkin maddelerin ikili ve lokalize salımını sağlayacak içerisinde nanopartikül içeren kompozit polimerik nanofiber hazırlamaktır. Bu amaçla nöroprotektif etkin maddeler olarak alfa lipoik asit (ALA) ve atorvastatin kalsiyum (ATR) kullanılmıştır. İlk aşamada ATR içeren nanopartiküller nanopüskürterek kurutma cihazında hazırlanmış; ikinci aşamada ise hazırlanan bu nanopartiküller, içerisinde ALA ve poli(laktik-ko-glikolik)asit içeren çözeltide süspande edilmiştir. Oluşturulan bu karışım, elektrospindleme ile kompozit nanofiberlerin hazırlanmasında kullanılmıştır. Formülasyonların karakterizasyon aşamasını takiben, seçilen uygun formülasyon, siyatik sinir hasarı oluşturulmuş hayvan modelinde implante edilmiştir. Sprague-Dawley cinsi sıçanlarda kompresyon yöntemi ile siyatik sinir hasarı oluşturulmuştur. Cerrahi sonrası dönemde gerçekleştirilen motor fonksiyon ve duyuusal testler sonucunda deney hayvanlarında sinir hasarında anlamlı bir düzelme olduğu görülmüştür. Buna ek olarak yapılan ultrastrüktürel incelemeler sonucunda da tedavi gruplarında normal miyelinli akson sayılarındaki artış tespit edilmiştir. Pro-inflamatuvar sitokinler üzerinde yapılan incelemelerde elde edilen bulgular geliştirilen formülasyonun etkinliğini destekleyici nitelikte sonuçlanmıştır. Sonuç olarak içerisinde ATR yüklü nanopartiküller gömülmüş olan ALA içeren nanofiberlerin periferik sinir hasarı sonrasında nöroproteksiyon amaçlı, özellikle de travma sonrasındaki ilk 15 günlük dönem içerisinde, kullanılabilecek uygun ilaç taşıyıcı sistemler olduğu düşünülmektedir.

Anahtar Kelimeler: Atorvastatin Kalsiyum, Alfa Lipoik Asit, Nanopartikül, Nanofiber, Periferik Sinir Hasarı, Hücre Kültürü

Ek Bilgi: Bu çalışma TÜBİTAK tarafından 115S202 numaralı 1001 Bilimsel Araştırma Projesi ile desteklenmiştir.

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SYMBOLS AND ABBREVIATIONS

ACN	Acetonitrile
ALA	Alpha Lipoic Acid
ATR	Atorvastatin Calcium
AUP	Area Under The Peak
BBB	Basso, Beattie, and Bresnahan
CH	Chitosan
CNS	Central Nervous System
Cx32	Gap Junction Beta-1 Protein (GJB1), Also Known As Connexin 32
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DSC	Differential Scanning Calorimetry
EDTA	Ethylenediaminetetraacetic Acid
EE	Encapsulation Efficiency
EPT	Extensor Postural Thrust
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
IL-1β	Interleukin 1 β
IL-1α	Interleukin 1 α
IL-6	Interleukin 6
LIF	Leukemia Inhibitory Factor
LOD	Limit of Detection
LOQ	Limit of Quantification
MeOH	Methanol
MIP-1	Macrophages Inflammatory Protein
MOG	Myelin Oligodendrocyte Glycoprotein

MTT	3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2H-Tetrazolium Bromide
Mw	Molecular Weight
P0 Protein	Myelin Protein Zero
PBS	Phosphate Buffered Saline
PDI	Polydispersity Index
PEG	Polyethylene Glycol
PLGA	Poly (lactic-co-glycolic acid)
PMP22	Peripheral Myelin Protein 22
PNS	Peripheral Nervous System
PTFE	Polytetrafluoroethylene
RSD	Relative Standard Deviation
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscope
SFI	Sciatic Functional Index
TEM	Transmission Electron Microscope
TGF-β	Transforming Growth Factor-B
TNF-α	Tumor Necrosis Factor Alpha
WD	Wallerian Degeneration
WRL	Withdrawal Reflex Latency
XRD	X-Ray Diffraction Analysis

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1. INTRODUCTION

The human nervous system is complex and sophisticated structure which contains a network of special cells called neurons. The primary function of the nervous system is to coordinate all activities of the body. Commonly, from the anatomical viewpoint, the human nervous system divided into two major parts which are called as Central Nervous System (CNS) and the Peripheral Nervous System (PNS).

The peripheral nervous system consists of everything outside the brain and spinal cord. PNS transfers information to the CNS and carries a signal out of the CNS. Peripheral nerves are complex structures which are comprised neuron, glial cells, connective tissues and blood supply. These nerves spread out all over the body (1).

In the PNS there are no protective structures like vertebral column or skull, they are distributed in different parts of the body and some of them are embedded into organs. Therefore, the peripheral nerve is more exposed to external damage and more vulnerable than CNS. PNS can sustain injury from physical trauma and a number of diseases (Diabetes, infection, metabolic problems). The PNI may be mild or a fully severed nerve injury (2).

PNI is a worldwide social and clinical serious problem. For instance, over 8.5 million restricted activity days and almost 5 million bed/disability days, just in the United States. In addition in Europe 300,000 cases occurring annually (3, 4). These data clearly show the adverse outcomes of the PNI in the lives of patients and repercussion in society.

Therefore, considering the importance of PNI, researchers have been investigated several approaches to find effective treatment methods. Regarding the nature and severity of the injury, several treatments approach exist.

Peripheral nerve injury causes motor, sensory and autonomic dysfunction of an associated organ. Despite the capability of the PNS to regenerate the axon, regeneration proceeds slowly as only a few millimeters per day.

Hence, when associated organs are located far from the damaged site, functional outcomes after PNI are often negligible and gradually reduce. Therefore, in order to recover the motor and sensory function of the denervated organs, additional treatment for increasing curative effects and acceleration of the regeneration process are required.

The objective of this thesis was to develop a novel and implantable nanocomposite electrospun PLGA/CH sheet for treatment of PNI. In formulation studies alpha lipoic acid (ALA) (a strong antioxidant) and atorvastatin calcium (ATR; an inhibitor of HMG-CoA reductase) were used as neuroprotective drugs. In order to provide dual and sustained local delivery of ALA and ATR, we utilized nanofibers and nanoparticles. ALA was incorporated on PLGA nanofibers and ATR was loaded chitosan nanoparticles. Finally, ATR loaded chitosan nanoparticles were embedded in PLGA nanofibers. After characterization, the effectiveness of the formulations was studied in a rat sciatic nerve crush injury model.

2. GENERAL INFORMATION

2.1. Human Nervous System

The nervous system is extremely complex and sophisticated system which contains a network of special cells called neurons. It is the control center of the body and the primary function of the nervous system is to coordinate all activities of the body and enable the body to respond and adapt to changes that occur both inside and outside the body (5). Totally, neural tissues make 3% of body weight (6) and 20% of the blood circulate in the brain (7). Commonly, from the anatomical viewpoint, the human nervous system can be broken up into two major parts which are called Central Nervous System (CNS) and Peripheral Nervous System (PNS) (8).

The CNS is made up of the brain and spinal cord. The brain is located inside the skull cavity. Spinal cord is the link between the brain and the nervous system existing in the rest of the body and encased in the cavity of the vertebral column. The peripheral nervous system comprises everything outside the brain and spinal cord. PNS transfers information to the CNS and carries a signal out of the CNS (9).

2.2. Peripheral Nervous System

PNS is a part of the nervous system containing all neural structures (nerves and ganglia) that are not part of the brain or spinal cord. It allows the CNS to receive information from rest of the body and transmit messages to the organs of the body. In the PNS there are no protective structures like vertebral column or skull, they are distributed in different parts of the body and some of them are embedded into organs. PNS is a vast network of 31 pairs of spinal nerves and 12 pairs of cranial nerves including their branches and roots which is attached to the brain and spinal cord. In the view of the fact that, PNS is a collection of nerves and ganglia, it is necessary to describe these two parts of PNS.

2.3. Nervous System Cell

2.3.1. Neuron

At the cellular level, neural tissue consists of two major type of nerve cells which are named as neurons (nerve cell) and neuroglial cell. The glial cells protect, provide physical support, supply nutrients and remove waste materials of the neuron. Neuroglial cells are not directly involved in the processing of information, but their functions are vital for normal activities of the brain (10).

Neurons are the basic functional unit of the nervous system and play a vital role in transmission of impulses (Figure 2.1.). They have the ability to process and transmit information (11). In general, the nervous system utilizes different type of neuron to communicate and regulate all body's activities. The communications between neurons are carried out by electrical and chemical signals. The neurons are irregular shaped in nature and structurally have three parts: Axon, Dendrites and Cell body

Axon is the longest outgrowth of the cell body and recognizable by its length. Axon typically conveys an output signal to other neuron or by other means, axon propagates the nerve impulse away from the soma. A single axon begins from a cone shape structure of cell body, where the axon emerges and known as axon hillock. The impulses are accumulated at the axon hillock and then transmitted along the axon. Eventually, the axon terminates in several branches which are called synaptic terminals. The axon may connect to the target cells by a tiny gap called synapse. The neurotransmitters which carry chemical messages, are released by presynaptic neurons through the synapse and bind to particular receptors on postsynaptic neurons.

Dendrites makes the input system of neurons. They are short branches that come out of the cell body. They connect the neuron to the other surrounding neurons and receive signals in the form of neurotransmitters and conduct it towards the soma or cell body.

Cell body or soma contains the nucleus and most of the organelles (Lysosome, Mitochondria, Endoplasmic reticulum, Golgi apparatus etc.) (12).

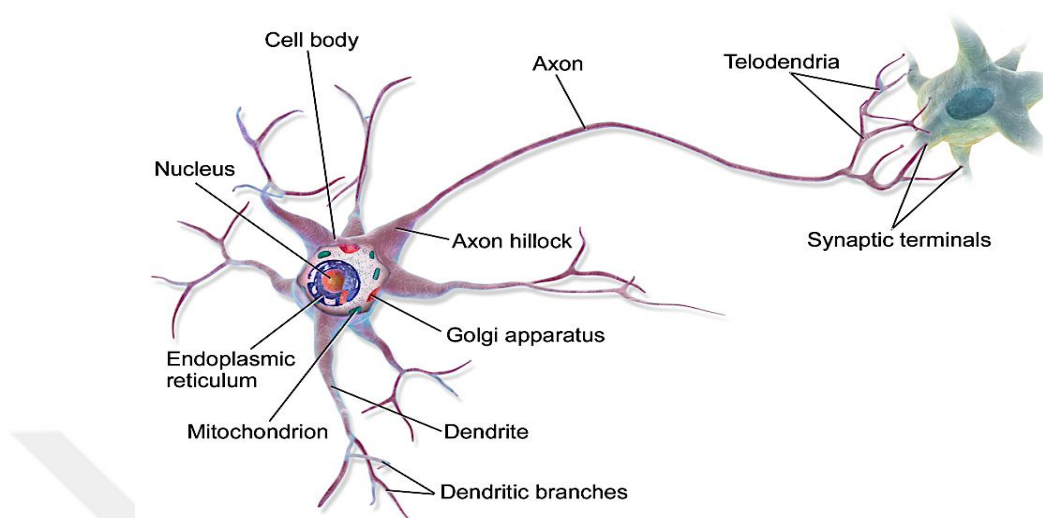


Figure 2.1. Different parts of a neuron (13).

2.3.2. Types of Neurons

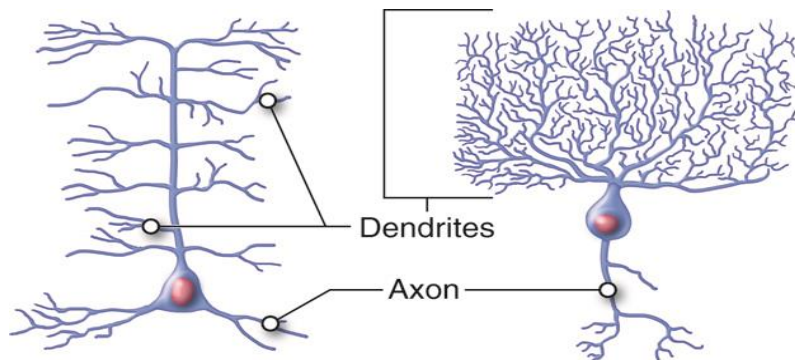
In consideration of different criteria like structure, function and location of neurons there are various types of neuron in the nervous system. In respect of structure, neurons are classified into four categories: multipolar neuron, bipolar neuron, unipolar neuron and anaxonic neuron (Figure 2.2.).

Multipolar neurons are commonly found in CNS and have more than two branches coming from the cell body. There is still just one axon and several dendrites. The dendrites emerge from cell body individually.

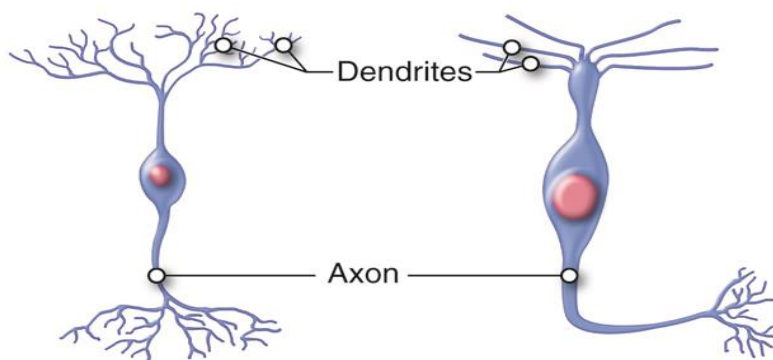
Bipolar neurons are quite rare in the human nervous system. In this kind of nerve cells, two branches extending from two opposite site of cell body exist. One of these extensions act as an axon and another is dendrite.

Unipolar neurons have just one protrusion from the cell body and it splits into two branches. Unipolar neurons are always sensory neurons and their somas are located in ganglia.

Anaxonic neuron is small in size and star shape neurons. Apparently, they are without axon and mostly found in CNS, retina and adrenal medulla. Their function is not clear yet.



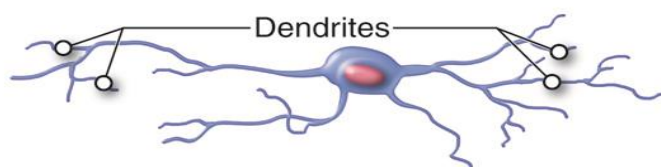
Multipolar neurons



Bipolar neurons



Unipolar neuron



Anaxonic neuron

Figure 2.2. Neuron classification in respect of shape (13).

In terms of function, the neurons may be classified as motor, sensory and interneurons (Figure 2.3.). Sensory and motor neurons make up the nerve fiber in PNS

Sensory neurons are responsible for collecting and transmitting sensory information (temperature, pain, light, etc.) to the CNS. The sensory neurons act as a transducer to change the physical and chemical stimulation to the electrical signal and transmit them for processing to CNS. The soma and dendrites of sensory neurons are outside of CNS, cluster in an elliptical structure named dorsal root ganglion. Sensory neurons have two axonal branches (unipolar), one branch attached to the periphery and the others connect to the CNS.

Interneurons are morphologically, multipolar and the most abundant type of neuron. They are entirely found in the CNS, around 90% of CNS occupied by interneurons. It is sandwiched between two pathways, the axon of sensory neurons from one side and motor neuron from another side and do facilitate communication between these two pathways in the spinal cord. Interneurons are involved in a simple to very complex brain neural circuits.

Motor (efferent) neurons transfer commands or transfer messages from CNS to organs (e.g. muscles and glands). The cell body and dendrites of motor neuron are located in CNS. The axons of PNS terminated to the target organs. They are multipolar in shape (14-16).

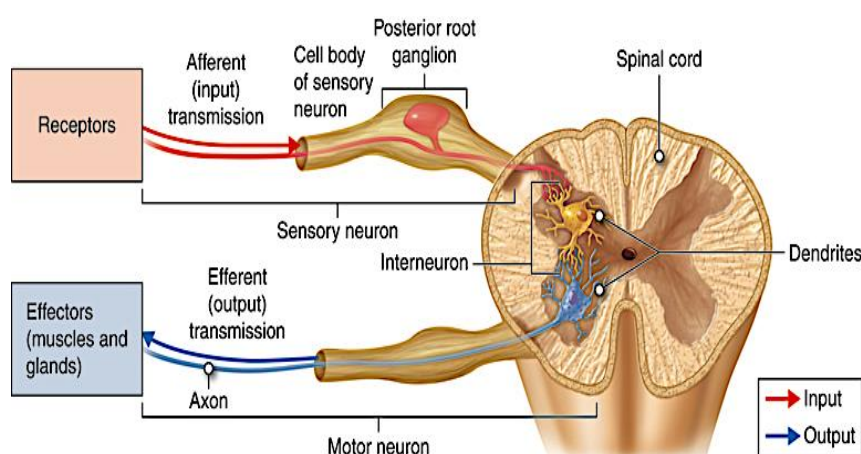


Figure 2.3. Classification of neuron in terms of function (17).

2.3.3. Neuroglia

The neurons are not the only cells which exist in the nervous system. There are many other supportive cells that either provide support in different ways and increase transmission along the length of the axon. In spite of very distinguished functional role of neurons in CNS, glial cells are existing 10 times more than neurons in CNS and make up 50% of the volume of the nervous system. They are electrically non-excitabile cells and non-responding to external stimulation (18). The nervous system can be divided into two parts CNS and PNS, these two sections themselves are contained in their own neuroglia (Figure 2.4.).

▪ Supporting Cells of The Central Nervous System

Astrocytes are star-shaped and multifunctional cells and that occupy the largest space in the CNS. They provide structural support or forms the scaffold for the other cells. Furthermore, they generate glial scar tissues after injuries, maintain brain homeostasis, contributing blood-brain barrier and clearing out the neurotransmitters.

Microglia are basically functioning as a cleaner. They are activated after injury in the nervous system and remove all apoptotic cells from the environment.

Oligodendrocytes are specialized cells wrapped around the axon of neurons and made up a multi-layer sheath which is called myelin sheath. Myelin sheath acts as an electrical insulator and accelerates the speed of signal along the axon.

Ependymal cells are one layered ciliated cuboidal epithelium cells that are lined with the cerebral spinal fluid-filled cavities within CNS. The ependymal cell's cilia move wavelike fashion that helps the cerebral spinal fluid to circulate inside the ventricles of the brain and central canal of the spinal cord.

▪ Supporting Cells of The Peripheral Nervous System

Within the PNS, apart from the neuron, two types of glial cells (Satellite cells and Schwan cells) are found.

Satellite glial cells, from the functional viewpoint, are the counterpart of astrocytes in CNS. They provide structural and nutrient support for the neurons. They do not produce myelin. In the ganglia of the sensory dorsal root, sympathetic and parasympathetic nerves, each cell body with primary part of its axon is covered by satellite cells separately. It maintains the optimum exchange rate of nutrient between neuron cell body and extracellular fluid.

Schwan Cells are very similar to oligodendrocytes in CNS. They carry out the same functions but are different in structure. Unlike to oligodendrocytes, Schwan cells physically attached to the surface of the neuron's axon and create myelin sheath around every single axon of neurons. (18, 19).

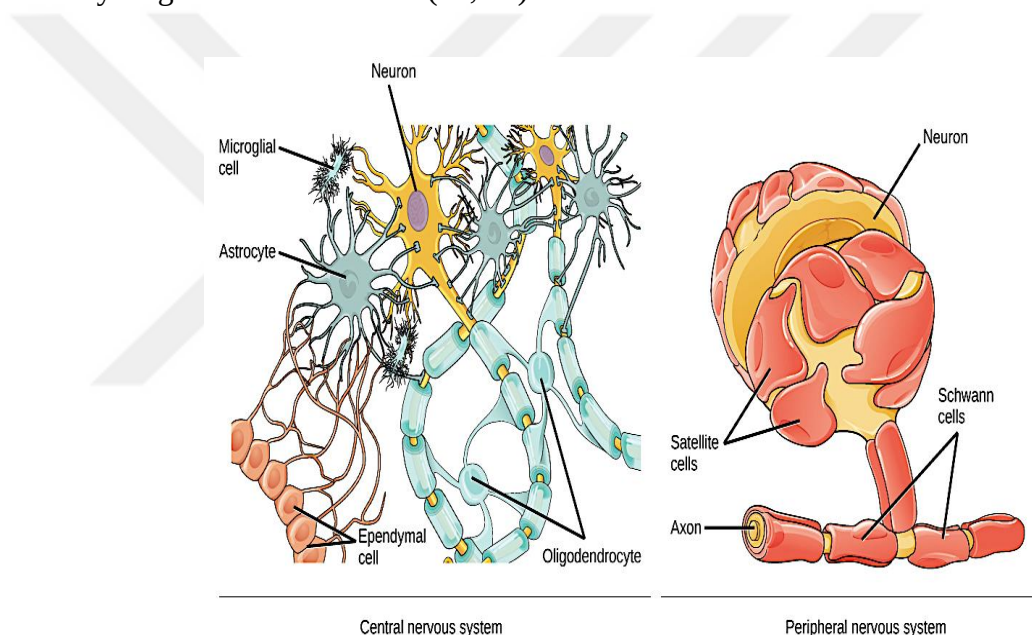


Figure 2.4. Neuron and glial cell in the CNS and PNS (20).

2.4. Peripheral Nerve Fibers

A nerve is a bundle of neuron's axons and its related Schwan cells in some certain cases, myelin sheath, together packaged up into a unit structure. Regarding the number of neurons, a nerve may be very thick or hardly visible. In addition to neuron and connective tissue, there are blood vessels supporting nervous tissue with nutrients. Within a nerve, there are bundles of the cluster of axons which are called a fascicle

(Figure 2.5.). The connective tissues that support a peripheral nerve trunk are as follows:

Endoneurium: Within a fascicle, each individual fiber is surrounded by a shapeless layer of loose connective tissue (10-20nm thickness), which is called endoneurium, and these nerve fibers could be either myelinated or unmyelinated. Endoneurium is without lymphatic vessels, therefore the pressure fluctuation of fluids in the endoneuria space has no impact on the conduction of impulse in the cytoplasm of an axon. Furthermore, it plays a significant protective role as well.

Perineurium: Every bundle of nerve fibers is enclosed in a permeable connective tissue that is called perineurium. This compartment of peripheral nerve tissue composed of perineurial cells and collagen. It isolates the endoneurium from extrafascicular tissues. It maintains the endoneurium optimum hemostasis, protects the constituent of the endoneurium and works as a barrier against external physical forces.

Epineurium: Finally, the entire nerve bundles or fascicles including blood vessels surrounding by a collagenous connective tissue called epineurium sheath. The internal epineurium retains the fascicles away from each other, at the meanwhile the external epineurium made a sheath around the cluster of fascicles within a nerve. The epineurium is a loose texture and lengthwise extended along the nerve. Epineurium itself rich of nerve and vessel and provide smooth motion of fascicles between each other. Epineurium is the main factor in expandability of the nerve but does not form a nerve-tissue barrier (21-23).

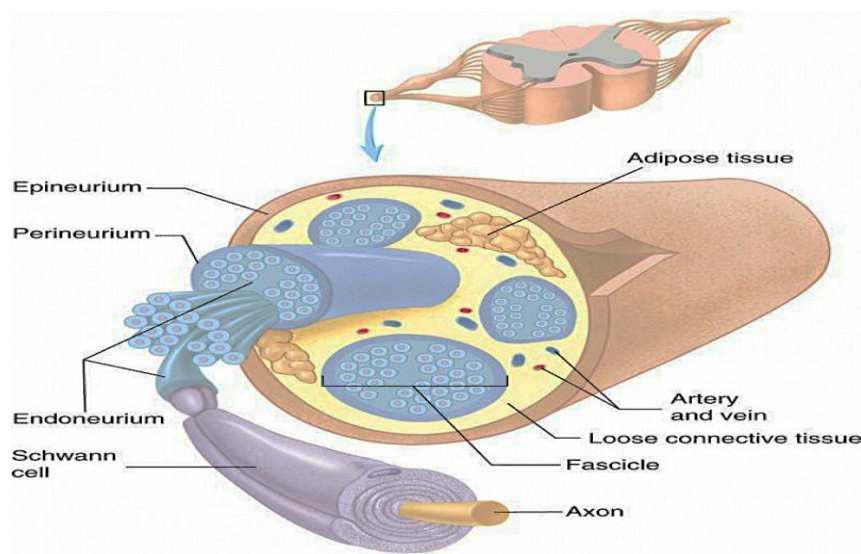


Figure 2.5. Anatomical structure of a nervous fiber (20).

The organization of fascicles within a nerve is considerable. In terms of numbers and thickness of fascicles, three type of nerves namely monofascicular, oligofascicular or polyfascicular exist in the peripheral nerves (24).

Schwan cells are abundant all over the PNS, mainly within the nerve stem. It is closely linked to neuron's axon and has vital importance for the neurons. Because of that reason, among the all glial cells, it has broadly studied. Schwan cells support the neuron in myelination of axons, lead the axons and remove the cellular residue. Unlike the neuron, they do not have the ability to transmit the messages. After the injury, uninterruptedly they enter mitosis phase (25). Unlike the oligodendrocytes in the CNS, Schwan cell myelinate only one fragment of a single axon.

Myelin sheath: Myelin (lipid-rich) sheath remarkably increase the conductivity of nerve, act as an insulator. Principally, it is required for quick saltatory impulse transmission along the axon. It protects the axon against physical stress and infection. Myelin is a multilamellar structure of plasma membrane of Schwan cell which is wrapped repeatedly around the axon. 60-75% (dry mass) of myelin sheath consists of lipid and 20-25% of this dry mass composed of proteins as the second essential constituent. Cholesterol is the basic ingredients of the lipid part and forms

more than 25% of total amount of lipid (26). The composition of the myelin sheath in CNS and PNS is similar but the number of some particular proteins are different. MBP and PLP are the main proteins in CNS, whiles MBP and PLP are existing in PNS but their functions are still unknown. The main protein in PNS is P0, and more than 50% of PNS myelin's protein is composed of P0 protein. It is the essential element in the synthesis and consolidation of the myelin sheath. PMP22 and Cx32 are also the major proteins in synthesis of the myelin sheath in PNS. In addition, there are some other proteins like MOG which is particularly found in CNS (18).

Myelin sheath is broken up by a small split called node of Ranvier. The length of the node of Ranvier is depended on the axon diameter. It is vary from approximately $1\mu\text{m}$ in the small optical nerve fibers up to $5\mu\text{m}$ in large fibers. At this region, the diameter of axon become narrower and naked (unmyelinated) but covered by protrusion of Schwan cell's cytoplasmic membrane (27). The myelinated segments between two nodes of Ranvier are called internodal segments. The thickness and length of internodal segments are directly depended on axon diameter (for instance, from $1\mu\text{m}$ in the case of a small axon and $5\mu\text{m}$ for thicker axon) (18).

In myelination process, the diameter of an axon is important, only the axon diameter which is larger than $1\mu\text{m}$ is myelinated by Schwann cells, whereas small diameter axons are just placed inside the troughs on the surface of Schwan cell. In this process, a single Schwan cell wraps around the several small axons and isolate them with a thin cytoplasmic membrane. The recent process is called as ensheathment and the axons are non-myelinated neurons (28).

In unmyelinated axon, the conduction of impulses is continuous and the upper limit of speed is 15m/sec . But in the myelinated axon, the nerve impulses move faster (120m/sec) and jump from one segment to another segment which is separated by the node of Ranvier. Electrical insulator (myelin sheath) prevents the conduction of electrical impulse to adjacent tissues. In addition, the node of Ranvier accelerates the speed of conduction (29).

Like other cells, a neuron is surrounded by a bilayer membrane which is composed of phospholipids and proteins. Due to lipidic nature of the membrane, charged molecules and ions cannot pass through this impermeable layer. Ions can enter or out the neurons through particular proteins which are called ion channels. Some of these channels are always open, regulate the exchange of particular ions and are named ungated channels. Some others are called as voltage-gated ion channels, and in response to alteration in electrical potential between inside and outside of the membrane, they are open and closed (8).

2.5. Action Potential

An action potential is a part of the signaling mechanism that starts from axon hillock and continues along the axon end. During this process, the membrane potential momentarily raises and subsequent falls. It requires the transport of the Na^+ and K^+ ions through voltage-gated ion channels along the axon membrane. Conduction of a nerve signal can be represented in three stages.

Normally, a nerve fiber or axon is maintained in the extracellular fluid. The axoplasm of the axon is surrounded by axolemma. Basically, the membrane (axolemma) is polarized, it means that the axon interior space is negativity charged and the outer space is positively charged. The interior side of the neuron is more negative (due to high concentration of K^+ ions) in contrast to the exterior side. In an unstimulated neuron (resting neuron), the voltage across the neuron membrane is about -70 mV (millivolts) and this is called resting potential. At this stage, the concentration of K^+ (inside neuron) and Na^+ (extracellular fluid) ions are not at the equilibrium level equilibrium. This inequality is maintained by Na-K pump channels, which regularly transport Na^+ out and K^+ inside the neuron.

Basically, in a neuron, stimulation begins from dendrites, where the stimulus is received and afterwards signals are propagated through the soma. This excitatory impulse at the dendrites activates the ligand-gated Na^+ channels and permits Na^+ to enter into the interior space of the cell. As a result, due to neutralization of some

negative charge ions in the interior space of the cell, the voltage becomes less negative on the cell membrane; and this phenomenon is called depolarization. The invasion of Na^+ into interior space of the neuron, make a stream that moves toward the axon hillock. If the excitatory signals are strong enough or by another meaning reaches to the threshold (-55mV), an action potential will be produced and the ligand-gated Na^+ channels are activated. Simultaneously the K^+ channels are also activated, but with a relatively slower performance. Because of the Na^+ influx, the charge become more positive on the inside space of the neuron and the neuron membrane becomes depolarized. At this stage, the voltage increases (around $+30\text{mV}$) and when the action potential reaches to its peak voltage, the Na^+ channels initiate closing. Instead, the K^+ channels are entirely opened and K^+ ions diffuse out of the neuron and the charge suddenly attenuates (repolarization stage) and becomes more negative (less than -70mV) and finally reaches to resting potential again (5, 8, 30-32).

2.6. Peripheral Nerve Injury

According to a published statistical report on Neuronal Regeneration Research journal on Year: 2015/Volume:10/Issue:11/ Page: 1777-1798 “*there are 10-15 million new trauma cases reported worldwide every year, of which 15-40% involve peripheral nerve injury. In China, 600,000-900,000 new cases of peripheral nerve injury are reported annually*” (33).

Peripheral nerves are complex structures which are comprised of neuron, glial cells, connective tissues and blood supply. These nerves spread out all over the body likewise an electrical network. They connect sensory organs, all voluntary and involuntary muscles to the central nervous system. These peripheral nerves are located outside the CNS, therefore, more exposed to injury. In PNS, three major types of nerves exists, which are classified as 1- motor nerves that govern the motion of

voluntary muscles; 2- sensory nerves that transfer information from sensory receptors (in skin, internal organs etc.) to the CNS; 3- autonomic nerves that govern the performance of autonomic organs (like heart, kidney, small and big intestine), are affected by some neuropathies in different degrees with a distinct range of symptoms (34). Peripheral nerve injury not only affects the neurons, but also damages the auxiliary cells like Schwann cell, satellite cells, and local blood vessels. Peripheral nerve injury causes motor, sensory and autonomic dysfunction of an associated organ. Therefore, it extremely affects the lives of an injured person. Sometimes these sensory and motor defects facilitate the determination of the injured side.

In general, the causes of peripheral nerve injury include an extensive range of physical trauma that can happen in various type of accident and surgical interventions. Nerve trauma may also be due to exposure to chemicals like alcohol overdose, agriculture, industrial and environmental chemicals. Furthermore, in some cases diseases, metabolic and vascular disorders and venom toxin events are also cause trauma (35). The results of the estimates show that more than 500,000 people are affected by peripheral nerve injury annually (36).

2.6.1. Classification

For a better management, understanding of peripheral nerve injury's pathological basis and recognition of the severest peripheral nerve injury that needs surgical treatment, a precise classification is required.

For the first time in 1943, Sir Herbert Seddon has proposed a classification for peripheral nerve injury. This classification was on the base of loss of nerve functionality, scale of damage to the nerve's components and whether nerve has the ability to recover. Considering the above-mentioned items, there are three main categories of nerve injuries:

Neuropraxia (demyelination): Neuropraxia is the minor compression of the nerve. The myelin sheath is compressed, axon and connective tissue maintain their structure, but the transmission of signal block is reversible. Because of that, motor and

sensor functionality of nerve may be lost temporarily. The recovery of nerve takes a few days to several weeks.

Axonotmesis: Axonotmesis is more severe than neurapraxia. All layers of connective tissues remain unscathed; however, demyelination and segment loss of axon may occur. The distal end will undergo Wallerian degeneration. The most outstanding syndromes are motor, sensory and autonomic dysfunctions. This type of injury is commonly seen in crush injury (bone fraction displaced).

Neurotmesis: Neurotmesis is the severest form of injury and contains complete discontinuity of the nerve. In this degree of nerve injury, all layers of connective tissue and axon are cutoff. Without surgical intervention, spontaneous recovery of nerve is not expected (37). In the same way, after Sir Herbert Seddon in 1951 Sunderland an Australian neuroanatomist, classified the nerve injury into five grads. Actually, he elaborates the work of Sir Herbert Seddon.

First- degree injury: Similar to neurapraxia, the nerve is just compressed and three layers of connective tissues and axon maintain their intact.

Second-degree injury: The axon is lost and endoneurium, perineurium, epineurium is preserved.

Third-degree nerve injury: Axon, Schwann cell and endoneurium are affected by injury but perineurium and epineurium keep their intact structure. Surgical intervention is needed.

Fourth-degree: In addition to axon, Schwan cells and endoneurium, perineurium are damaged as well. Only epineurium is left intact. It does not recover without surgery.

Fifth-degree: This type of injury is similar to neurotmesis (38).

Lastly, McKinnon and Dellon have suggested a combination classification and called the sixth degree of nerve injury. It indicates several degrees of injury that may

happen within the same nerve stem at the different parts of a nerve trunk. Moreover, each degree of nerve injury has special complexity and particular clinical symptoms. When the degree of injury is increased, this implies the intensity of lesion; and perhaps reduction of the rate of recovery (39).

According to some clinical experiences, the above classifications contain some diagnostic restrictions. The nerve damage may affect different segments with various intensity along the lengths of one nerve or several regional nerves trunk. Therefore, in the clinic, nerve injury may not match the proposed above models (40).

2.6.2. Response of Nerve After Injury

Peripheral neurons are one of the longest and the most complex cells in the body. For this reason, they are not able to perform all their vital activities alone. Therefore, the supportive cells must provide structural and metabolic support for them ten times more than common cell. On the other hand, if the injury occurs centimeters away from the soma, the cell response involves the rest of the neuron and affiliated cells (41). The response of peripheral nerve against injury is a very complex process, it involves several molecular and cellular alterations at the level of the neurons (including cell body, upper side and underside of the nerve cord, proximal and distal segments of the injury), Schwann cells, and other non-neuronal cells.

A couple of degeneration process accomplish at the site of injury before regeneration. In fact, this process can be a background for the start of the regeneration process. The rate of regenerative process outcome mainly depends on the severity or degree of nerve injury. In the first-degree injury, which contains temporary blocking of impulse conduction, pathological changes do not occur at all or happening slightly.

In the axonotmesis injury, at the injury site, Wallerian degeneration (WD) occur. WD is a primary response of PNS against injury. It happens when nerve losses continuity because of traumatic, metabolic, and toxic incidents. This process is initiated by entrance of Ca^{2+} ion from extracellular space, which causes the activation of calpains proteases family (42). Consequently, levels of neurotubules and

neurofilaments are reduced (43, 44). Within the first hours of injury, axon and myelin may start to degrade physically. After 48 to 96 hours, because of the discontinuity of axons, signals are not transmitted longer anymore (45). It suggests that, before the accumulation of macrophages to the injury region, the breakdown of axon and degradation of neurofilament proteins are in forwarding movement.

Schwann cells act a fundamental role in WD. When the axon losses its continuity, which is followed by an axonal fraction, Schwann cells start to divide myelin sheaths to small fragments. At the initial stage, the residues of axon and myelin sheath are eliminated by Schwann cells. Simultaneously, macrophages start to move to the injured site. When the macrophages' number reach to maximum, together with Schwann cells they begin to phagocyte the debris, and clean the region of injury. This process takes one week to several months (46).

After severing of axon either proximal or distal segments of damaged nerve undergoes some molecular and morphological changes. The transected segment of an axon, distal to the injury region is detached from the cell body and face WD. Thus, this part of axon slowly degenerates and finally gets absent and leave a complete endoneurial tube for Schwann cell growth. Axon degradation is an inherent exclusive active self-destruction process which proceed, completely independent of surrounding glial cell and connective tissue (47).

When Schwann cell loses its contact with axon, it vigorously proliferates and proceeds along the preformed endoneurial tube and makes Schwann cell column or band of Bügner (48). This generates an indispensable ambient that is full of trophic factors, that provides guidance for axonal regeneration (49). Furthermore, Schwann cells have the ability to separate the small spiral rings of myelin and produce lipid droplets (50).

In some studies, it has been demonstrated that a relationship may exist between excitation of Schwann cell proliferation and release of mitogens by macrophages and the degradation of neuron membrane (axolemma) fragments as well (51).

Cell body and proximal segment of injured nerve: The changes in soma and proximal segment of the injured nerve depend on the severity of injury and closeness of injured region to the cell body. In mild injuries, degeneration of proximal segment of the injured region is not unlimited and continuous up to the first node of Ranvier, but in severe injuries, it may go further towards the cell body. If the soma degenerated, the entire proximal portion will be facing degeneration.

Depending on the proximity of focal injury to the soma, apoptosis may also happen, whilst in intense injury, chromatolysis-change may occur (52). In this process the neuron switch from transmitting mode to regeneration mode. Morphological, molecular, and physiological changes are the features of this alteration. The most noticeable and steady morphological changes of soma are the loss of Nissl granules and endoplasmic reticulum degraded and scatter, nucleus moving to the outer edge of the neuron, nucleolar enlargement, and cell swelling. These changes in cell body are associated with some alterations in the proximal nerve. If the functional link to the relevant organ does not re-establish, the diameter of the proximal segment of the axon is decreased and in proportion to this, the intensity of conduction is also diminished and re-establishment of functional connections may be prolonged (53).

2.6.3. Inflammatory Response

Instantly after nerve injury, macrophages appear at the injured site and assist degenerated myelin debris removal. Schwann cells and macrophages eliminate the debris, moreover, they are responsible for secretion of required proteins (cytokines and neurotrophic factors) for axonal regeneration. The presence of macrophages in the affected area represents the existence of inflammatory reaction (54, 55).

Within the initial hours following injury, activated immune-like glial cells (Schwann cells) secrete pro-inflammatory cytokines. Interleukin 1α (IL- 1α), IL- 1β , IL-2, IL-6, leukemia inhibitory factor (LIF), macrophages inflammatory protein-1 (MIP-1), monocyte chemoattractant protein-1 α (MCP-1 α) and tumor necrosis factor (TNF- α) are represented at the primary phase of WD (41, 56). Particularly, TNF- α and IL-

1α levels begin to increase in the distal segment of nerve. Among inflammatory mediators, only $\text{TNF-}\alpha$ can be found in detectable levels in the uninjured nerve (57).

At the same time, anti-inflammatory cytokines (IL-10 and transforming growth factor- β (TGF- β)) are released by activated immune cells, like T cells, B cells, macrophages, and mast cells. The inflammatory process may be reduced through anti-inflammatory cytokines or prevention of the release of pro-inflammatory cytokines (58).

Naturally, in PNS Schwann cells produce myelin sheath around an axon, furthermore, resident macrophages and fibroblasts exist in a healthy nerve. These are the first cells that respond to nerve injury.

2.6.4. Nerve Regeneration

Glial cells of PNS and CNS show different reactions in case of injury. For instance, in CNS, several proteins that inhibit the axon growth are left in the axonal microenvironment and are not cleaned efficiently by the macrophage. Inversely, in PNS this critical myelin debris for neuron regeneration are removed properly by Schwann cells and macrophages. Schwann cells do facilitate the growth of axon. But in the CNS, glial cells do not support axonal regeneration. Thereby, PNS has a superior capacity for regeneration (59). Considering the results which have been obtained from various studies, the regeneration potential of CNS and PNS is related to various variables like glial cells, immune cells, effector molecules, passed time after injury, and extent of the injury (60).

After nerve lesion, successful nerve regeneration and functional connection of damaged nerve depend on various factors. These variables include, age of a patient, time after injury, level of injury, the intensity of injury, the gap between nerve ends, type of nerve, and associated injuries (61, 62). In relatively mild injuries, nerve regeneration begins almost immediately after an injury. On the other hand, in severe injuries, it begins after completion of the WD process. Nerve regeneration process

takes place at different levels of injured nerve including soma, the proximal segment of the injury site, injured region, and distal segment of injury (63).

After a nerve is damaged, recovery or reinnervation of targeted organs or skin occurs through three mechanisms: collateral sprouting of preserved axons, regeneration from lesion region, and remyelination. Collateral sprouting initiates within first 4 days and could be effective in the relatively mild lesion, with more than 70-80% surviving axons. The recovery may take approximately 2-6 months. When the degree of injury is increased with a ratio of 90%, the regeneration of affected axons may take longer time (64, 65). Successful functional and sensory recovery of organs depends on WD, axonal regeneration, and reinnervation of organs, where a damaged nerve must undergo.

Regeneration process may be represented into four anatomical zones: 1-cell body; 2-the lesion region; 3-the proximal segment of the injury; 4-the distal portion of axon between a damaged site and end organ (66). Regeneration and repairing of an injured nerve may take several months. After a while, some major changes in the cell body are clearly visible, that indicate the reversal of chromatolysis. The return of the nucleus to the original location, rearrangement of nucleoproteins into the compact Nissl granules are the remarkable signs in the soma (67).

Axonal regeneration is a complex and precise process. Attempt to begin the reconstruction of proximal root depends on the severity of the injury. It may start within 24 hours after injury, but in more severe lesions it may also start after weeks. The process leading to growth cone formation at the tip of the proximal trunk begins within hours post-injury. Locally available materials in the axon have a significant role in the formation of the growth cone and occur independently of the cell body. At this moment, cytoskeletal elements are transformed into daughter axonal extensions from proximal nerve stump (68).

Growth cone by bundling and extending actin filaments make a network called filopodia. They are finger-like and fine extensions of the growth cone. Growth cone is

very sensitive and has great capability to detect navigational cues. Axonal directional guidance is an important step in axon proximal tip regeneration process (64).

For the proper guidance of growing axons to reach their appropriate targets, the response of motile growth cones to environmental causes is very important. It is well established that growth cone acts a significant role in this process. Growth cone facilitates and guide the axonal growth by structures named as filopodia. Filopodia is covered by a membrane which is equipped with receptors and cell adhesive molecules that are important for axon growth and guidance (68).

Four mechanisms are involved in Growth cone guidance: contact-mediated attraction, chemoattraction, contact-mediated repulsion, and chemo-repulsion. These mechanisms are performed by different families of extracellular molecules. These molecules are either attractive and repulsive or inhibitory and including the Netrins, Slits, Semaphorins, and Ephrins. An example for inhibitory guidance molecules like Collapsin-1 that can lead to growth cone collapse (69, 70).

The rate of axonal growth depends on nerve location, regeneration process of proximal segment (2mm/day to 3mm/day) is faster than distal segments (1mm/day to 2mm/day). Growing axon that approaches the endoneurium tube in the nerve section distal to lesion region, have a higher opportunity of reaching the target organ (59, 64).

Soon after nerve lesion, the dissected axons close their ends and form end-corpuscle, thereby preventing the loss of axoplasm. Endcorpuscle secretes some constituents like calcitonin gene-related peptide (CGRP), nitric oxide and some other element to the local environment. CGRP acts as a local vasodilator of nerve micro vessels (71).

2.7. Nanofiber

The materials, having at least one dimension less than 100 nm are known as nanomaterials, their processing and use are under the perspective of nanotechnology. In some instances, a nanomaterial refers to a nanosized material while in other, a nanomaterial is a bulk material with nanoscaled structure. Nanomaterials include

nanoparticles, nanoclusters, nanocrystals, nanotubes, nanofibers, nanowires, nanorods, nanofilms etc (72). From geometrical viewpoint, nanofibers may place into category of one dimensional nanostructures that includes nanotubes and nanorods, as well (73).

According to national science foundation definition, nanofibers have at least one dimension of 100 nanometer (nm) or less (74) . By another mean, these are solid fibers with diameters from a few tens up to 1000 nm (75). The length of nanofibers can be as large as thousands of meters (76).

These amazing characteristics include very large surface area to volume ratio (for instance, nanofibers with ~ 100 nm diameter have a specific surface of $\sim 1000\text{m}^2/\text{g}$), small pore size, low density, remarkably high porosity, flexibility in surface functionalities and superior mechanical performance (77). These outstanding properties make polymeric nanofibers as good candidates for many applications. For instance nanofibers mats are now being considered for composite materials reinforcement, nanosensors (78), filtration, protective clothing, biomedical applications (including wound dressing, scaffolds for tissue engineering, implants, membranes and drug delivery systems) (79).

2.7.1. Fabrication of Polymeric Nanofibers

Several techniques have been developed for preparation of polymeric nanofibers. In general, the methods for nanofiber preparation may be classified as physical, chemical, thermal, and electrostatic fabrication techniques. Following methods are commonly discussed in literature:

- a) Electrospinning
- b) Self-assemble
- c) Phase separation
- d) Drawing of nanofibers
- e) Template synthesis

The general principles and applications regarding electrospinning methods are overviewed and existing literature knowledge is summarized in the following sections.

2.7.2. Electrospinning Technique

Anton Formhals was described the electrospinning method in a sequence of patents from 1934 to 1944 for the fabrication of textile yarns (77). In this method, the production of filaments is achieved by using electrostatic force (80). In other words, electrospinning is a process that creates nanofibers with diameters varying from 3 nm to greater than 5 μm , through an electrically charged jet of polymer solution or melted polymer (81). Electrospinning is a cost-effective, fast and straightforward method. It seems to be the only method, which is feasible for large-scale production of nanofibers for industrial applications. Basically, in electrospinning technique, a fluid either molten polymer or polymer solutions are drowned by an electrical force that applied to the fluids.

Electrospinning apparatus consists of four major components, *i*-high-voltage power supply, *ii*-flow control pump, *iii*-spinneret and *iv*-collector (Figure 2.6.). Regarding to the requirements, the collector can have different shapes. One electrode is placed to the spinneret where polymer's solution/melt is ejected and another electrode is attached to the collector part. In order to maintain electrospinning, high voltage (30 – 50 KV) is applied to the end of a spinneret containing polymeric solution/melt. Hereby, electric field between polymer solution or melt and the collector is created (82). By using syringe pump, an electrically charged polymer solution or melt is delivered to the tip of spinneret at a constant flow rate. As soon as the electric field overcomes a threshold value, the repulsive forces govern the surface tension forces within the solution or melt. Afterwards, a charged jet of the polymer solution is ejected from the tip of the Taylor (Taylor who has made essential studies on the jet formation Taylor 1969) cone. The trajectory of the ejected solution may easily be controlled by the created electric field. During the travel of jet in the air, the solvent evaporates from the polymer solution and continuous non-woven fibers are collected (83-85).

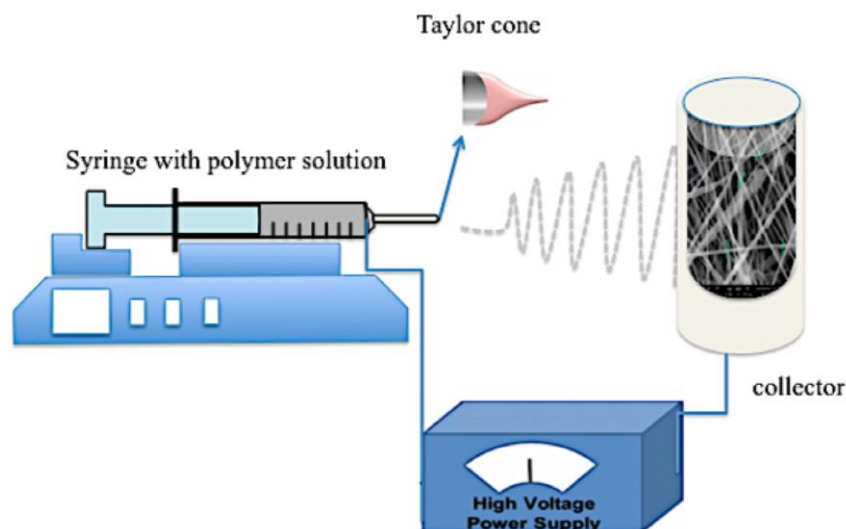


Figure 2.6. Schematic representation of electrospinning system (86).

2.7.3. Electrospinning Variables

Both extrinsic and intrinsic variables are known to affect the structural morphology of the nanofibers and they must be considered during electrospinning process regarding the final characteristics of the nanofibers. These method variables can be listed as polymers properties (the corresponding polymer molecular weight and concentration), solution properties like surface tension, concentration and viscosity, charge density, solution conductivity, equipment parameters (applied electric voltage, flow rate and the distance between the tip and collector) and environmental factors (temperature and humidity) (84-87).

▪ Polymer Properties

Polymer concentration and molecular weight are the most important factors, which are related to polymer solution properties. Polymer solution viscosity will increase with increasing either polymer concentration or molecular weight. Consequently, polymer solution will contain a great number of polymer chain entanglements, which are necessary to prevent the breakup of the jet during the electrospinning process (88).

In the low concentrations and molecular weight of polymer, only beads will be formed during the electrospinning process. By increasing these two factors, beads shape elongates and it changes to beadless fibers. A further increase will cause the formation of micro ribbon and thicker fibers (89). Thereby, electrospinning needs an optimum polymer viscosity; if the viscosity is too high, it is difficult to pump polymer solution through the nozzle. Moreover, polymer solution may dry at the nozzle tip before electrospinning process initiated.

▪ **Solvent System Properties**

The intrinsic solubility of the polymer is different in various single or blend solvent systems. Therefore, polymer solution may have different viscosity. Mixing solvents, having different surface tensions and dielectric constants, may be a suitable choice to optimize polymer solution properties for electrospinning. Thus, morphology and electrospinnability of polymers could be affected by type of solvent (90).

Surface tension, solution conductivity and dielectric constant of solvents are key factors in the electrospinning process, which cause either beaded or facilitate the electrospinning process of the polymer solution. Since conductivity and surface tension of polymer solution is adjustable, it could be one of the determining parameters for controlling fibers diameter (91).

▪ **Process Parameters**

In an electrospinning method, applied voltage and feed rate are related to each other. A stable “Taylor Cone” requires a suitable equilibrium between feeding rate and applied voltage. At high voltage, the tendency for bead formation will increase. It is also reported that, by increasing voltage, fiber diameter will be reduced (40). On the other hand, another study reported that fibers diameter increase non-linearly with the increasing voltage (92).

Electric field strength and jet flight time depend on the distance between nozzle and collector. Distance does not have any significant effect on fibers' diameter but reducing distance cause formation of elongated beads along fibers (93).

Depending on polymer chemical nature and fluctuation of humidity, fiber diameter may either decrease or increase. In lower humidity, solvent evaporates rapidly and fiber diameter becomes thicker, whereas in the higher value of humidity solvent evaporates slowly, and fiber diameter decreases. Temperature may change evaporation rate of solvent and viscosity of polymer solution, which are two main factors that have effects on formation and diameter of fibers (94, 95).

Different types of numerous polymers may be used for fabrication of fine nanofibers. Natural polymers, synthetic or mixture of both are used in electrospinning method. Water soluble, insoluble, synthetic and natural polymers, especially biodegradable biocompatible polymers like poly (N-vinylpyrrolidone) (PVP), poly (ethylene glycol) (PEG), polyvinyl alcohol (PVA), polyethylene oxide (PEO), poly-(glycolic acid) (PGA) and their copolymers, are ideal candidates for fabrication of nanofibers. Natural polymers, such as collagen, gelatin, elastin, silk fibroin, spider silk, fibrinogen, chitosan, hyaluronic acid and alginate, are more suitable for electrospinning nanofibers (82-84).

▪ **Environmental Variables**

Moisture and temperature are among the environmental factors that have remarkably high impact on nanofiber morphology. For instance, when the humidity increases, the water vapors will condense on the surface of the jet. Eventually, after evaporation of both water and solvent, some pores will form on the surface of the fibers.

The rate of evaporation of the solvent in the polymer solution is also related to environmental humidity. When the humidity reduces, the solvent may dry quickly, the solvent will evaporate before Taylor cone formation. Eventually, the nozzle tip may clog (96).

Solution temperature extremely effects on the viscosity of polymer solution. On the other hand, by increasing temperature the surface tension of liquid would

reduce. When the viscosity of polymer solution decreases the fiber diameter will also decrease (97).

2.7.4. Physical Strength of Nanofibrous Scaffold

The physical strength of the polymeric electrospun nanofibrous scaffold is related to some factors, like diameter of a single nanofiber, the pattern of nanofibers in the scaffold (alignment and random), density of nanofibers, composition and molecular structure (98). For instance, poly(d,l-lactic-co-glycolic acid 50:50) nanofibrous scaffold is more rigid than poly(epsilon-caprolactone) structured scaffold (99). By reducing fibers diameter both strength and stiffness may be increased. As reported by Chew and colleagues, kind of encapsulated drugs is another factor which has an impact on the mechanical strength of the individual fiber. The mechanical strength of a single fiber composed of poly(epsilon-caprolactone), and its copolymer, poly(caprolactone-co-ethyl ethylene phosphate) were increased as the diameter of fiber reduced to 200-300 nm. In addition, the mechanical strength of fiber composed of poly(caprolactone-co-ethyl ethylene phosphate) was increased proportionally when the concentration of retinoic acid was increased (1 to 20%; w/w). Whereas, an inverse result was obtained by increasing the concentration of bovine serum albumin (10–20%; w/w) (100). Alignment of nanofibers in a scaffold has a significant effect on the mechanical strength of nanofibrous scaffold. It was previously reported that aligned nanofiber scaffolds have higher tensile strength than random nanofibrous scaffolds (101).

2.7.5. Degradation of Nanofibers

Nanofiber degradation can be assessed from two different aspects. Firstly, degradation of an individual nanofiber is directly related to polymer properties. The majority of synthetic polymers have either crystalline and amorphous parts (semi-crystalline). In the hydrolysis process, when the water penetrates into the nanofibers, the long polymer chains are broken down from the weak portions (amorphous region). The long polymer chain becomes shorter and converted into water-soluble species. As hydrolysis continues, the nanofiber gradually begins to disintegrate and eventually

disappear. In the case of enzymatic degradation, enzyme digest the nanofibers and as a result, the mass of nanofibers rapidly falls down (102).

Secondly, in the form of a scaffold, the electrospun nanofibrous scaffold has higher porosity when compared with a thin film which is made from the same polymer. Accordingly, the degradation agents diffuse away more rapidly. Otherwise, acidic degradation agents, play a catalyst role and accelerate the degradation process. Consequently, because of the difference in porosity, electrospun nanofibrous scaffolds need longer time to degrade than the bulk film that is made of same polymer and mass (103, 104).

2.7.6. Drug Loading into Nanofibers

Drugs molecules can be incorporated within electrospun nanofibers or adsorbed on the surface by using different ways, which also include post-treatment of nanofibers (105), immobilization of drug-loaded nanocarriers to nanofibers (106), co-electrospinning of drug-polymer blends (107), coaxial electrospinning (108), and emulsion electrospinning (109). These methods provide different loading ratios, but the main reason for the selection of loading method is the physicochemical properties of the drug and polymer molecules.

2.7.7. Post-Treatment of Nanofibers

Drugs can be easily immobilized on the modified surface of the prefabricated nanofibrous matrix. Drugs are bound or conjugated to the fiber surfaces by hydrogen bonding, physical adsorption, electrostatic, hydrophobic and van der Waals interaction forces. The additional requirements for this method include modification of the polymer properties in three different pathways, which are named as *i*-plasma treatment, *ii*- chemical treatment or *iii*- surface graft polymerization.

Controlling the amount of immobilized drug, preservation of the functionality of immobilized biomolecules, prevention of the problem for initial burst release and short release time are the advantages of this strategy (110, 111).

2.7.8. Electrospinning of Blend Drugs and Polymer

In comparison with the other loading techniques, electrospinning of drugs with polymers as a blend is simple, rapid and one step manufacturing process. Drugs, either as solution or suspension/emulsion form are prepared in a single step with polymer solution and electrospun together for the manufacturing of nanofiber formulations. Physicochemical properties of polymers and compatibility with drugs molecules are important factors for a successful encapsulation of drugs into nanofibers (112).

Various therapeutic agents such as antibiotics, anticancer drugs and NSAIDs may be encapsulated into nanofibers with this preparation method. Presence of organic solvents causes denaturation, especially for sensitive proteins and cytokines. Whilst, Zeng et al., used water-soluble polymers such as polyvinyl alcohol (PVA), polyoxalate (POX) for embedding of sensitive proteins into nanofiber matrices (113).

2.7.9. Coaxial Electrospinning

By the development of a new electrospinning technique that contains a specialized nozzle system, it is achievable to overcome the burst release drug from nanofibers. This modified electrospinning process includes coaxial electrospinning (114) and side-by-side electrospinning (Layer-by-layer nanofibers) (115).

Coaxial electrospinning enables to produce core and sheath nanofibers for loading multiple drugs or by another mean, facilitate the encapsulation of a component within another. Coaxial nozzle contains inner and outer needle that separate two different solutions. This system is suitable for simultaneous electrospinning of two immiscible polymers solutions containing different drugs. By this method, it is possible to minimize the interaction possibility of biologic therapeutic molecules with the organic solvent of the polymers. The shell not only provides prolonged release of the drug but also protects the core ingredient from direct exposure to the biological environment (116). Hongliang et. al. (117), focused on encapsulation of bioactive agents (DNA, growth factors) and even living organism like cells, virus, bacteria, cell organelles into core-shell nanofibers.

2.7.10. Emulsion Electrospinning

The emulsion electrospinning contains water phase (micron or sub-micron sizes droplet of drug solution), which is referred as drug aqueous solution, and the oil phase, which is obtained by dissolving polymer in organic solvents. After electrospinning of w/o emulsion, water phase (dispersed droplet in the emulsion) could be elongated and converted into core and oil phase of emulsion (continuous phase) become the shell of fibers. Emulsion electrospun fibers can be manufactured by a single needle (118).

In conventional electrospinning, polymer concentration acts a key role to obtain continuous and uniform nanofibers. The polymer solution's chain entanglement density must be at a high level that is enough to form nanofiber structures. Increasing polymer concentration could reduce bead formation, but further increasing cause clogging of the nozzle tip and prevent electrospinning process. Unlikely, electrospinning of polymers with low molecular weight or dilute solution of polymers is possible by using emulsion electrospinning.

Surfactants, which are used in the preparation of nanofibers, significantly affect the morphological structure and mechanical characteristics in emulsion electrospinning method. Due to the significant impact of surfactants on rheological or electrostatic properties of the polymer solution, regulation and control of the process of emulsion electrospinning are possible. Thereupon, the existence of suitable and adequately amount of surfactant as a significant component of nanofiber formulation is required (109).

2.7.11. Electrospinning Setup Modification

A typical electrospinning setup that has mostly been used in the research laboratories is generally composed of simple equipments. It is designed for utilizing a polymer solution or melt, while the basic electrospinning is not suitable for spinning off some polymers and tailoring the structure of manufactured nanofibers. As far as electrospinning technique is a flexible method, it could easily be modified for different

purposes. Accordingly, the modifications which are carried out especially on nozzle and collector parts.

Fibers structure could be controlled by modification of spinneret. For example, as in side-by-side electrospinning, two different polymers solutions may be separately pumped to the spinneret, where the electrospinning process begins (115). A single jet with slow flow rate is time-consuming and express inefficiency of electrospinning technique. To solve this drawback, multiple needles or multiple-jet equipment were developed by researchers. Shiratori et al. have prepared a nanofibrous mat from the blend of poly(vinyl alcohol) and cellulose acetate through multi-jet electrospinning (115). Coaxial electrospinning and emulsion electrospinning are other approaches to modify the structure of nanofibers that has been already explained.

The second approach to control the structure of nanofibrous mats is the modification of the collector. Normally, fibers are collected on the surface of flat collector randomly and the obtained mats would be nonwoven. The alignment of fibers in a scaffold is a key factor in tissue engineering. Modification of collector could lead to production of highly ordered and aligned polymeric nanofibrous mats. Rotating cylinder, frame collector, wheel-like-disk, are the examples of a modified collector (119, 120)

2.7.12. Drug Release Kinetics

Controlled release drug delivery is an effective process to reduce toxicity and side effects, balance the release profile of drugs and consequently, improve patient convenience. In electrospun nanofiber formulations, generally drug release is maintained by one of these mechanisms: *i*- desorption from the surface of nanofiber structures, *ii*-diffusion through the channels of pores existing on nanofibers or matrix degradation of the nanofibers (121).

With a view to drug loading techniques into nanofiber matrix, two main drug distribution geometries are distinguished. In the first one active ingredient is homogeneously distributed into the matrix of polymers such as drugs and polymer

blend electrospun nanofibers. The second one is the reservoir type in which drug core is surrounded by polymeric matrix. This option is generally observed in nanofibers that are manufactured by coaxial electrospinning.

In most of nanofiber-based drug delivery systems, drug release occurs by diffusion-controlled mechanism, which contains a matrix-type system and the reservoir-type system. In the first system (matrix), drug release starts with an initial burst release, which is further followed by a slow and controlled release. On the other hand, in a reservoir type system, it provides a relatively constant release of a loaded drug across the barrier.

Furthermore, in the case of biodegradable polymers, polymers are subjected to degradation that is followed by erosion. Degradation takes place either by surface erosion of electrospun polymeric nanofibers or bulk erosion type, in which degradation occurs all over the matrix structure (122).

Drug release kinetics, strongly depend on many factors like, nature of polymer, polymer swelling and erosion behavior, fiber geometry, factors related to drug (drug dissolution and diffusion characteristics, drug distribution in the polymer matrix, drug matrix interaction, initial amount of loaded drug), and drug-polymer ratio (82).

2.7.13. Application

Because of unique characteristics, electrospun polymeric nanofibers cover a wide range of application. The application area of nanofibers includes filtration, affinity membranes and recovery of metal ions, energy storage, sensors, catalyst and enzyme carriers, tissue engineering, wound healing and controlled release drug delivery. In the following sections; drug delivery applications of polymeric electrospun nanofibers have been focused on (123).

2.7.14. Drug Delivery Applications

Drug delivery is one of the main and important areas of research in the biomedical application of nanofibers. Therapeutic efficacy, safety and attenuated

toxicity are the outstanding objectives of controlled drug delivery systems. Improving therapeutic efficacy of the drug is feasible by delivering a predetermined amount of the drug in a controlled release manner to the site of action over an adequate period.

During the treatment, dose frequency of conventional dosage forms causes fluctuation of drug concentration in systemic circulation; therefore, the concentration can exceed the recommended maximum value $C_{\max}$, or fall below the minimum effective concentration, $C_{\min}$. In order to minimize these drawbacks and improve therapeutic response of drugs, retaining a desired concentration of drug through various delivery systems have been investigated.

Nanoscale formulations, which are designed for controlled delivery of drugs, offer promising techniques to achieve optimized therapeutic outcome and improved patient compliance. Nanofibers are one of these forthcoming nanoscale formulations. They are very attractive depending on their significant characteristics as high drug loading capacity, high encapsulation efficiency, simultaneous delivery of divers drugs having different physicochemical characteristics, simplicity of manufacturing, cost effectiveness and structural similarities to the extracellular matrix. For this reason, nanofiber structures became one of the most interesting areas that many researchers have been recently focusing on. Within the last decade, nanofiber structures are being widely used in tissue regeneration and drug-delivery applications having a constantly increasing trend.

Researchers have conducted many approaches to improve loading capacity of drugs in nanoscale systems, however it still keeps to be a challenge. Large specific surface area and porosity of nanofibers could increase the total portion of loaded drugs. In addition to that, physical flexibility, which is controllable by changing process parameters and material variables, provides a regulated drug release from nanofiber formulations.

Local delivery of antibiotics and anti-cancer drugs attenuate cytotoxicity and improve the pharmacological effect of incorporated drugs. In the following sections,

literature findings on the combination of nanofiber dosage forms with the drug delivery options have been overviewed.

2.7.15. Local Delivery of Antibiotics Other Than Wound-Dressing

Purposes

In local drug delivery, the usage of polymeric electrospun nanofibers as a carrier for antimicrobial agents is a promising approach for the treatment of various disorders. Among these, infection due to bacterial contamination after orthopedic implantation and surgery is one of the significant areas in which nanofibers take place. Usually, for preventing infections after implantation and surgery, antibiotics are administered systemically that is likely associated with bacterial resistance, toxicity and need for an optimum concentration of drug (124, 125). For this reason, localized controlled released antibiotics delivery is suggested.

Zhang et al. (126), developed an antibiotic coated titanium implant by electrospinning technique. In this study, Vancomycin was used as a model drug and poly(lactide-co-glycolic acid) (PLGA) (50:50) with a molecular weight of 80,000 Da was used to prepare antibiotic-loaded nanofibers. Similarly, in another study, Kuo-Sheng Liu et. al. (127), presented a novel biodegradable (PLGA) vancomycin-eluting nanofiber-loaded vascular prosthetic grafts.

2.7.16. Antibiotic Loaded Nanofibers for Wound Dressings Applications

An ideal wound dressing material should protect the wound from contamination, accelerate the healing process and prevent skin disfiguration, absorb exudate of lesion's environment, and cosmetically improve skin appearance (128). Chronic wounds require a sustained delivery of antibiotics for an effective treatment. Local delivery of antibiotics for a certain period of time is more effective, safe and preferable than systemic administration.

Nanofibers, which are prepared by electrospinning, showed great influence on the wound healing acceleration because of maintaining the release of antibiotic in a controlled manner directly at the injury site (129). Furthermore, antibiotic-loaded

fibrous membrane may provide enough mechanical strength, antibacterial activity to minimize infection (130). Nanofibrous wound dressings offer superior advantages such as hemostatic properties, absorbability and semi permeability for respiration of cells, better conformation to the wound surface and the potential for leaving no scar.

A variety of antibiotics like Neomycin (131) Streptomycin (132) Tetracycline hydrochloride (133) Nitrofurazone (134) Enrofloxacin (130) have been loaded into natural and synthetic polymers for preparation of electrospun nanofibrous scaffolds.

2.7.17. Local Delivery of Chemotherapeutic Agents

Currently, the removal of solid tumor is achieved by surgical resection and to minimize the risk of recurrence, this procedure is generally followed by chemotherapy or radiation therapy. Accordingly expanding the tumor exposure time and local delivery of chemotherapeutics drugs may improve the outcome of chemotherapy either for resectable and unresectable tumors

For this reason, electrospun polymeric nanofibers loaded with anticancer drugs, especially in postoperative local chemotherapy, not only provide high local concentration with incorporation of small amounts of the drug but also reduce the administration frequency, toxicity and side effects. Therefore, local chemotherapy by applying electrospun polymeric nanofibers may be suggested as a promising method in the treatment of cancer (135).

Liu et al. (107), have proposed one-step easy and feasible electrospinning of doxorubicin and polylactide solution, aiming to investigate whether doxorubicin-loaded polylactide electrospun nanofibers may be an option for a local chemotherapy system against unresectable liver cancer. Hereby, a remarkable antitumor effect was observed from direct implantation of doxorubicin loaded fiber mats on the liver surface of mice.

Unique properties of electrospun polymeric nanofibers allow the simultaneous or combination therapy of more than one active substances with similar or different therapeutic effect for improvement of the treatment result and therapeutic outcome.

The independent controlled release of drugs is the most important challenge, particularly in cancer or other complex diseases (136). Regarding to this subject, Luo et al. (137), has combined combretastatin A-4 (CA4) (a vascular disrupting agent) and hydroxycamptothecin (HCPT) (a chemotherapeutic drug) to observe their cooperative or synergistic effect in a nanofiber formulation. The release of CA4 and HCPT were extended through the structure of fibers for dual drug loadings and the inoculation of 2-hydroxypropyl- β -cyclodextrin in fiber matrices. In an orthotopic breast tumor model, all the CA4/HCPT-loaded fibers showed significant antitumor effect and higher survival rate than fibers with loaded individual drug.

2.8. Preparation Techniques of Polymeric Nanoparticles

In drug discovery area, investigation of various new therapeutic moieties is in ascending path. Therefore, the demand for a delivery system which guarantees the stability, high loading efficiency and targeted delivery of therapeutic agent also increased in accordance to that.

Because of small size and unique properties, polymeric nanoparticles have been widely investigated for delivery and sustained release of these chemical, biological and genetic new therapeutic moieties. Regarding the requirement of the application, nature of polymers and physicochemical properties of the therapeutic substances, several preparation techniques have been proposed for nanoparticles. These techniques mainly divided into two sections. *i*- Polymerization or bottom-up methods *ii*- Top-down methods (138).

In the first technique, monomers are joined together by the chemical reaction, and they generate a long polymer chain. The polymerization process can be performed in several manners, emulsion polymerization, interfacial, and precipitation polymerization. The presence of some toxic residues and insufficient biodegradability are the shortcomings of this method which confined the use of this method for preparation of polymeric nanoparticles.

Several variables associated with polymeric nanoparticle preparation methods have effects on physical characteristics like particle size, surface morphology, drug loading and entrapment efficiency, and release profile of drug molecules. Therefore, a good understanding of these parameters could be leading to modify and characterize the nanoparticles.

Basically, polymeric nanoparticle preparation methods contain two different approaches. In the first approach, nanoparticles form spontaneously, whereas in the second approach the principle is based on emulsification and emulsion formation is needed as a start point of the process (139, 140).

2.8.1. Nanoprecipitation (Solvent Displacement)

One of the requirements of this method is the usage of a miscible organic solvent with the aqueous phase. A hydrophobic preformed polymer is dissolved in the organic phase and together with an intended drug is added dropwise into the aqueous medium that contains a stabilizer along with gentle stirring. After diffusion of the organic phase in the aqueous phase, and immediate precipitation of hydrophobic polymer and drug, the nanoparticles are formed. This method is not efficient for water-soluble drugs because the drug may remain in the aqueous phase and as a result encapsulation efficiency may decrease. (141).

2.8.2. Coacervation Method

Coacervation is a self-assembly method and based on the difference in protein solubility in the aqueous solution and desolvating agent. A desolvating agent (the material which causes the precipitation of a protein like alcohol, sodium sulphate etc.) is added on a protein solution and afterwards, the protein starts to shrinking and become smaller and smaller that results in the formation of protein nanoparticles slowly. Coacervation methods mostly used for the preparation of protein nanoparticles. In this regard, gelatin nanoparticles were prepared by coacervation technique having a typical diameter 45 ± 5 nm (142). In addition, this method was implemented for preparation of human serum albumin nanoparticle (143).

2.8.3. Ionic Gelation Method

This method is based on the ionic crosslinking interaction between oppositely charged molecules. Ionic gelation consists of two aqueous phases, one of them containing a biodegradable hydrophilic polymer like (chitosan⁺, sodium alginate⁻) and the second aqueous phase that is comprised of a polyanion material such as (sodium tripolyphosphate, calcium chloride). After the combination of two phases, a physical crosslinking is formed between positively charged groups of the polymer and the negatively charged group of the polyanion and it leads to the generation of nanoparticles (144). Ionic gelation method is mostly used for production of chitosan nanoparticle (145). Hydrophilic nanoparticles which are prepared by ionic gelation have great potential as a carrier for delivery of hydrophilic protein. They have an outstanding capacity for the entrapment of big therapeutic molecules (proteins) and provide an extended release of loaded drugs (146).

2.8.4. Preparation of Polymeric Nanoparticle Based on The Emulsification

Formation of an emulsion is the start point of this approach which is followed by homogenization. At this step of this method, homogenization, which is conducted by a high-pressure homogenizer, microfluidizer and sonicators, determines the size of resulting particles. Organic solvent miscibility and properties of the polymer to be used, are two fundamental phenomena in emulsification-based approach.

In some techniques (double emulsion, emulsification solvent evaporation) a water-immiscible organic solvent utilizes for the dissolving of a hydrophobic polymer that facilitates the separation or evaporation of organic solvent from the medium and leading to precipitation of the polymer. In some other techniques (salting out, emulsification diffusion) relatively or total water-miscible solvents are utilized. After diffusion of organic solvent into the aqueous phase, precipitation of polymer occurs (147). These techniques are as follows:

2.8.5. W/O Emulsification

W/O emulsion is produced with emulsification of an aqueous solution of hydrophilic macromolecules like albumin, in the oil phase (vegetable oil). Afterwards, w/o emulsion is added drop by drop into the preheated ($>120\text{ }^{\circ}\text{C}$) oily medium under stirring. The heat causes rapid evaporation of water in the droplet and coagulation of hydrophilic polymer. In the end, the dispersion is allowed to cool down and the nanospheres are gradually solidified (148). Doxorubicin loaded PLGA nanoparticles were also prepared by single and double emulsion methods. Doxorubicin encapsulation efficiency and release profiles were evaluated comparatively for those nanoparticles formulated by single and double emulsion method. These results indicate that the encapsulation efficiency of doxorubicin was increased in nanoparticles formulated by single emulsion and the burst release was decreased (149).

2.8.6. Emulsification-Solvent Evaporation

This technique was firstly developed by Gurny et al. in 1981 and the preformed polymers were used for the production of nanoparticles. The preparation of organic phase involves the dissolution of hydrophobic polymer and drug in a volatile and water-immiscible organic solvent. Afterwards, this phase is emulsified in an aqueous phase containing a stabilizer. Emulsification is performed by sonication or by any other type of homogenizer. The size of droplet directly depends on the homogenization process. After removing the volatile organic solvent through evaporation or under vacuum, polymer precipitation occurs that leads to nanoparticles formation (141-150).

2.8.7. Emulsification-Solvent Diffusion

In this method, polymer and drug are dissolved in a partially water-miscible organic solvent. As result, the oily or organic phase of the emulsion is produced and following to that it is emulsified in an aqueous phase comprising an emulsifying agent. In order to disturb the miscibility ratio of organic solvent, a significant amount of water must be added to the emulsion system. Consequently, organic solvent diffuses into the

continuous phase (external phase) and after polymer precipitation, nanoparticles are formed. This technique is mainly appropriate for hydrophobic drugs (141-148). PLGA nanoparticles containing estrogen were prepared by the emulsification-diffusion method and the effect of processing conditions on the mean particle size was evaluated. The size of the resulted nanoparticles was less than 100 nm (142).

2.8.8. Salting-Out Method

The salting-out method could be the modified form of emulsification-solvent diffusion method. Unlike the previous method, in the salting-out method, a water-miscible organic solvent like acetone is used. Primarily, polymer and drug are dissolved in a water-miscible organic solvent (acetone, ethanol etc.) and subsequently emulsified in the aqueous phase that comprises either salting-out agent or and stabilizer. In order to increase the diffusion of organic solvent into the aqueous phase, the o/w emulsion system is diluted with an adequate amount of water, thus leading to nanoparticle formation. Magnesium acetate, magnesium chloride, calcium chloride may be used as salting-out agents and polyvinyl pyrrolidone, hydroxyethyl cellulose could be mentioned as a stabilizer (141).

2.8.9. Nano Spray Drying Method

Considering the physical and chemical properties of the polymer and drug, nanoparticles synthesis has been designed. Endless efforts are being made to prepare an ideal nanosystem. For the preparation of nanoparticles with a characteristic of interest, recently researchers have proposed very attractive and sophisticated techniques. These methods have technical bases; hence they are more suitable for industrial production.

In addition to preparation of nanoparticle in liquid media (solvent displacement and emulsification solvent evaporation), a mechanical method (Nano Spray Dryer) have been utilized to synthesize nanoparticles. Organic or aqueous solution of polymers and drugs or nanoparticles suspension may be converted to nanopowders. Basically, this method consists of the production of a liquid droplet and subsequently

exposure to the hot air stream that is followed by evaporation of utilized solvent resulting in solid product form (147).

Nano Spray drying is a liquid atomization-based method that uses high energy to produce ultrafine particles. Nano Spray Dryer B-90 was developed by Büchi (in 2010) that opened a new door in spray drying technology (Figure 2.8.). Three technological novelties were involved within this device, which were 1-A vibrating mesh spray with precise micron size holes (4 μ m, 5.5 μ m, 7 μ m), 2- Electrically charged collector, 3-Generating a laminar air flow in spray chamber differentiate Nano Spray Dryer B-90 from conventional spray dryer.

The laminar flow is produced by crossing air through a well-set and condensed porous metal bed. This heating system has a fast heat up rate and provides an optimal energy input. Furthermore, vertical setup of spray dryer simplifies the downward movement of particles and subsequent accumulation into the extremely charged collector, which aid to reduce sticking of particles to the inner surface of the glass chamber, as a result, the production yield is increased.

The separation of manufactured particles from air flow is performed in an electrostatic collector, where electrostatic force causes particles to absorb independently of their mass on the inner surface of the collector. Basically, collector consists of two parts 1- a grounded star shape electrode (cathode), 2- a cylindrical collector as an anode (Figure 2.7.).

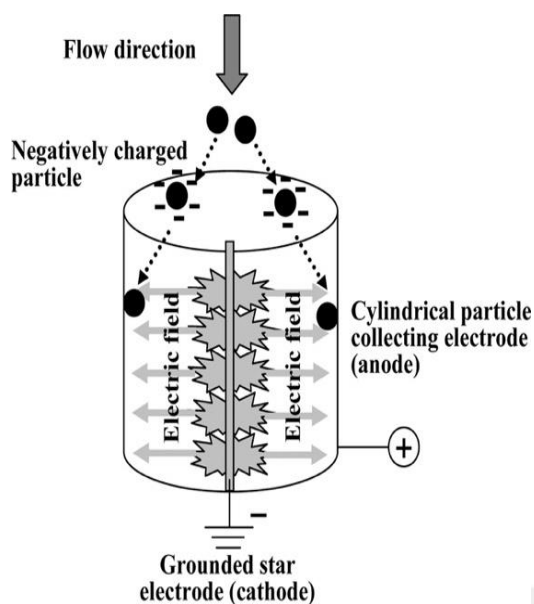


Figure 2.7. A schematic representation of Nano Spray Drying collector (151).

These technological novelties of Nano Spray Dryer B-90 highlight numerous advantages, like producing particles with small size and high yield, processing of small volumes in the primary phase development of a drug candidate, over the classic one (152).

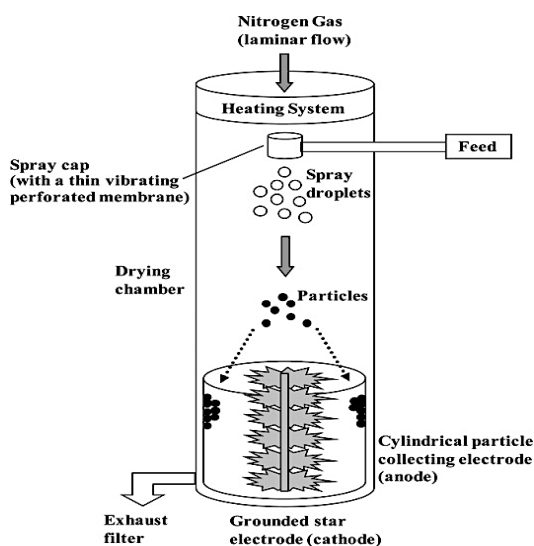


Figure 2.8. A schematic representation of Nano Spray dryer (151).

Material solution or dispersion is fed by a peristaltic pump, and afterward millions of homogeneous droplets with small size distribution are generated by vibrating mesh spray. Up and down movement (vibration) of spray mesh provided by a piezoelectric driven actuator with ultrasonic frequency. Generated droplets follow the stream of a laminar drying air flow in the spray chamber, after drying fine particles are separated by an electrically charged collector from the stream of drying air (153).

Moreover, Nano Spray drying method is a one-step, continuous and flexible process that allows the manipulation of particle's characteristics by adjustment of process and formulations variables.

For instance, Mulla et al. (154), have used Nano Spray drying method for preparation of rifampicin-loaded nano-lipomer composites of palmitic acid and poly caprolactone. PLGA loaded phosphodiesterase V inhibitor sildenafil nanoparticles, through the similar technique, were characterized by Broichsitter et al. (155). Furthermore, Bürki et al. (156), optimized process parameter and formulation to produce particles of the highly sensitive material (biopharmaceuticals)

2.9. Neuroprotective Drugs

The relative maintenance of neuronal structure and function is referred to as neuroprotection. In other words, neuroprotective agents inhibit neurodegeneration and prevent progression of the disease/condition. The term "*neuroprotective agents*" contains numerous drugs that are relevant to different pharmacological groups.

A significant number of neuroprotective agents are under investigation and are being evaluated for their effectiveness to reduce neuron damage after injury models. The neuroprotective agents are pharmacologically comprised of calcium channel blockers, corticosteroids, glutamate antagonists, and free-radical scavengers (157).

2.9.1. Atorvastatin Calcium

Atorvastatin calcium (ATR) is a member of statin family; and from the physical viewpoint, it is a white and crystalline powder. It shows the highest solubility

in methanol, chloroform and acetone 232.8 mg/mL, 216.8 mg/mL, 215 mg/mL respectively. Conversely, it shows the lowest amount of solubility in hexane and water (0.3 mg/mL, 0.8 mg/mL). ATR is not soluble in aqueous solutions at pH 4 and very slightly soluble in distilled water and PBS at pH 7.4.

The stability of ATR in the crystalline form was evaluated at 80°C and 40 °C, 75% RH over a time period of 8 weeks. After two months, no considerable variation in the initial assay value was reported (158).

ATR has a fast oral absorption rate that approximately happens within 1-2 hours and its concentration in plasma reaches to steady state. The absolute bioavailability of ATR is around 14%. First-pass metabolism and relatively high intestinal clearance are the major reasons of low bioavailability. The rate and extent of ATR absorption are extremely affected by foods (159).

ATR is high bound to plasma proteins with value of $\geq 98\%$. The 70% of HMG-CoA reductase inhibition is achieved by active metabolites of ATR. Ortho and para-hydroxylated derivatives and different oxidation product are the metabolites of ATR. The plasma half-life of ATR is 14h and the half-life of the active metabolite is 20-30h (160).

Statin family has both hydrophilic or lipophilic members. The hydrophilic group includes fluvastatin, mevastatin, and cerivastatin, while lipophilic members are simvastatin, lovastatin and ATR, considering having a lipophilic nature makes them easily cross the blood-brain barrier.

Statins exert their neuroprotective effects in different mechanisms. The substantial mechanisms consist of modulation of endothelial nitric oxide synthases, vasoactive endothelial protection, antithrombotic, antioxidants, anti-inflammation, and immune-modulation (161).

It is well-known that ATR a BCS class II drug has been suggested as first-line therapy of hyperlipidemia and atherosclerosis by competitively inhibiting of HMG-

CoA reductase interfere with the biosynthesis of cholesterol and subsequently reduce the plasma lipid level (162).

The result of many researches indicated that ATR a member of statins groups exerts anti-inflammatory, antioxidant and neuroprotective effects beyond its anti-cholesterol effect. In this context, clinical trial and animal models in the literature have been addressed the neuroprotective effect of ATR. In this regard, Nacar et al. (163), have investigated the anti-inflammatory effect of ATR on rat spinal cord injury model. Similarly, Eroglu et al. (164), have evaluated the neuroprotective efficiency of ATR by an experimental spinal cord injury model. Furthermore, Pan et al. (165) also proved the neuroprotective effect of ATR in an experimental model of nerve crush injury; and the results indicated that ATR improved injury induced neurobehavioral-electrophysiological changes and axonal loss.

In a similar study, Nazli et al. (166) demonstrated the considerable neuroprotective effect of atorvastatin on the neurological, biochemical and histopathological status of rabbits with ischemia-induced spinal cord injury.

Ewen et al. investigated the anti-inflammatory effect of ATR on rat brain injury model. They found that ATR reduces the level of TNF- α and increase the level of IL-10 in rats (167). ATR shows anti-excitotoxic effects on neurons in in-vitro cell injury model (168). Besides that, ATR showed an antioxidant effect in aged beagles as a mammalian model of Alzheimer's Disease (169). Furthermore, ATR blocks the opening of mitochondrial permeability transition pore and reduced the release of cytochrome-C in a cerebral ischemia-reperfusion rat model (170).

2.9.2. Alpha Lipoic Acid

Alpha lipoic acid (1,2-dithiolane-3-pentanoic acid) (ALA) is naturally present in animal and plant cells. It is synthesized in small quantities in mitochondria through the enzymatic reaction of octanoic acid and cysteine. It has two optical isomers R-lipoic acid and S-lipoic acid and in biological system; however, just R isomer is active (171).

For the first time, ALA has been discovered in 1937 under the name of potato growth factor. Subsequently, it was isolated and purified from bovine liver and in 1951 it was identified as an active ingredient (172).

ALA is an intrinsic segment of each cell in the human body and as an essential co-enzyme it is involved in the energy synthesis of proteins, carbohydrates and fats. ALA has a fundamental role in energy production, since it is an essential part of mitochondrial multi-enzyme complexes like pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase. These two enzyme-complexes are parts of the Krebs cycle and related to energy production (173).

Due to the fat and water solubility of ALA in the biological condition, it can be easily absorbed and crosses through the cell membrane. ALA is not stable at high temperatures and immediately at temperature above its melting point (59 -62 °C), it is polymerized (174). ALA is a potent antioxidant and capable to neutralize the free radicals either in the fat and aqueous area of cells.

Due to the unique properties of ALA, many experimental and clinical studies have been done to investigate biological functions of ALA. In view of these studies, attention and interest in the use of ALA as a therapeutic agent has been extremely increased. Therefore, it is believed that ALA has various set of biological functions.

ALA acts as an antioxidant, metal ion chelator, decreasing the oxidized form of other antioxidants, reparation of oxidized proteins, regulation of gene transcription, reducing oxidative stress etc. (175).

It is believed that an increase in the production of reactive oxygen species (ROS) is a major underlying mechanism of many neurological diseases. As it has been proved in numerous studies, ROS damages a number of key molecular elements that play a potential role in the cellular structure and vital activity of the cells such as DNA, lipids, enzymes and cell membrane.

Under natural circumstances in the cell, ROS is neutralized by the action of some antioxidant vitamins like C, E and glutathione; however, under abnormal conditions (pathologic), due to the increase in the generation and defective removal of ROS, the intracellular level of ROS may be increased. In this case, the presence of an excess of ROS causes the destruction of the cell. Therefore, the administration of an appropriate antioxidant could lead to preventing neuronal damage and restoring ordinary cellular function (176).

Oxidative stress which is the result of the cell's incapability to scavenge the excessive production of ROS is related to several nervous diseases. Since the majority part of the myelin sheath in CNS and PNS is composed of lipids, nervous tissues are more vulnerable to oxidative stress with respect to the other tissues in the body (177)

Toklu et al, have evaluated the neuroprotective effects of ALA in the rat model of spinal cord injury. It has been reported that ALA exerts its neuroprotective effect by inhibiting lipid peroxidation, glutathione depletion and DNA fragmentation (178). In another study, the antioxidant effect of ALA in the sciatic nerve crush injury of rat model has been investigated and as a result, it was found that ALA significantly reduced the oxidative stress (179).

3. MATERIALS AND METHODS

3.1. Materials and Equipment

3.1.1. Chemical Reagents

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)	AppliChem(Darmstadt, Germany).
Acetic acid (99-100%) glacial	J.T. Baker, USA
Acetonitrile (HPLC grade)	Merck, Germany
Alpha Lipoic acid	Sigma Aldrich, USA
Atorvastatin calcium	Cadila Pharmaceuticals Ltd. India
B35 cell line (Neuroblastoma Cells)	Prof. Dr. Ismet Deliloglu Gurhan laboratory Ege University.
Chitosan (Low molecular weight and Medium molecular weight)	Sigma Aldrich, USA
Dichloromethane (HPLC grade)	CARLO ERBA, France
Dulbecco's Modified Eagle Medium (DMEM)	Sigma Aldrich, USA
EDTA	Capricorn Scientific, Germany
Ethanol, absolute, for HPLC 99,8%	Sigma Aldrich, USA
Fetal Bovine Serum (FBS)	Capricorn Scientific, Germany
Isopropyl alcohol (HPLC grade)	Sigma Aldrich, USA
L-929 cell line (NCTC clone 929)	American Type Culture Collection (Manassas, VA)
N,N-Dimethylformamide	Merck, Germany
Penicillin	Biochrom (Berlin, Germany).
Poly(D,L-lactide-co-glycolide) 50:50, Mw 38,000-54,000	Sigma Aldrich, USA
Poly(ethylene glycol), average M_n 4,000	Sigma Aldrich, USA

Streptomycin	Biochrom (Berlin, Germany).
Tetrahydrofuran (HPLC grade) 99.8%	Lab-Scan Analytical Science (Ireland)
Trypsin.	Capricorn Scientific, Germany

3.1.2. Equipment

Differential Scanning Calorimeter	Q 100 DSC (TA Instruments, USA).
Electrospinning Device	Ne 300 Inovenso, Turkey
FTIR Spectrometer	Thermo Nicolet is50, USA
High Pressure Liquid Chromatography System	Agilent 1200 Series system HP1200, Hewlett Packard GmbH, Germany
Nano Spray Dryer B-90	Büchi, Switzerland
Porosimeter	Quantachrome Corporation Poremaster 60, USA
Scanning Electron Microscope	SEM, FEI, Quanta 400F, Holland
Texture Analyzer	TA-XT plus, United Kingdom
Transmission Electron Microscope	JEM- 1010 transmission electron microscope (JEOL, Tokyo, Japan)
Ultrasonic homogenizer	SONOPULS HD 2200 (Bandelin, Germany)
X-Ray Diffractometry	Rigaku Ultima IV (Texas, USA)
Zetasizer Nano ZS Particle Size and Zeta Potential Analyzer	Zetasizer Nano ZS, Malvern, Britain

3.2. Methods

3.2.1. Development and Validation of Revers Phase-High Performance Liquid Chromatography System Method for ALA and ATR

Validation of an analytical method is conducted to prove the reliability and reproducibility of obtained data and to ensure that the data acquired is sufficient for the predesignated purpose. Precise reviews of the articles that currently exist in literature indicated that there is no analytical method for simultaneous quantitative determination of ALA and ATR. Therefore, an optimum RP-HPLC conditions for simultaneous quantitative analysis of ALA and ATR has been developed as a part of this thesis.

3.2.2. Selection of Initial RP-HPLC Conditions for Simultaneous Quantitative Analysis of ALA and ATR

A diverse number of chromatographic conditions were examined, in development of an optimum RP-HPLC method for simultaneous quantitative analysis of ALA and ATR. The HPLC system (HP1200, Hewlett Packard GmbH, Germany) was used for quantitative analyses of ATR and ALA. The pH of mobile phase (2.5 and 4.5), type of organic solvent (acetonitrile, methanol or blend of both), the ratio of organic solvents mixture and buffer, flow rate, and reverse phase analytical column as main chromatographic parameters were evaluated to optimize the most favorable separation condition for ATR and ALA.

3.2.3. Preparation of Buffer and Stock Solution

The buffer solution was prepared by weighing certain amount of sodium dihydrogen phosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and dissolving it in double deionized water to maintain 50mM buffer strength and afterwards, the pH was adjusted to 4.5 using 0.1N sodium hydroxide solution. The buffer was then filtered through 0.45 μm mixed cellulose ester membrane filter.

A mixture stock solution of ATR and ALA at concentrations of 1 mg/mL in methanol was prepared. Working standard solutions were prepared by diluting the stock solution with mobile phase in different concentrations (0.01, 0.05, 0.1, 0.5, 1, 2, 4 and 8 µg/mL).

3.2.4. Method Validation

The method that is used for quantitative analysis of ATR and ALA was validated with regard to following parameters: linearity, specificity, accuracy, precision and system suitability. The validation procedure performed according to ICH guideline (180).

System Suitability

To ensure the system is working properly, the system suitability test was conducted. The system suitability was evaluated by ten independent replicate analyses of the mixed standard solution of ALA and ATR at a concentration of 4 µg/mL. The accepted criterion for the percentage relative standard deviation (RSD%) of peak area for both drugs were 2%.

Linearity

The linearity of HPLC method was analyzed by using working standard solutions. Six series of eight different mixed standard concentrations of ALA and ATR in the range of 0.01-8 µg/mL were prepared and injected into the HPLC system. A calibration curve was generated by plotting the values of peak area versus the corresponding concentrations. The linearity was assessed by calculating the linear regression coefficient.

Specificity/Selectivity

Selectivity refers to the ability of an analytical method, which it can measure accurately a specific analyte in the presence of other potential complex components within the sample. The selectivity of the developed method was investigated by

preparing a placebo sample (unloaded nanofiber composite formulation), a standard solution containing 4 µg/mL ALA and ATR, and mobile phase. In order to verify the specificity of the method, the obtained chromatograms were investigated for any possible interference peaks with the API peaks.

Precision and Accuracy

Instrument Precision (Injector Repeatability)

It shows the ability of HPLC injector to draw the same amount of sample in multiple injections from a single vial. Instrument Precision (Injector Repeatability) was determined through 10 successive injections of ALA and ATR mixed solution with the concentration of 4 µg/mL. The acceptance criterion for RSD% of peak area was 2 %.

Method Precision and Accuracy

Three different concentrations levels (1, 2, 4 µg/mL) of ALA and ATR mixed standard solutions as low, medium, and high concentrations were selected. Each sample was analyzed by HPLC in three replicates, three times within the same day over a short period of time (intra-day precision) and three consecutive days (inter-day precision). The precision of the method was assessed by using the repeatability (intra-day) and intermediate precision (inter-day) assay and calculated in terms of relative standard division (RSD%). The accuracy (bias) of the developed method was determined for intra and inter-day variations and calculated according to following equation.

$$Bias(\%) = \left\{ \frac{\text{Calculated concentration} - \text{Theoretical concentration}}{\text{Theoretical concentration}} \right\} \times 100 \quad (3.1.)$$

Sensitivity (Detection and Quantitation Limits)

The sensitivity of the proposed method was determined by calculating the value of the limit of detection (LOD) and limit of quantitation (LOQ) for ALA and ATR from the linear curve. LOD and LOQ were calculated by using the signal-to-noise ratio approach. LOD and LOQ were calculated based on Signal-to-Noise. S/N ratios were assessed at the lowest reliably visible concentration separately for ALA and ATR. LOD and LOQs were calculated from the average of S/N as follow:

$$LOD_{\mu g/mL} = \left(\frac{\text{lowest known concentration of analyte}}{\frac{S}{N}} \right) \times 3 \quad (3.2)$$

$$LOQ_{\mu g/mL} = \left(\frac{\text{lowest known concentration of analyte}}{\frac{S}{N}} \right) \times 10 \quad (3.3)$$

Robustness

Robustness refers to the potency of an analytical method that, by introducing small but intentional variations in the method parameters, analytical method stays unaffected. Robustness of the analytical method for ALA and ATR was determined by a small changes in several parameters like buffer pH (4.5 ± 0.2), detection wavelength (212 ± 2 nm), flow rate (1 ± 0.1 mL/min), the ratio of ACN and buffer in the mobile phase (53:47, 55:45, 57:43 v/v) around the actual values of the method. The analyses were done in triplicate for each level. Only one parameter was changed at a time, while the other parameters were kept constant. The RSD% values were calculated for peak area of each level.

Stability Test

The stability of ALA and ATR in analytical solution (under HPLC auto-sampler condition) and in stock solution (under refrigerator condition $4 \pm 0.5^\circ\text{C}$) over

the analysis time was investigated. In the case of HPLC auto-sampler stability test, the sample was stored in the HPLC auto-sampler for 48h and at the different time points (0, 4, 24, 48h) sample was injected in triplicate into the HPLC. But in the case of stock solution stability test, samples were withdrawn at mentioned time intervals and analyzed by HPLC. The RSD% values of determined concentrations at different time points were calculated.

3.2.5. Formulation of Atorvastatin Loaded Chitosan Nanoparticles by Nano Spray Drying Method

3.2.6. Preparation of Atorvastatin Calcium/Chitosan Nanoparticles

Preparation of nanoparticles was carried out by the Nano Spray dryer B-90 (Büchi, Switzerland). For preparation of drug loaded nanoparticles, 1% (w/v) chitosan (CH) solution with two molecular weights (low and medium) were prepared by dissolving 1g CH in 100mL 1% (v/v) acetic acid solution. These solutions were used as stock solutions for preparation of final diluted CH solution containing drug solutions with different concentrations.

At the second step, different amount of ATR was weighted and dissolved in methanol and then polyethylene glycol (0.5%-w/v, as a surfactant) was added and vigorously mixed with vortex. ATR solution was mixed with deionized water under the mild magnetic stirring (250-300 rpm) in a separate beaker. At the end, certain amount of CH from stock solution was added dropwise and the final solution was spray dried. The composition of different formulations are shown in Table 3.1 and spray drying process parameters which are selected on base preliminary experiments were set as follows: 4, 5.5 and 7 μ m spray cap size was used, inlet temperature was fixed at 120 °C and the outlet temperature was 56 °C, polymer solution flow rate was kept at 4 %, aspirator:100, spray head temperature: 107 °C, compressed gas flow:140 L/min and pressure: 55 bar)

Table 3.1. Formulation variables for ATR loaded CH nanoparticles.

Variables					Formulation code	Formulation Contents							
						CH (mg)	ATR (mg)	MeOH (mL)	PEG (mg)	Distilled water (mL)	CH (Mw)	Total solid material (mg)	
%CH concentration(w/v)	0.02	%ATR concentration (w/w)	20	Spray cap size (µm)	4	F1	60	12	1	1.25	300	Low	73.25
	0.02		30		4	F2	60	18	1	1.25	300	Low	79.25
	0.02		40		4	F3	60	24	1	1.25	300	Low	85.25
	0.03		20		4	F4	90	18	1	1.25	300	Low	109.25
	0.03		30		4	F5	90	27	1	1.25	300	Low	108.25
	0.03		40		4	F6	90	36	1	1.25	300	Low	127.25
	0.04		20		4	F7	120	24	1	1.25	300	Low	145.25
	0.04		30		4	F8	120	36	1	1.25	300	Low	156.25
	0.04		40		4	F9	120	48	1	1.25	300	Low	169.25
	0.03		20		4	F10	90	18	1	1.25	300	Medium	109.25
	0.03		20		5.5	F11	90	18	1	1.25	300	Low	109.25
	0.03		20		7	F12	90	18	1	1.25	300	Low	109.25

CH= Chitosan, ATR= Atorvastatin calcium, Mw= Molecular weight, MeOH= Methanol, PEG= Polyethylene glycol

3.2.7. Nanoparticle Characterization

Production Yield of Nanoparticles

The Nano Spray drying production yield was calculated by comparing the amount of spray dried powders with the total amount of initial solid contents in the formulations as follows:

$$\text{Production yield (\%)} = \frac{\text{Weight of nano spray dried powders}}{\text{total initial solid weight of formulations}} \times 100 \quad (3.4.)$$

Determination of Particle Size, Polydispersity Index and Zeta Potential

The average particle size, size distribution and zeta potential of ATR loaded CH nanoparticles were measured on Zetasizer Nano ZS Particle Size and Zeta Potential Analyzer (Zetasizer Nano ZS, Malvern, Britain). Samples were prepared by suspending the CH nano spray dried powders in isopropyl alcohol with aid of an ultrasonic homogenizer, model SONOPULS HD 2200 (Bandelin, Germany), equipped with a converter UW 2200 and SH 213 G horn as amplifier (titanium flat tip TT 13, dia. 13 mm). Samples were sonicated at 20% power for 20 sec. and subsequently analyzed on Zetasizer. The analysis was performed at 25°C, the dispersant refractive index and viscosity were defined as 1.37 and 2.4cP, respectively. Each nanoparticle dispersion was measured in triplicate over 12 repeated measurements and results were reported as the mean $\pm$ standard deviation.

Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM)

The surface morphology of the spray dried ATR nanoparticles was investigated with Scanning Electron Microscopy (SEM, FEI, Quanta 400F, Holland). Prior to analysis, the samples were mounted on aluminum stubs and sputter-coated with gold palladium (AuPd) under an argon atmosphere. For TEM observation, certain amount of spray dried nanoparticles was suspended in ethanol solution and a drop of this

suspension was placed on the surface of carbon coated grid. After evaporation is completed, TEM investigation was carried out at 120 kV.

Encapsulation Efficiency (EE)

The EE of ATR in the nanoparticles was determined as follows:

Samples of 2mg nano spray dried powder were accurately weighed and dissolved in 1 mL acetic acid 1% (v/v) solution by vortex. Afterwards, 1mL methanol was added and vortexed it again for 1 minute which was followed by sonication in ultrasonic baths for 5 minutes. After filtration of samples, resulting solution was analyzed by previously developed HPLC method. The experiment was carried out in triplicate (n = 3). The data has been presented as the mean $\pm$ standard error of three independent experiments. The percent of ATR encapsulation efficiency was calculated by using following equation.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Practical amount of drug within nanoparticles}}{\text{Initial drug amount}} \times 10 \quad (3.5.)$$

Differential Scanning Calorimetry Analysis (DSC)

DSC analysis of pure ATR, CH and prepared nanoparticles formulations were investigated using Q 100 DSC (TA Instruments, USA). In brief, accurately weighed (3-5mg) sample placed in a standard aluminum pan and sealed with aluminum cap heated under nitrogen atmosphere (50mL/min) at a rate of 10°C/min over a temperature range of 30-350°C.

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared (FTIR, Thermo Nicolet is50) analyses were evaluated in order to confirm encapsulation of ATR inside the CH nanoparticles. For this purpose, samples in contact with ATR crystal were scanned within IR range from 650 - 4000 cm^{-1} and the background spectrum was subtracted from the IR signal.

3.2.8. In-Vitro Release Studies ATR loaded CH Nanoparticles

In-vitro release characteristics of ATR from nanoparticles were investigated in phosphate buffer saline solution (PBS, pH 6.8 prepared according to the USP monograph). In details, 5 mg of ATR loaded chitosan nanoparticles were dispersed in 20 mL of PBS in sealed vials to prevent evaporation of the medium. All the vials were kept in a shaking water bath that was maintained at $37\pm0.5^{\circ}\text{C}$ and 100 rpm. At predetermined time intervals (1h, 4h, 8h, 19h, 29h, 77h, 101h, 154h, 200h) 1 mL of medium was withdrawn and replenished with equal volume of pre-warmed and fresh medium to maintain the original volume of medium for maintaining sink conditions. Drug content was quantified by the previously described HPLC method. The cumulative released percentage of ATR was calculated and plotted over time.

3.2.9. Cell Culture Studies

L-929 mouse fibroblast cell line and B35 neuroblastoma cell lines were used for dose and time dependent biocompatibility assessment of the prepared nanoparticles. The cells were grown in DMEM (High Glucose, 4 mM L-glutamine and sodium pyruvate) supplemented with 10% FBS and 1% Penicillin-Streptomycin (10 000 U/10 000 mg/mL) in an incubator with 5% CO_2 at 37°C . L-929 and B35 cells were seeded in 96 well plates at concentration of 5×10^3 cells/well and cultured overnight for cell viability studies. For the direct method; L-929 and B35 cells were incubated either with 2.5, 5 and 10 $\mu\text{g/mL}$ of ATR solution, ATR loaded CH nanoparticles in equivalent ATR doses or blank nanoparticles following the extraction of both blank and ATR loaded CH nanoparticles in PBS, prepared by sonication and extensively vigorous stirring overnight. The samples were filtered through membrane filter (0.22 μm pore size) and diluted with DMEM to give predetermined ATR concentrations. After incubation for 24 and 48 h, cell viability was assessed by the addition of MTT reagent in concentration of 5 mg/mL (1:5 dilution in cell culture media) per well and after 4h of incubation, the formazan crystals were dissolved by overnight incubation with 23% SDS. The optical density was measured with ELISA

microplate reader (Molecular Devices, Silicon Valley, CA) at 570nm. All cell viability results were expressed as normalized values against the controls of the used solvents.

3.3. Optimization of Electrospinning parameters for PLGA Nanofiber

PLGA was determined to be the most appropriate polymer for fabrication of composite nanofibers. In order to produce uniform, bead-free nanofibers with the desired diameter, optimization of electrospinning parameters including polymer molecular weight, solvent system, polymer solution concentration, applied voltage, nozzle to collector distance and the polymer solution flow rate were investigated and other variables were set at constant values in all the experimental runs. For the optimization of electrospinning process in total, 18 experimental runs were performed and for each spin, 3mL of polymer solution was electrospun (Table 3.2). The software Image J was used to measure the fibers' diameter. At least 100 fibers were selected randomly in each samples and analyzed. The fiber diameter result was reported as mean $\pm$ SD.

Table 3.2. Different electrospinning conditions for optimizing electrospinning variables for preparation of PLGA nanofibers.

Experimental Runs	Electrospinning Parameters					
	Polymer Molecular Weight	Polymer Solution Concentration (w/v%)	Solvent System	Applied Voltage (kV)	Nozzle To Collector Distance (cm)	Flow Rate (mL/h)
1	24,000-38,000	25	THF +DMF (65:35)	20	12	1.5
2	38,000-54,000	25	THF +DMF (65:35)	20	12	1.5
3	54,000-69,000	25	THF +DMF (65:35)	20	12	1.5
4	54,000-69,000	15	DMF: THF (70:30)	20	12	0.5
5	54,000-69,000	20	DMF: THF (70:30)	20	12	0.5
6	54,000-69,000	25	DMF: THF (70:30)	20	12	0.5
7	54,000-69,000	25	DMF: THF (65:35)	20	12	1.8
8	54,000-69,000	25	THF : DMF (65:35)	20	12	1.8
9	54,000-69,000	25	CHL : DMF (60:40)	20	12	1.8
10	38,000-54,000	25	THF+DMF(70:30)	19	14	0.8
11	38,000-54,000	25	THF+DMF(70:30)	21	14	0.8
12	38,000-54,000	25	THF+DMF(70:30)	22	14	0.8
13	54,000-69,000	25	THF +DMF (65:35)	20	12	1.5
14	54,000-69,000	25	THF +DMF (65:35)	20	16	1.5
15	54,000-69,000	25	THF +DMF (65:35)	20	18	1.5
16	54,000-69,000	20	THF +DMF (65:35)	20	16	1
17	54,000-69,000	20	THF +DMF (65:35)	20	16	1.5
18	54,000-69,000	20	THF +DMF (65:35)	20	16	2

THF= tetrahydrofuran, DMF= dimethylformamide, kV= kilovolt

3.4. Fabrication of Poly (Lactic-Co-Glycolic Acid) (PLGA) and CH Electrospun Composite Nanofiber

In the first step of the nanofiber preparation process, the amount of PLGA 20% (w/v) and ALA 5% (w/w) with respect to polymer portion, were weighed accurately. The amount of ALA was dissolved in the blend organic solvent system of THF:DMF:Ethanol (50%:35%:15%). The stirring was followed at ambient temperature and in the absence of light on the magnetic stirrer until complete dissolution of ALA. Subsequently, PLGA was added to the solution, and the stirring was continued until the complete dissolution of polymer and obtaining a homogenous state.

In the second step of the process, CH nanoparticles (Formulation 4) with 5% (w/w) concentration on the base of PLGA amount, were dispersed in the separated part of the solvent system with aid of an ultrasonic homogenizer probe at 20 % power for 20 sec. Then the resulting nanosuspension was added dropwise to the PLGA and ALA solution by vigorous stirring on the magnetic stirrer. Stirring was continued until CH nanoparticles distributed uniformly in PLGA and ALA solution. The resulting solution containing nanoparticle was transferred in a 10mL syringe which is fixed horizontally on the syringe pump (NE-1000 Programmable single syringe pump) and the electrospinning process parameters (Inovenso Ne300 Electrospinning Machine) were adjusted as *flow rate = 0.5mL/h, applied voltage = 22kV and nozzle tip to collector distance = 18cm*. Randomly orientated fibers were collected on the surface of rotating drum collector (300 rpm) covered by aluminum foil. Depending on composition of the PLGA/CH electrospun nanocomposite four formulations were prepared (Table 3.3).

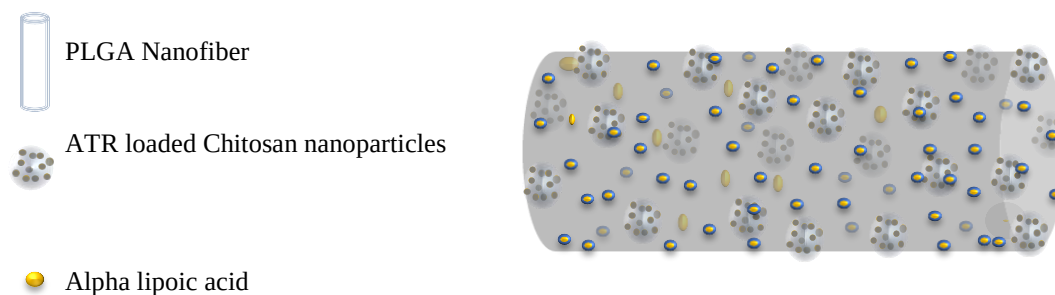


Figure 3.1. Schematic representation of composite nanofibers.

Table 3.3. Composition of Electrospinning Solution .

Formulations code	Formulation Composition
A	PLGA/CH electrospun composite nanofiber formulation containing ALA and ATR
B	PLGA/CH electrospun composite nanofiber formulation containing ALA without ATR
C	PLGA/CH electrospun composite nanofiber formulation containing ATR without ALA
D	PLGA/CH electrospun composite nanofiber formulation without ALA and ATR

3.4.1. Characterization of PLGA/CH Electrospun Composite Nanofiber Formulation

Morphology

The surface morphology of the PLGA/CH electrospun nanocomposites were observed with SEM (FEI, Quanta 400F, Holland) at a voltage of 20kV. Prior to analysis, the samples were mounted on aluminum stubs and sputter-coated with gold palladium (AuPd) under an argon atmosphere. The average of resulting nanofibers' diameters was measured using Image J software. Minimum 100 nanofibers were randomly selected from SEM images and analyzed. The distribution of CH nanoparticle in PLGA nanofibers were characterized by TEM analysis at 120 kV.

Encapsulation Efficiency

In order to determine the EE of ALA and ATR, 5 mg of formulation A was precisely weighed and dissolved in 1 mL DCM in a vial with 10 mL volume. Then 1 mL acetic acid solution 1% (v/v) and 3 mL MeOH were added to the vial and followed by vortexing for 5 min. The extraction process was followed by 10 min sonication in water bath sonicator. Afterwards, DCM was evaporated by mild stirring on the magnetic stirrer at room temperature. The solution was filtered through 0.45 µm PTFE filter. The amount of ALA and ATR in PLGA/CH electrospun nanocomposite were determined through analyses of the samples with the developed HPLC method. The EE values of drugs were calculated by following equation:

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Practical Amount of Drugs Within Nanocomposite}}{\text{Initial Drug Amount}} \times 100 \quad (3.6.)$$

Differential Scanning Calorimetry Analysis (DSC)

DSC analysis was accomplished for the evaluation of thermal characterization of all components of PLGA/CH electrospun nanocomposite formulation and the possible interactions between them. DSC analysis was performed using DCS Q 100 (TA Instruments, USA) instrument. For this purpose, a standard aluminum sample pan was used. In brief, accurately weighed (3-5mg) samples placed in a standard aluminum pan and sealed with the aluminum cap. During the analysis, thermograms were recorded by heating the samples under the nitrogen atmosphere (50 mL/min) at a rate of 10°C/min over a temperature range of 30-350°C.

X-Ray Diffraction Analysis (XRD)

In order to characterize the solid-state transformations, nanoparticles containing ATR, ALA, and ATR/ALA loaded electrospun nanocomposite formulation were analyzed using X-ray diffractometry, which is one of the crystallographic methods. Rigaku Ultima IV (Texas, USA) brand diffractometer was used for this

purpose. The XRD analysis was performed at 40 kV and 30 mA and using Cu Ka ($\lambda = 1.5406 \text{ \AA}$) radiation, the scan was performed from 3 to 90° with a speed of 1 min^{-1} .

Fourier Transform Infrared Spectroscopy-FTIR

Fourier Transform Infrared (FTIR, Thermo Nicolet is50) analysis was performed to confirm that ALA and ATR used as active ingredients in the formulations were encapsulated in PLGA nanofiber structures. For this purpose, samples were scanned at an IR wavelength range of $650\text{-}4000 \text{ cm}^{-1}$ and the results were expressed by subtracting the background spectrum from IR signals.

Porosity Measurements

The porosity of the final nanofiber formulation, prepared with PLGA polymer and containing ATR loaded nanoparticle with ALA, was measured with Quantachrome Corporation Poremaster 60 (Florida, USA) convex porosimeter. This device operates according to the physical principle that an unabsorbable and unreactive liquid enter into small pores unless adequate pressure is applied. The relationship between the applied pressure and the pore diameter is obtained by the Washburn equation:

$$D = (-4\gamma \cos\theta)/P. \quad (3.7)$$

Where P is the applied pressure, D is the pore diameter, γ is the surface tension of the mercury, and θ is the contact angle between the mercury and the pore wall (usually 140°). It is used to generate pore size/volume distributions from mercury intrusion doses.

Texture Analysis

The Texture Analyzer was utilized with the aim of measuring the mechanical resistance of the PLGA/CH composite nanofiber formulations. For the measurements of the mechanical strength of formulations, samples with $1\text{ cm} \times 3\text{ cm}$ (width $\times$ length) size were cut off from nanofiber sheets $n = 3$. Then the samples were vertically placed

between vice grips Texture Analyser (Analyser (TA.XTPlus, Stable Micro Systems, Surrey, UK). equipped with 500 g load cell. The samples were then pulled with a rate of 1 mms^{-1} to a distance of 60 mm before returning to the initial point and the force required to break the formulations was determined.

3.4.2. In-Vitro Release Studies of PLGA/CH Composite Nanofiber Sheet

In-vitro release experiments of nanofiber formulations were carried out on nanofiber sheets (formulation A) which were cut into regular square pieces (1 cm^2 ; $n=3$). The release characteristics of drugs (ALA and ATR) from PLGA/CH composite nanofiber sheet were evaluated by placing of nanofiber sheet pieces (2.2 mg) in 20 mL of phosphate buffer solution with $\text{pH} = 6.8$ (prepared according to USP) as release medium. The test was conducted in a horizontal shaker water bath and the vials were kept in the water bath at $37 \pm 0.5^\circ\text{C}$ and 100 rpm. 1mL of samples were withdrawn, at the predetermined time intervals (1, 4, 8, 19, 29, 77, 101, 125, 150, 175, 200, 225h) and the same amount of fresh medium was added back to the release medium to maintain the sink condition. After filtration, the samples were analyzed by developed HPLC method. The experiments were done in triplicate. The results were presented in terms of cumulative release percentage as a function of time.

3.4.3. Cytotoxicity Studies of PLGA/CH Composite Nanofiber Sheet

L-929 mouse fibroblast and B35 neuroblastoma cell lines were used for biocompatibility assessment of the prepared nanocomposites. The cells were grown in DMEM (High Glucose, 4 mM L-glutamine and sodium pyruvate) supplemented with 10 % FBS and 1 % Penicillin-Streptomycin (10,000 U/10,000 mg/mL) in an incubator with 5% CO_2 at 37°C . L-929 and B35 cells were seeded in 96 well plates at a concentration of 5×10^3 cells/well and cultured overnight for cell viability studies.

The biocompatibility of prepared formulations was assessed through indirect method; L-929 and B35 cells were incubated with extracted solutions of formulations A, B, C,D and separate solutions of ALA and ATR. The extraction of formulation A, B, C and D in PBS were prepared by sonication and extensively vigorous stirring on

magnetic stirrer for three days. After extraction the samples were filtered (0.45µm pore size) and analyzed by HPLC to determine the amount of ALA and ATR in the samples. The samples were filtered through membrane filter (0.22µm pore size) and diluted with DMEM to give predetermined ATR/ALA concentrations.

After incubation for 24 and 48h, cell viability was assessed by the addition of MTT reagent at a concentration of 5 mg/mL (1:5 dilution in cell culture media) per well and after 4h of incubation, the formazan crystals were dissolved by overnight incubation with 23% sodium dodecyl sulphate (SDS). The optical density was measured with ELISA microplate reader (Molecular Devices, Silicon Valley, CA) at 570 nm. All cell viability results were expressed as normalized values against the controls of the used solvents.

3.4.4. Stability Test

The stability of PLGA loaded ALA/ATR loaded CH electrospun nanocomposite final formulation (Formulation A) was monitored according to ICH guideline (181). After preparation of formulation, the nanofiber composite was removed from the surface of aluminum foil and was cut into regular square pieces (1cm²). Then every piece was placed individually in amber colored glass vials. The samples were stored in the different conditions as explained in (Table 3.4). At each time point 6 sample withdrawn and analyzed for the amount of ALA and ATR

Table 3.4. Different storage conditions for stability test (182).

Study	Storage Condition	Time Period	Testing Frequency (month)
Intermediate	30°C ± 2°C/65% RH ± 5%	6 Months	0-1-2-3-4-5-6
Long term Storage in the Refrigerator Condition	5°C ± 3°C	6 Months	0-1-2-3-4-5-6
Normal Condition at Room Temperature	25°C ± 2°C/ 60% RH	6 Months	0-1-2-3-4-5-6

3.5. In-Vivo Experiments

3.5.1. Experimental Groups and Surgical Procedure

In this study, 126 healthy adult Sprague-Dawley male rats that were about 8 weeks old (weighing 250 -300 g) were used. 36 rats were assigned for assessment of motor function recovery, 30 rats for histomorphology and 60 rats for biochemistry analysis. All animal experiments were approved by the Local Ethics Committee of Kobay Deney Hayvanları Laboratuvarı A.Ş. with the protocol number of 127 (20/01/2015). The rats were classified into six groups as follows (Table 3.5).

Table 3.5. Experimental groups.

Groups number	Sciatic nerve crush	Intervention after injury
1	+	Animal received no treatment; n=24
2	+	Formulation A* implanted; n=24
3	+	Formulation B* implanted; n=24
4	+	Formulation C* implanted; n=24
5	+	Formulation D* implanted; n=24
6	–	No intervention (healthy animal); n=6

* Formulation A = PLGA/CH electrospun composite nanofiber containing ALA and ATR

* Formulation B = PLGA/CH electrospun composite nanofiber containing ALA without ATR

* Formulation C = PLGA/CH electrospun composite nanofiber containing ATR without ALA

* Formulation D = PLGA/CH electrospun composite nanofiber without ALA and ATR

3.5.2. Surgical Procedure

The rats were anaesthetized with a combination of ketamine (80mg/kg) and xylazine (5 mg/kg) and placed in prone position. The nerve crush was achieved on the right hind limb for motor function assessment, and on both hind limbs for histomorphology and biochemistry analysis. The thigh area of hind limbs was shaved and sterilized with polyvinylpyrrolidone iodine solution. An incision (approximately 2cm) parallel to the femoral bone was made on the skin of the hind limb thigh.

Afterward, to reach the nerve, the underlying muscles were split, the sciatic nerve was exposed. The sciatic nerve has been carefully dissected from the surrounding tissues.

The nerve was crushed 5 mm proximal to trifurcation point using an aneurysm clip (Yasargil FE 693 transient aneurysm clip-Aesculap) with a holding force of 50 g/cm² with 1 mm in width for 2 times (30 sec. each time) (Figure 3.2)

After the sciatic nerve injury was exposed, different formulations which have been already cut in appropriate dimension (1cm²) and sterilized under UV light for 30 min were implanted and wrapped around the crushed area. Then the incision was closed anatomically. After surgery, all animal housed in separate cages (n=6) and provided free access to standard food and water.

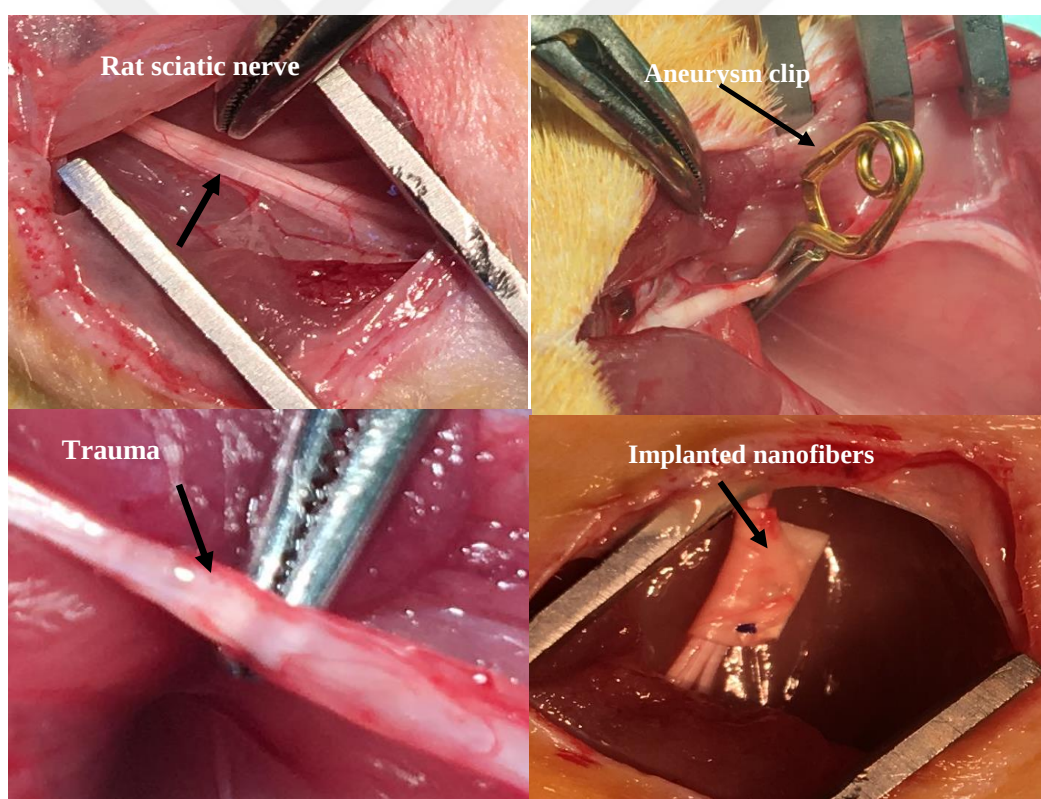


Figure 3.2. Surgical procedure and sciatic nerve crush.

3.5.3. Assessment of Functional Recovery

Sciatic Functional Index Measurement (SFI)

To evaluate the motor recovery after sciatic nerve crush, the SFI test was accomplished on 1, 3, 5, 7, 15 and 30 days post operation. The test was conducted in a narrow wooden walkway (9 cm wide, 65 cm length, 15 cm height) with a relatively small shelter at the end. A white paper strip was cut in the appropriate dimension and placed on the bottom of the walkway. The hind feet of the rats were painted by pressed down onto a blue stamp ink pad and the rats were allowed to walk along the walkway, leaving their prints of each paw on the paper.

At least 3 clear and measurable footprints of each the normal (N) and experimental (E) paws were selected from each run and the average of the measurements was used in SFI calculation. The sciatic functional index (SFI) was calculated as described in the following equation (183) (Figure 3.3).

$$SFI = -38.3 \left(\frac{EPL - NPL}{NPL} \right) + 109.5 \left(\frac{ETS - NTS}{NTS} \right) + 13.3 \left(\frac{EIT - NIT}{NIT} \right) - 8.8 \quad (3.8)$$

After sciatic crushed nerve injury in rats, the print length (PL), toe spread (TS), and intermediary toe spread (IT) were taken, and these were incorporated above formula. (E) experimental side, (N) normal side, (EPL) experimental print length, (NPL) normal print length, (ETS) experimental toe spread, (NTS) normal toe spread, (EIT) experimental intermediary toe spread, (NIT) normal intermediary toe spread.

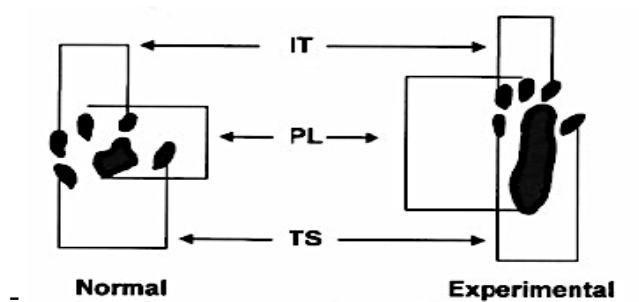


Figure 3.3. Rat foot print analysis (181).

Behavioral Testing BBB

For the evaluation of locomotor recovery of hindlimb after sciatic nerve crush, Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale was used on days 1, 3, 5, 7, 15 and 30 after surgery. The animals were observed individually for at least four minutes by two examiners who are blinded to the study in an open field and calm environment and BBB scores were recorded. The animals were scored according to BBB coding scheme (184). The scores rising from 0 to 21 it indicates no spontaneous movement to normal locomotion, respectively.

Extensor Postural Thrust Test (EPT)

Extensor postural thrust test was described by Thalhammer et al. (185), as an alternative test to evaluate performance of motor recovery after sciatic nerve injury in animal model. EPT is the force which is apply by the experimental animal to a digital balance (186). The animals were tested postoperatively at 1, 3, 5, 7, 15 and 30 days.

The upper part of the rat's body (except hind legs) was wrapped in a towel. The animal was held in the upright position and supporting by the thorax with the examiner's hand. During the test, the experimental hind-limb of the animal was lowered and allowed to touch the platform of the digital balance. After pressing, the extent of the thrust exerted by the experimental limb of the animal was recorded. The same procedure was repeated for the normal hindlimb as well. The percentage of motor deficit was calculated with the equation:

$$Motor\ deficit\ \% = \left(\frac{NEPT - EEPT}{NEPT} \right) \times 100 \quad (3.9)$$

where: NEPT: normal leg extensor postural thrust; EEPT: experimental leg extensor postural thrust.

Sensory Testing

The nociceptive function was evaluated by measuring withdrawal reflex latency (WRL). The sensory function recovery was evaluated at postoperative day 1, 3, 5, 7, 15 and 30 by measuring the response of operated and normal hind paws to the thermal stimulus. For this purpose, a modified form of hotplate test was performed. The rat was wrapped in a surgical towel and each paw was immersed in a 55°C water bath until withdrawal of the hind paw occurs. The withdrawal of the hind paw was recorded by stopwatch (187).

Tissue Harvesting

The rats were anaesthetized and the segment of sciatic nerve that have been subjected to injury was dissected with microscissors. Tissue specimens were fixated with 2.5% glutaraldehyde for electron microscopic analysis and for biochemical analysis, the tissues were kept in liquid nitrogen. After tissue harvesting, the animals were sacrificed.

3.5.4. Ultrastructural Examination

In order to investigate the regeneration process after surgery, one animal from each group at different time points (day 1, day 3, day 5, day 7, day 15 and day 30) was euthanized and the section of sciatic nerve that subjected to trauma was dissected with microscissors and 5 mm length sample of the traumatic area of the sciatic nerve of both legs was resected. The specimens were immersed immediately in 2.5% glutaraldehyde for 48 hours then washed in phosphate buffer (pH 7.4) for electron microscopic analysis

The scoring (Table 3.6), was conducted on samples of each group. During the grading procedure, 50 myelinated axons from each sample (two sciatic nerve samples per animal) were examined ultrastructurally.

Table 3.6. Scoring system for quantitative evaluation of ultrastructural findings (188).

Scoring for myelinated axons	
0	Ultrastructurally normal myelinated axons
1	Separation in myelin configuration
2	Interruption in myelin configuration
3	Honey comb appearance

3.6. Biochemical Analysis

3.6.1. Preparation of Samples

For this purpose, the sciatic nerve tissues obtained from the rats were weighed and homogenized in 50 mM Tris/HCl buffer with pH=7.4 (10 % w/v). The homogenization was performed in presence of ice with Pro-200 homogenizator in 30+30 seconds at 25,000 rpm. The rest of the homogenate was centrifuged at 14,000 rpm at + 4°C for 30 minutes, the supernatant was separated and used for ELISA and protein assays experiments.

3.6.2. Determination of TNF- α , IL-1 β and IL-6 Levels

Invitrogen Tumor Necrosis Factor- α (TNF- α) ELISA kit, Invitrogen Interleukin-6 (IL-6) ELISA kit and Invitrogen Interleukin-1 β ELISA kit were used to measure the levels of Tumor Necrosis Factor- α (TNF- α), interleukin 6 (IL-6) and interleukin 1 β (IL-1 β). The assays were conducted in accordance with the recommendations of the manufacturer. In brief, 50 μ L of dilution buffer, 50 μ L sample was placed in each well. Then, 50 μ L of biotinylated TNF- α , IL-1 β and IL-6 were added to each well and incubated for 2 hours at room temperature. Afterwards, the wells were rinsed 4 times with 200 μ L of wash buffer solution. After elimination of the liquid from the wells, 100 μ L of streptavidin-peroxidase conjugate was added per well and incubated for 1 hour. At the end of the incubation, wells were washed again four times with 200 μ L wash buffer solution. After removal of the liquid, 100 μ L of chromogenic substrate per well was added and incubated for 10 minutes. At the end of this run, 100 μ L solution of 0.5 N HCl per well was added to prevent the color

formation reaction and the results were immediately quantified with Molecular Devices Spectra max M2 microplate reader at a wavelength of 450 nm. The results were obtained from the standard graphic by 4-parameter regression analysis.

3.7. *In-Vitro* Models of Neurotrauma/Organotypic Spinal Cord Culture

Swiss Albino male mice (n = 3) were anaesthetized with high dose of chloral hydrate and spinal cord was isolated from connective tissue. Then, the vertebrae surrounding the spinal cord were dissected with bone scissors and the spinal cord was isolated. For oxygenation, the isolated tissue was introduced into an artificial cerebrospinal fluid (ACSF) at 4°C. The tissue was placed in the pre-cooled spinal cord matrix then under laminar flow, coronal sections were taken with 0.5 mm thickness and cultured.

The mechanical trauma model (by dropping of an impactor with weight of 0.2g) was created on the 7th day of the spinal cord section culture. Five minutes after the impactor was dropped, PLGA electrospun nanofibrous sheets containing ALA at different doses (5%, 10% and 20%) were applied. Tissues were incubated in the incubator for 1 night. The tissues were fixed into 4% PFA solution at 4 °C for 1 night. Afterwards, the samples were cryoprotected into 30% sucrose solution, which was prepared in PBS buffer at 4 °C incubated for one more night and finally, 12-µm thick cryostat sections were cut. The nucleus of tissue sections and inflammatory marker, IL-6 and IL-1beta were marked by Neun and primary and secondary antibodies and then rinsed (2X 5) with wash buffer (TBS + Triton X-100 (0.025%). After washing it was incubated with permeabilization buffer (TBS + Triton X-100 (0.2%)) for 10 min. The tissues sections were incubated for 1 hour with 1% BSA solution prepared with TBS and 10% NGS blocking buffer. Primary antibodies were diluted with 1% BSA solution prepared with TBS and incubated at +4 °C for 1 night. Then, tissues were rinsed with wash buffer (3 times; 5 replicates) and incubated with secondary antibody at room temperature for 1 hour. After incubation the tissues were sealed with Hoechst nucleus dye solution.

Tissues were investigated under the Nikon E-600 epifluorescent microscope using appropriate filters (10X) and images were taken from trauma regions and the tissues that are surrounding the trauma regions.

3.8. Statistical Analysis

All data are expressed as mean $\pm$ SD. Statistical analyses were performed using two-way ANOVA followed by the "Tukey's multiple comparisons" test for multiple comparisons. Statistical analyses were conducted with Prism 7 scientific software by GraphPad.



4. RESULTS

4.1. RP-HPLC Method Development and Validation

According to the results, the developed and fully validated RP-HPLC method was considered as a suitable method for simultaneous quantitative determination of ALA and ATR from the same sample. A good separation of ALA and ATR were achieved by using the Inertsil ODS-3 C₁₈, 250x4.6 mm, 5µm i.d column. Several chromatographic conditions were examined to ensure the optimum performance of the assay. The optimized chromatographic condition for ALA and ATR was mentioned in (Table 4.1). The retention time for ALA and ATR were recorded at 6.2, 9.3min respectively. The total time run for analysis was selected as 11min.

Table 4.1. The optimized chromatographic condition used for method validation.

Instrument	High Performance Liquid Chromatography
Method	Reverse Phase Chromatography / isocratic elution
Column	Inertsil ODS-3 C ₁₈ , 4.6X250mm, 5µm
Mobile phase	ACN:50mM NaH ₂ PO ₄ .2H ₂ O (pH 4.5) (55:45)
Flow rate	1 mL/min
Detector	DAD
Wavelength	212 nm
Temperature	Room temperature
Retention time (min)	6.2 and 9.3 minutes for ALA and ATR
Injection volume	50µL

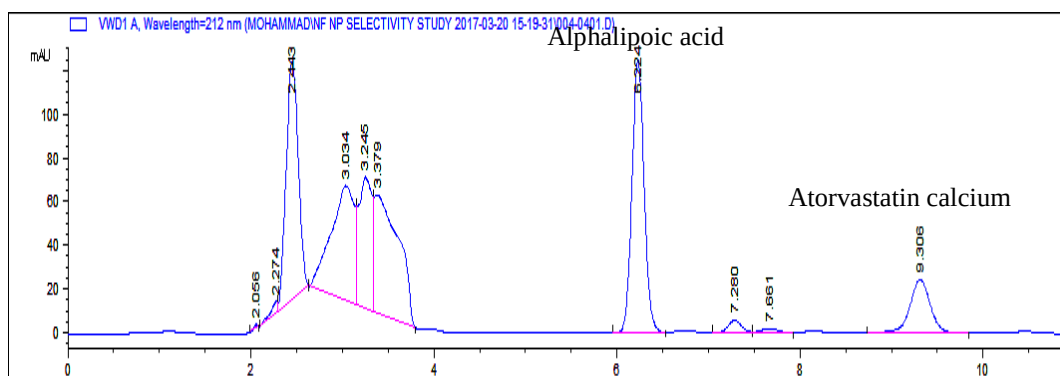


Figure 4.1. Chromatogram of ALA and ATR.

System Suitability Test

Before running method validation process, in order to ensure the performance of chromatographic system performance, system suitability test was conducted. Ten independent mixture solutions of ALA and ATR at the concentration of 4 $\mu\text{g/mL}$ was prepared from stock solution and injected into HPLC individually. The RSD% values for peak area were determined. The RSD% was 1.620 and 1.652 percent for ALA and ATR respectively (Table 4.2).

Table 4.2. System suitability test data.

Concentration ($\mu\text{g/mL}$)	ALA	ATR
	Area under peak	Area under peak
4	99.45	672.50
	99.25	696.70
	95.68	683.46
	98.29	694.96
	99.60	688.79
	99.78	690.34
	99.89	678.94
	99.29	659.08
	100.9	687.26
	96.55	687.79
Average	98.868	683.982
SD	1.157	11.297
RSD%	1.620	1.652

4.1.1. Instrument Precision (Injector Repeatability)

The injection precision (injection repeatability) was determined by 10 injections of single sample. The RSD% of peak area was obtained as 0.922 % and 0.163 % percent for ALA and ATR, respectively (Table 4.3).

Table 4.3. Injector repeatability data.

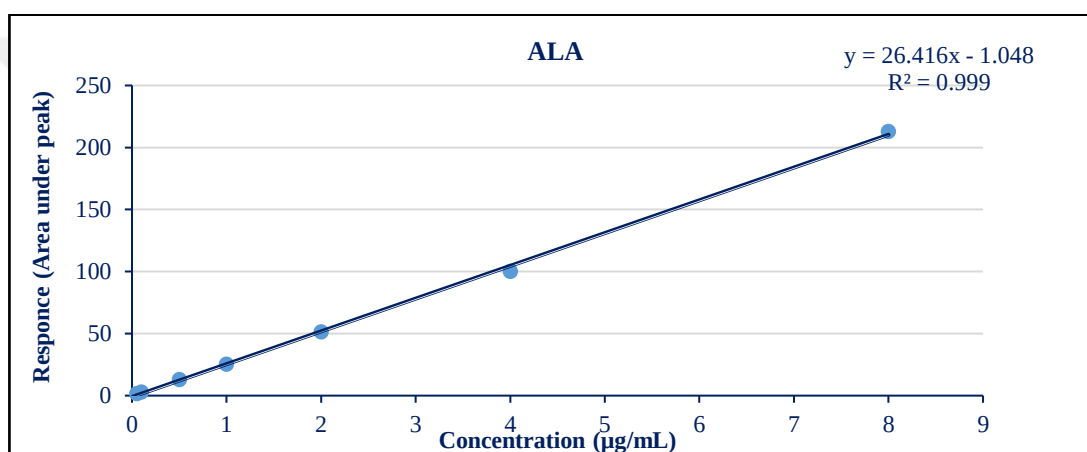
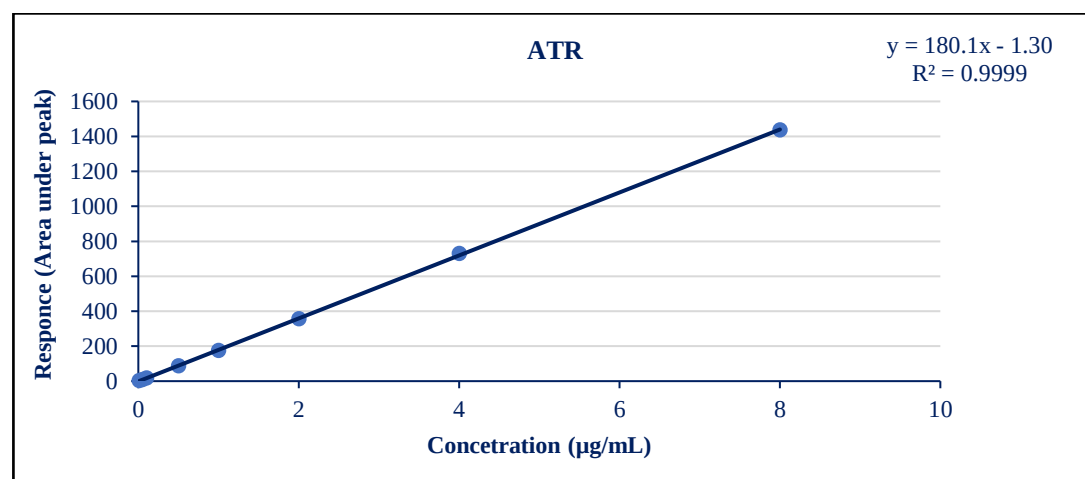
Concentration (µg/mL)	ALA	ATR
	Area under peak	Area under peak
4	98.97	686.19
	98.24	685.01
	99.28	684.78
	98.81	684.72
	99.87	685.84
	99.18	687.87
	99.66	686.88
	97.54	686.69
	98.17	687.25
	99.56	687.18
Average	98.628	686.241
SD	0.745	1.120
RSD%	0.922	0.163

4.1.2. Linearity and Range

Two calibration curves under optimized condition were evaluated for ALA and ATR by plotting the response (peak area) against the nominal concentrations of analytes. Linear least-square regression analysis was applied and as a result, calibration curves demonstrated linearity between responses and concentrations within the range of 0.01-8 µg/mL for both ALA and ATR as shown in (Table 4.4). Calibration curves for ALA and ATR are given in (Figure 4.2 and Figure 4.3).

Table 4.4. Linearity data of the ALA and ATR.

	ALA	ATR
Regression equation	$y = 26.416x - 1.048$	$y = 180.1x - 1.301$
Determination of coefficient (r^2)	0.9991	0.9999
Linearity range ($\mu\text{g/mL}$)	0.01-8.00	0.01-8.00
Number of data points	8	8
LOD ($\mu\text{g/mL}$)	0.020	0.005
LOQ ($\mu\text{g/mL}$)	0.080	0.016

**Figure 4.2.** Calibration curve for ALA.**Figure 4.3.** Calibration curve for ATR.

4.1.3. Sensitivity

The sensitivity of the proposed method was figured out by specifying the limits of detection (LOD) and quantitation (LOQ). LOD and LOQ values were calculated by the signal-to-noise ratios approach. The LOD value for ALA and ATR were 0.02 $\mu\text{g/mL}$ and 0.005 $\mu\text{g/mL}$, respectively; where the peak height was three times the baseline noise. In the same way, the LOQ values were 0.080 $\mu\text{g/mL}$ and 0.016 $\mu\text{g/mL}$ with the signal-to-noise ratio of 10:1. The sensitivity data of the analytical are summarized in (Table 4.4).

4.1.4. Precision and Accuracy

The precision of the analytical method was investigated with respect to repeatability (intra-day) and intermediate (inter-day) precision.

The repeatability of the developed HPLC method was investigated by analyzing 6 independent series of the 3 different concentrations (1, 2 and 4 $\mu\text{g/mL}$ in linear range) within the same day. After the calculation of concentration from the obtained peaks area, the RSD% values of the concentrations were found to be less than 2%.

The intermediate precision of the method was demonstrated by calculating RSD% of the data which were obtained from analyses of the three mentioned concentrations in three consecutive days. The RSD% was found to be less than 2 %. The results of the intra and inter day studies are given in (Table 4.5. and 4.6).

The accuracy of the method was demonstrated by estimating the percent of difference (bias %) of the observed mean concentrations, in contrast to theoretical concentrations. The relative error (bias) was calculated for within-day and between day precisions and the results are summarized in (Table 4.5 and 4.6).

Table 4.5. Intra-day precision (repeatability) results of ALA and ATR n=6.

Drugs	Concentration (µg/mL)	Mean± SD	RSD %	Bias %	Recovery %
ALA	4	3.921±0.041	1.053	-1.971	98.025
	2	2.018±0.034	1.691	0.931	100.9
	1	1.018±0.014	1.431	1.855	101.800
ATR	4	3.922±0.039	1.016	-1.934	98.05
	2	2.035±0.019	0.975	1.793	101.750
	1	1.016±0.16	1.540	1.696	101.600

Table 4.6. Inter day or intermediate precision and accuracy results of ALA and ATR n=6.

Drugs	Concentration (µg/mL)	Mean±SD	RSD%	Bias%
ALA	4	3.942±0.007	0.178	-1.900
	2	2.013±0.018	0.896	0.650
	1	1.013±0.008	0.886	1.333
ATR	4	3.973±0.65	1.642	-0.658
	2	2.030±0.008	0.398	1.517
	1	1.010±0.0125	1.238	1.033

4.1.5. Specificity (Selectivity)

The chromatograms of the placebo sample (unloaded nanofiber composite formulation), the standard solution containing 4µg/mL ALA and ATR, and mobile phase indicated that there were no interfering peaks at the same retention time of the active ingredients. These data indicated that chromatographic condition is specific for the detection and quantification of ATR and ALA (Figure 4.4).

4.1.6. Robustness

Robustness of the developed method was investigated by intentional changes of the analytical parameters. The variations on buffer pH (4.5 ± 0.2) and the ratio of the ACN in the mobile phase ($\pm 2\%$) did not have any remarkable effect on response. The overall RSD % was 0.747 % and 0.815 % respectively. On the other hand, small variations in flow rate ($1 \text{ mL/min} \pm 0.1$) and detection wavelength ($212 \pm 2 \text{ nm}$) had a significant effect on responses and the overall RSD% values were out of the acceptance limit (Tables 4.7. and 4.8).



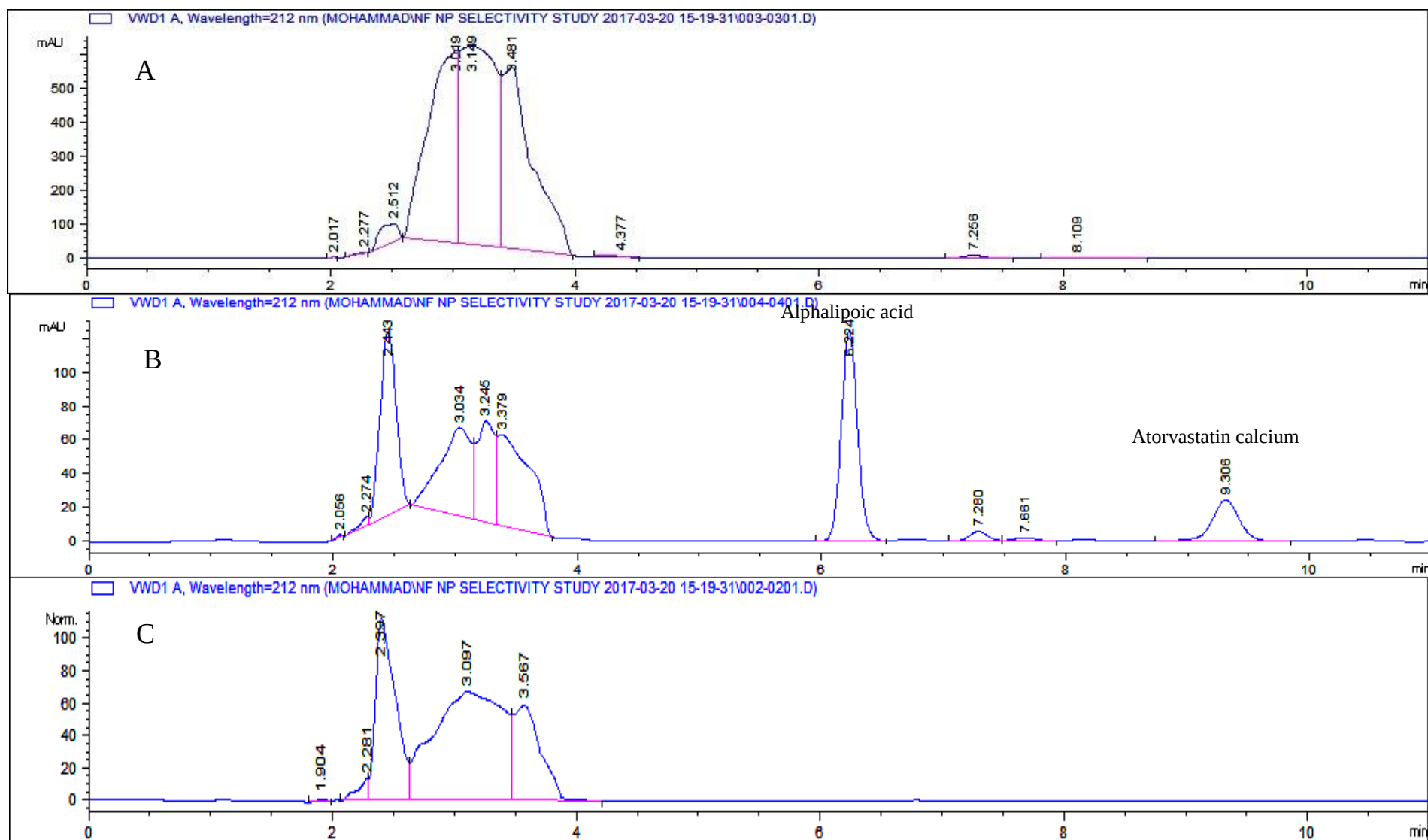


Figure 4.4. Representative chromatogram of the A) Mobile phase, B) Standard solution of ALA and ATR (4 µg/mL) and C) Placebo

Table 4.7. Robustness study of ALA.

ALA											
		Peak Area			Concentration (µg/mL)	Average	SD	RSD%	Overall average	Overall SD	Overall RSD%
Variables	Levels	1	2	3							
Wavelength (nm)	210	103.87	104.72	103.86	4.07	104.15	0.49	0.47	101.26	3.00	2.96
	212	102.32	102.83	101.33	3.99	102.16	0.76	0.74			
	214	97.144	97.891	97.429	3.81	97.49	0.37	0.38			
Flow rate (mL/min)	1.1	102.54	101.23	100.07	3.96	101.28	1.23	1.22	105.41	5.61	5.32
	1	102.32	102.83	101.33	3.99	102.16	0.76	0.74			
	0.9	112.04	113.27	113.13	4.42	112.81	0.67	0.59			
ACN: Buffer	57:43	101.22	102.3	102.6	3.99	102.04	0.72	0.71	101.91	0.8311	0.81
	55:45	102.32	102.83	101.33	3.99	102.16	0.76	0.74			
	53:47	100.47	102.75	101.39	3.97	101.54	1.15	1.13			
pH	4.3	102.05	101.53	101	3.97	101.53	0.52	0.51	102.04	0.76	0.74
	4.5	102.32	102.83	101.33	3.99	102.16	0.76	0.74			
	4.7	101.44	103.12	102.8	4.00	102.46	0.89	0.87			

Table 4.8. Robustness study of ATR.

ATR											
		Peak Area			Concentration (µg/mL)	Average	SD	RSD%	Overall average	Overall SD	Overall RSD%
Variables	Levels	1	2	3							
Wavelength (nm)	210	819.14	824.32	822.64	4.52	822.04	2.64	0.32	715.82	92.50	12.92
	212	717.65	717.98	715.23	3.94	716.96	1.50	0.20			
	214	606.34	611.73	607.34	3.34	608.47	2.87	0.47			
Flow rate (mL/min)	1.1	647.98	649.35	644.08	3.55	647.14	2.73	0.42	718.87	63.08	8.77
	1	717.65	717.98	715.23	3.94	716.96	1.50	0.20			
	0.9	786.26	799.77	791.57	4.36	792.54	6.80	0.85			
ACN: Buffer	57:43	711.75	719.77	711.43	3.92	714.32	4.72	0.66	715.26	2.85	0.39
	55:45	717.65	717.98	715.23	3.94	716.96	1.50	0.20			
	53:47	713.31	715.74	714.53	3.93	714.53	1.21	0.16			
pH	4.3	709.01	712.74	717.23	3.92	713.00	4.11	0.57	715.95	3.29	0.46
	4.5	717.65	717.98	715.23	3.94	716.96	1.50	0.20			
	4.7	719.15	718.91	715.65	3.94	717.91	1.95	0.27			

AUP = Area under the peak; ALA = Alpha lipoic acid; ATR = Atorvastatin calcium; ACN = Acetonitrile; SD = Standard deviation; RSD = Relative standard deviation

4.1.7. Stability Test

The stability test results demonstrated that ALA and ATR maintained stability in case their analytical solutions were stored at the auto-sampler condition and refrigerator condition ($5^{\circ}\text{C} \pm 3$) for 48h. The results of these tests are shown in (Table 4.9. and 4.10), where the RSD % were less than 1%.

Table 4.9. Stability test data in auto-sampler condition (n=3)

Drugs	Time Points				Statistical Analysis	
	0	4h	24h	48h	Average $\pm$ SD	RSD %
ALA	4.084 $\pm$ 0.036	4.081 $\pm$ 0.053	4.093 $\pm$ 0.018	4.135 $\pm$ 0.045	4.098 $\pm$ 0.024	0.603
ATR	4.033 $\pm$ 0.042	4.030 $\pm$ 0.057	4.094 $\pm$ 0.058	4.101 $\pm$ 0.36	4.065 $\pm$ 0.038	0.947

Table 4.10. Stability test data of stock solution in refrigerator condition ($5\pm 3^{\circ}\text{C}$) (n=3)

Drugs	Time Points				Statistical Analysis	
	0	4h	24h	48h	Average $\pm$ SD	RSD %
ALA	4.044 $\pm$ 0.110	4.089 $\pm$ 0.0101	4.073 $\pm$ 0.076	4.004 $\pm$ 0.078	4.053 $\pm$ 0.037	0.923
ATR	4.008 $\pm$ 0.053	4.043 $\pm$ 0.071	4.082 $\pm$ 0.054	4.089 $\pm$ 0.054	4.055 $\pm$ 0.037	0.931

4.2. Formulation of Atorvastatin Loaded Chitosan Nanoparticles by Nano Spray Drying Method

4.2.1. Characterization of Chitosan Nanoparticles

As shown in (Table 4.11), production yield values for all formulations have ranged from 58.30 % to 74.10 %. The highest production yield was obtained with formulations F12 and F11 that were prepared using spray cap ($7\mu\text{m}$ and $5.5\mu\text{m}$) and formulations with the highest amount of solid mass in initial solution (F7, F8, and F9 in case low Mwt CH is used). ATR loading efficiency was decreased by decreasing the molecular weight of chitosan (F10 and F4). The highest loading efficiency was

obtained in the case of formulation F9 (53.61 ± 4.52 %) containing 0.04% (w/v) CH and 0.04% (w/w) ATR.

The mean particle size values for all formulations were found to be in submicron range (510.5 ± 22.33 nm- 820.0 ± 98.71 nm) with almost a narrow particle size distribution (PDI) value and having positively charged. The smallest particles were obtained by 4 μ m spray mash diameter. As spray mash diameter increased, mean particle size also increased (F4: 516.5 ± 59.48 nm; F11: 602.3 ± 76.38 nm; F12: 779.6 ± 24.39 nm, respectively).

Chitosan molecular weight was found to be an effective variable on particle size. When molecular weight was the only parameter that has been altered (F4 and F10), mean particle size significantly increased in accordance with the increase in molecular weight ($p < 0.05$) (516.5 ± 59.48 nm and 683.4 ± 54.25 nm for F4 and F10, respectively).

Table 4.11. Results of the nano sprayed CH nanoparticles regarding product yield, loading efficiency, particle size and zeta potential $n=3$.

Formulations	Product yield (%)	Loading efficiency (%)	Average particle size (nm) $\pm$ S.D.	Polydispersity index (PDI) $\pm$ S.D.	Zeta potential (MV) $\pm$ S.D.
F1	63.89	38.07 ± 3.11	510.5 ± 22.33	0.26 ± 0.10	11.1 ± 1.09
F2	58.41	48.95 ± 3.26	566.1 ± 59.98	0.32 ± 0.18	11.9 ± 0.41
F3	62.38	49.62 ± 2.91	580.4 ± 93.96	0.22 ± 0.12	15.2 ± 1.64
F4	58.30	33.30 ± 4.01	516.5 ± 59.48	0.58 ± 0.38	18.5 ± 1.30
F5	67.30	36.54 ± 1.13	628.7 ± 150.80	0.1 ± 0.02	20.8 ± 0.73
F6	67.20	45.16 ± 2.45	708.9 ± 124.80	1	18.7 ± 1.66
F7	67.30	23.37 ± 2.71	656.4 ± 147.70	1	26.6 ± 1.65
F8	69.80	47.98 ± 2.42	630.3 ± 61.75	0.20 ± 0.13	21.7 ± 0.41
F9	67.60	53.61 ± 4.52	820.0 ± 98.71	0.30 ± 0.28	24.9 ± 4.95
F10	73.27	36.65 ± 2.98	683.4 ± 54.25	0.48 ± 0.15	20.6 ± 1.65
F11	69.40	31.66 ± 2.29	602.3 ± 76.38	0.22 ± 0.12	15.0 ± 2.11
F12	74.10	40.93 ± 3.97	779.6 ± 24.39	0.43 ± 0.12	21.6 ± 1.95

Morphology

Surface morphology investigation of ATR chitosan nanoparticle was evaluated by SEM and TEM investigations. The visual observations of SEM images indicated that, ATR nanoparticles were almost spherical with a wrinkled surface for all formulations (Figure 4.5). Nearly in all cases, small particles presented smoother surface with respect to bigger particle. TEM investigations also confirmed that the CH nanoparticles containing ATR were in spherical shape and in nanosized (Figure 4.6).

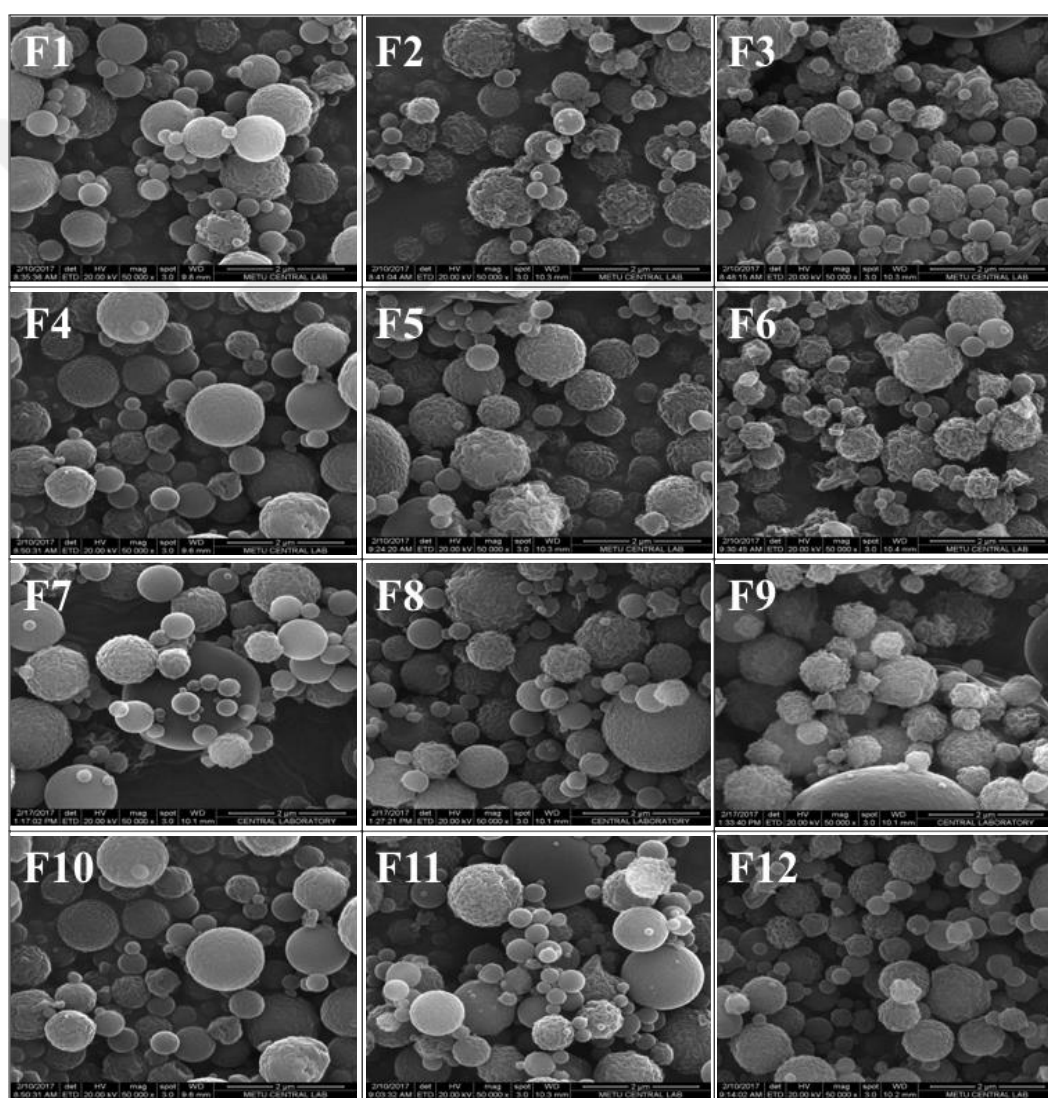


Figure 4.5. SEM images of CH nanoparticles containing ATR for F1 to F12 formulations (magnification ratio: X50.000; voltage 20 kV).

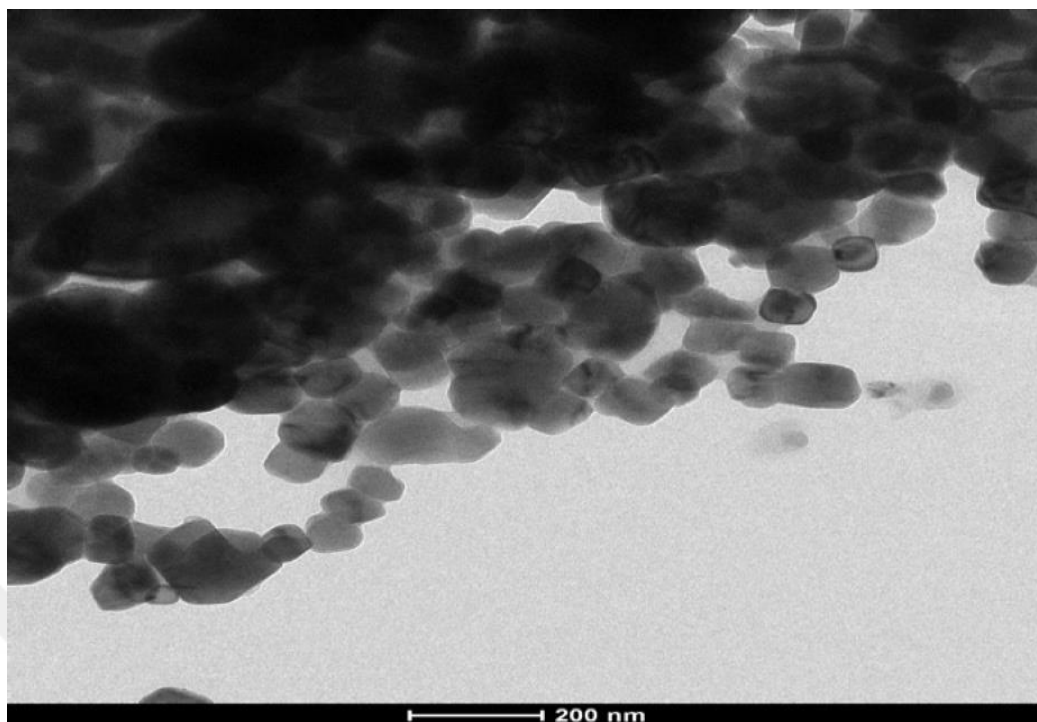


Figure 4.6. TEM image of CH nanoparticles containing ATR (formulation code F4; voltage 120 kV).

Differential Scanning Calorimetry Analysis

The thermograms of ATR, CH, blank chitosan nanoparticles and loaded nanoparticles are shown in (Figure 4.7). The DSC thermogram of ATR demonstrated three endothermic peaks; first at 97.54 °C, which was formed during the loss of water molecules; the second endotherm peak depicting the melting of ATR which took place at 256.39°C and followed by a broadened endotherm peak centered at 228.84 °C which was attributed to complete loss of water of calcium salt of ATR. The decomposition exothermic peak of ATR was observed at 285.62°C. With respect to the CH thermogram, a broad endothermic peak centered at 80.16 °C indicated loss of absorbed water and the exothermic peak at 303.76 °C can be link to polymer thermal decomposition. The endothermic peaks of ATR disappear in DSC curve of ATR loaded chitosan nanoparticles, which indicated successful encapsulation of ATR.

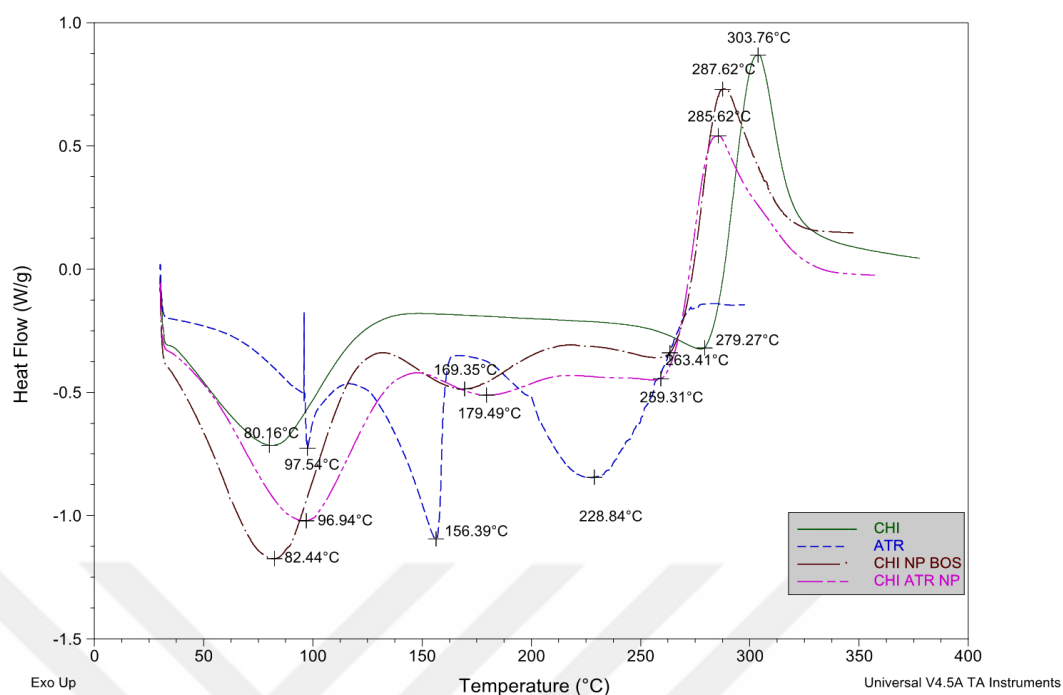


Figure 4.7. Differential Scanning Calorimetry (DSC) thermograms for CH, ATR, unloaded CH nanoparticles and ATR loaded CH nanoparticles.

FTIR Results

Fourier transform infrared spectra (FTIR) was adopted to characterize the potential interactions among the constituents of nanoparticles formulations. Figure 4.8. presents IR spectra of ATR, CH, unloaded CH nanoparticle and ATR loaded CH nanoparticles. In the spectra of CH, the broad band -OH stretching at 3362 cm^{-1} ; the carbonyl (C=O) stretching band at 1656 cm^{-1} and N-H bending vibrations at 1561 cm^{-1} were observed. The ATR exhibits characteristic peaks at 3362 cm^{-1} (-OH stretching at carboxylic acid), 3222 cm^{-1} (-OH stretching), 1651 cm^{-1} (C=O stretching), 1315 cm^{-1} (C-N stretching) and 1215 cm^{-1} (C-F stretching). In the IR spectrum of ATR loaded CH nanoparticles, the specific bands at fingerprint area and -OH stretching of carboxylic acid band disappeared. These results indicated that ATR was successfully embedded in the CH nanoparticles.

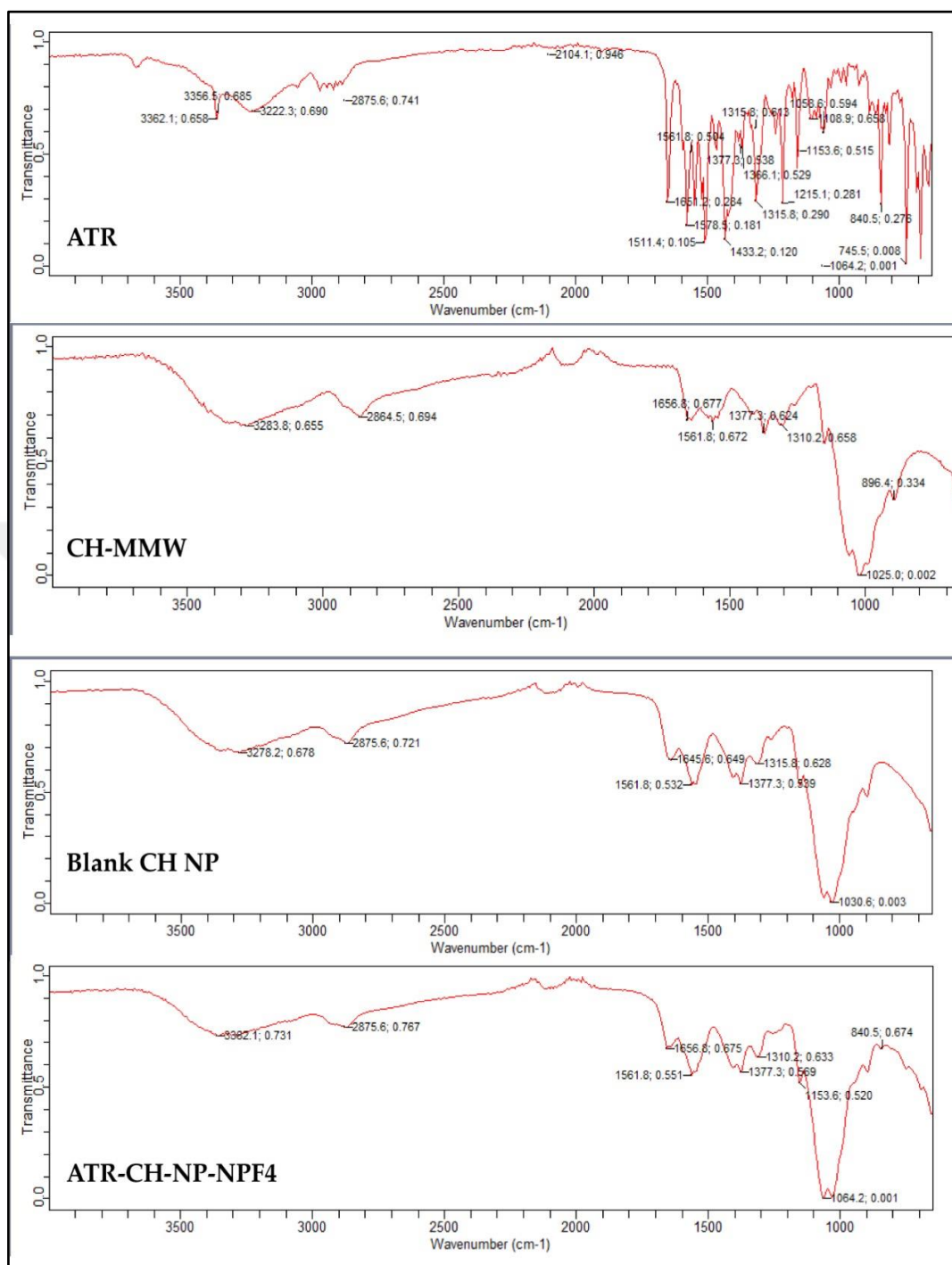
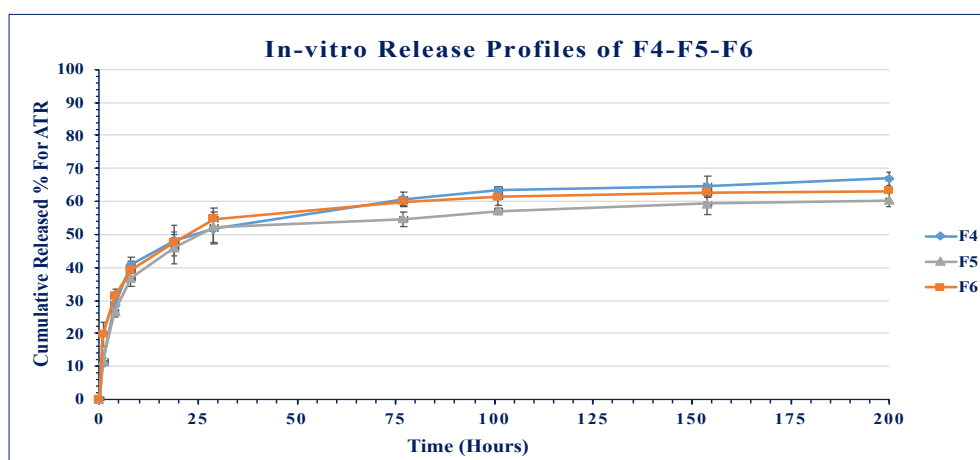
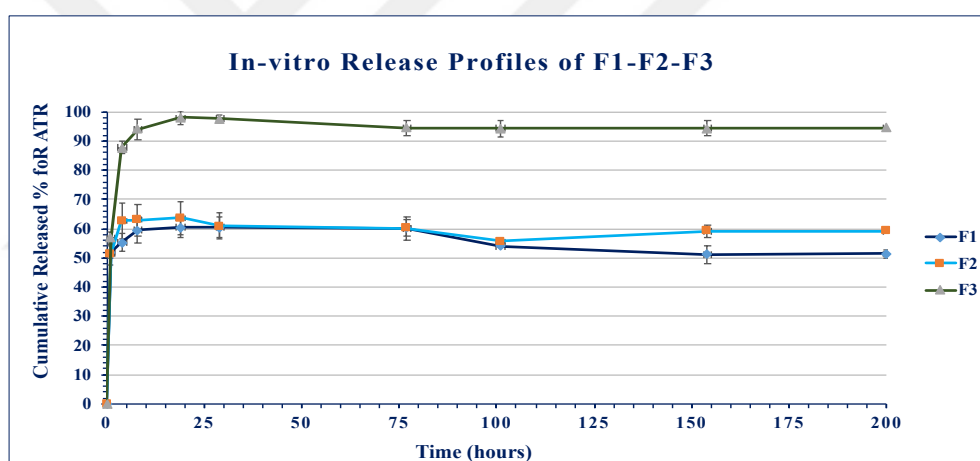


Figure 4.8. FTIR spectra of ATR; CH; unloaded CH nanoparticles and ATR loaded CH nanoparticles. *ATR = Atorvastatin calcium; *CH-MMW = Chitosan medium molecular weight; *Blank CH NP = Blank chitosan nanoparticles; *ATR-CH-NP-NPF4 = Chitosan nanoparticles containing atorvastatin calcium formulation 4

4.2.2. In-Vitro Release Studies

In-vitro release studies results indicated that, all formulations displayed biphasic release profile that has started by an initial burst release and followed by a sustained release of ATR.

Figure 4.9 shows the plotting of cumulative percent release profile of ATR from chitosan Nano Spray dried formulations. Formulations F1, F2, F3 exhibited the highest initial burst release (51.96, 51.49 and 56.85 %) during the first one hour whereas, significantly lower burst release (9.88-19.62 %) was observed for other formulations. Among all formulations, F3 demonstrated a remarkable highest release (97.97 %) of the entrapped drug in 200 h and F7 showed a maximum (44.73 %) of cumulative drug release.



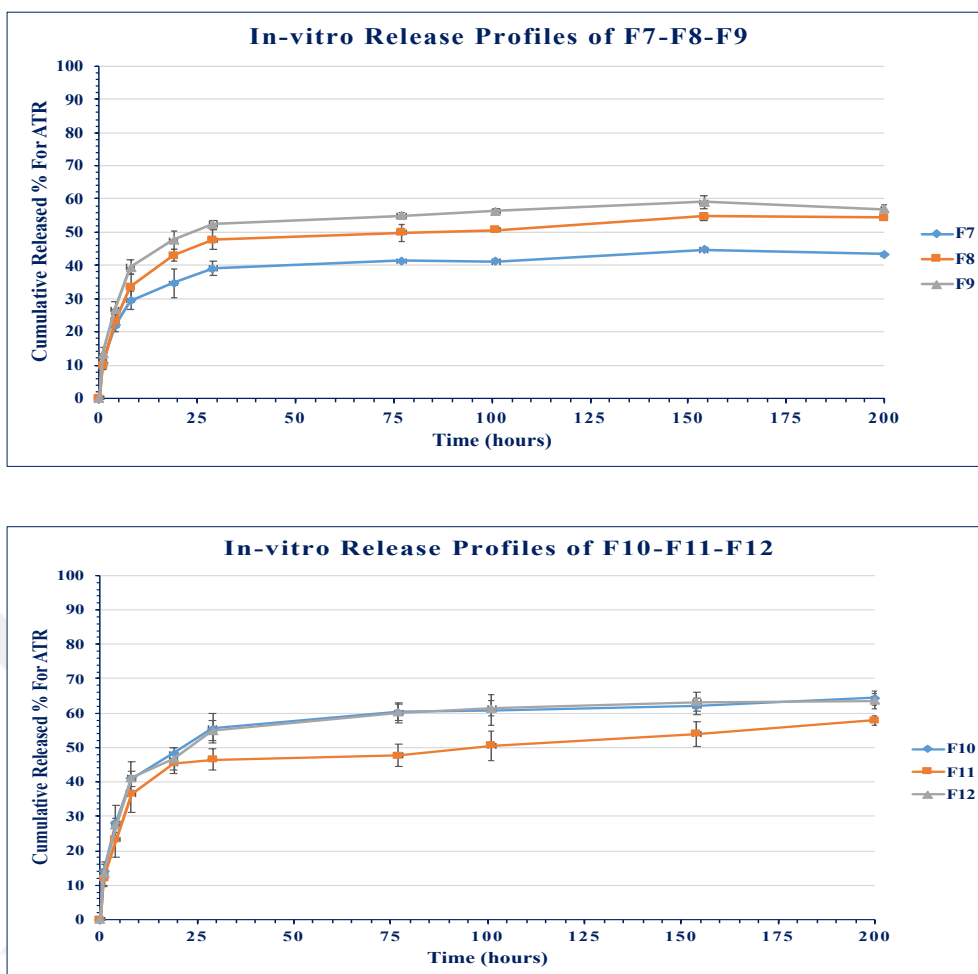


Figure 4.9. In-vitro release profiles of ATR from CH nanoparticles. Release data for every time point is presented with average of 3 replicates with standard error bars.

4.2.3. Cell Viability

According to cell viability results, blank and ATR loaded nanoparticles were found to have no cytotoxic effect on both L-929 and B35 cell lines up to 48h showing similar dose dependent tendency. The viability of B35 cells at 24h and 48h were between 95-113 % and 96-98 %, respectively; showing the bio-compatible nature of the blank nanoparticles. The positive impact of the encapsulation of ATR on neuroblastoma cell line was significant when compared to that of blank nanoparticles at concentrations of 10, 5 and 2.5 $\mu\text{g/mL}$, after 48 hours of incubation ($p < 0.1$, $p < 0.0001$, $p < 0.01$, respectively) (Figure 4.10.A).

For L-929 fibroblast cell line, viability was observed between 90-97% and 76-85% with blank nanoparticles after 24 and 48 h of incubation, respectively. No significant difference was found for both blank and ATR incorporated nanoparticles between the same applied dose groups during 24 and 48h periods. The difference became significant by time, implying the positive effect of atorvastatin in chitosan nanoparticles. The addition of ATR increased the cell viability after 48 h, and the viability was observed as approximately 126% ($p<0.0001$) for 10 $\mu\text{g/mL}$. In addition, the proliferative effect of ATR was slightly increased during 24 h in a dose dependent manner (Figure 4.10 B).

The effect of encapsulation of ATR in nanoparticles as compared to the solution form was observed for 24 and 48h (Figure 4.11). For L-929 cell line, no cytotoxic effect was observed at any concentration with more than 96% cell viability up to 48h. The use of chitosan was observed to increase cell viability in B35 cell line both in the absence and presence of the drug, and the effect became significant with the addition of ATR. The results have shown that although there was no significant difference between different doses of ATR, encapsulation of ATR in chitosan nanoparticles has increased the activity of ATR when compared to the solution form in B35 cell line, which implies the neuroprotective potential of ATR nanoparticles. The unfavorable effect of the ATR solution at high concentration (10 $\mu\text{g/mL}$) concentration on B35 cell line was prevented by increasing the viability 1.65-fold with the administration of ATR in chitosan nanoparticles, which shows the potential of the drug delivery system in decreasing the undesirable negative effects of the drug in solution.

The cytotoxicity results revealed the potential of ATR loaded chitosan nanoparticles in terms of biocompatibility and bioactivity in two different cell lines. The safe use of ATR loaded chitosan nanoparticles were shown in the range of 2.5 to 10 $\mu\text{g/mL}$ in-vitro, giving the opportunity to change the profile of the drug when incorporated in nanoparticles.

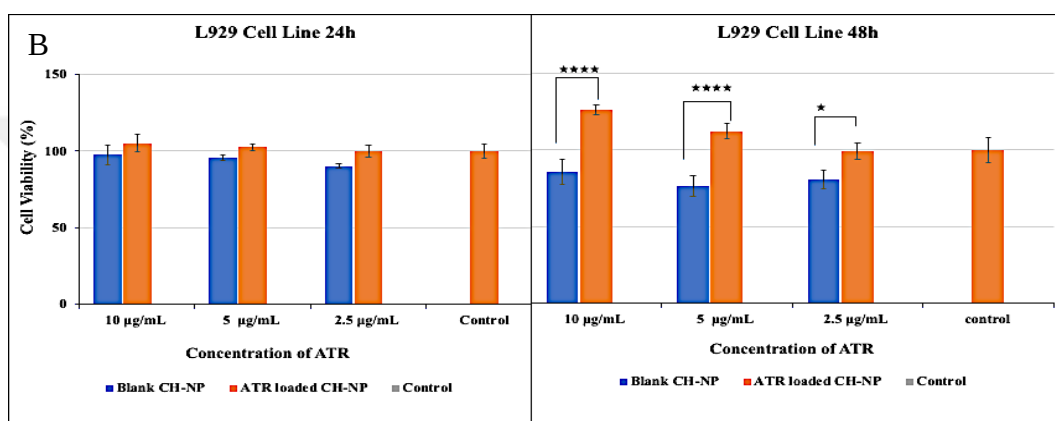
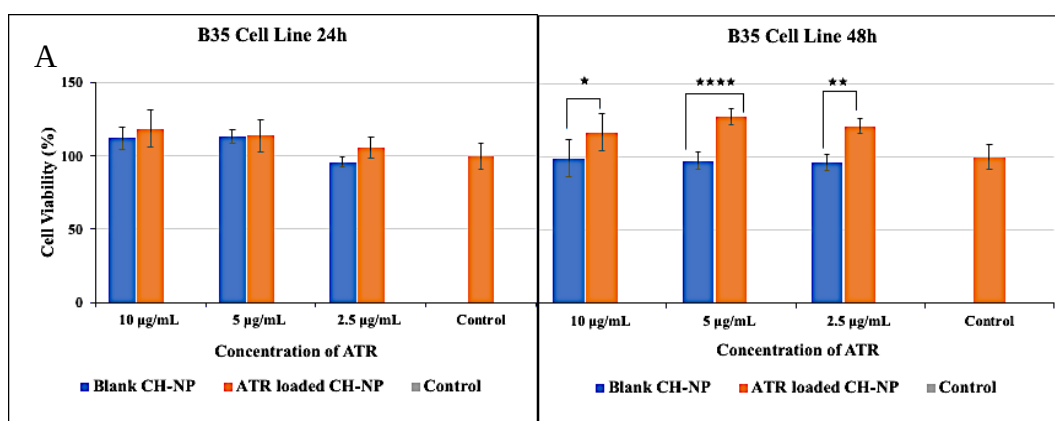
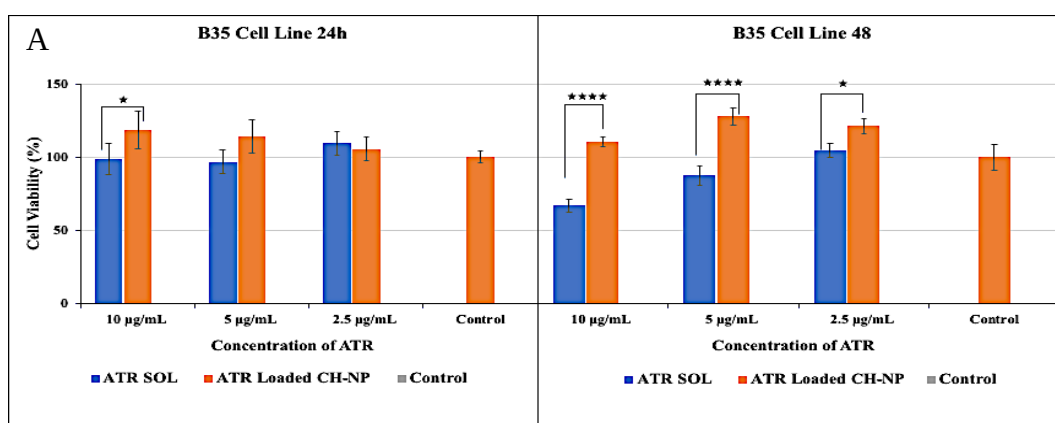


Figure 4.10. The cell viability of CH nanoparticle formulations in: A) B35 cell line; B) L-929 cell line. * $p < 0.1$; ** $p < 0.01$; **** $p < 0.0001$



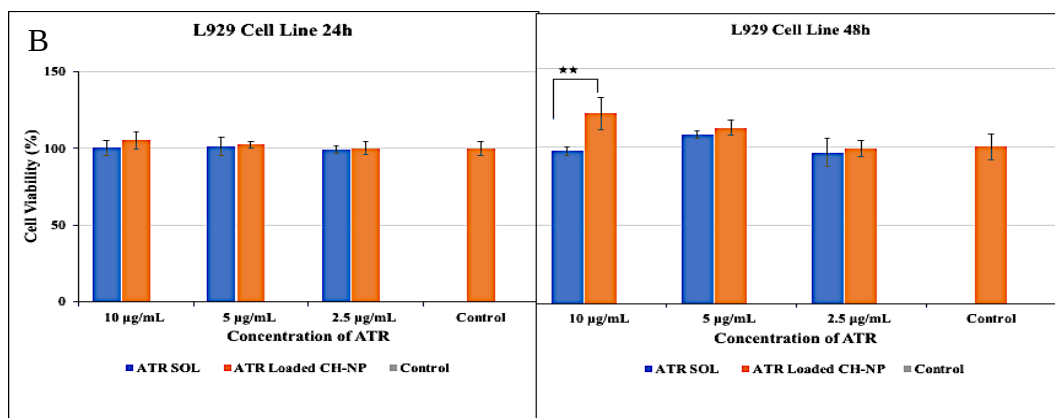


Figure 4.11. The cell viability of ATR in: A) B35 cell line; B) L-929 cell line n = 4.

* p<0.1; ** p<0.01; **** p<0.0001

4.3. Optimization of Electrospinning parameters for PLGA Nanofiber

With a low molecular weight of PLGA ($M_w = 24,000-38,000$), instead of nanofiber, electro nanoparticles or microparticles were formed (Figure 4.12.A). Uniform fibers were obtained with the increase in PLGA molecular weight ($M_w = 38,000-54,000$) (Figure 4.12. B). With a further increase in the polymer molecular weight, the diameter of nanofiber was also increased (Figure 4.12. C).

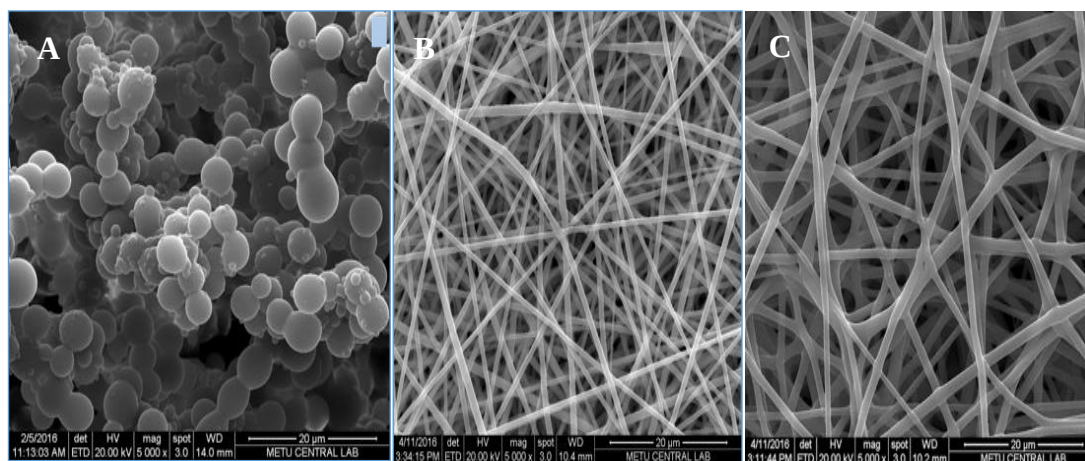


Figure 4.12. SEM image PLGA nanofiber: A) PLGA ($M_w = 24,000-38,000$); B) PLGA ($M_w = 38,000-54,000$); C) PLGA ($M_w = 54,000-69,000$).

The lowest concentration of PLGA (15wt%) had the highest tendency to form beads. Concentration of 15% resulted in nanofibers with a morphology primarily composed of polymer beads with small amounts of fibers (Figure 4.13.A).

PLGA concentrations of 20% resulted in fibers containing beads (Figure 4.13.B). In the case of 25% PLGA, the number of beads decreased, but beads were still rarely visible (Figure 4.13.C).

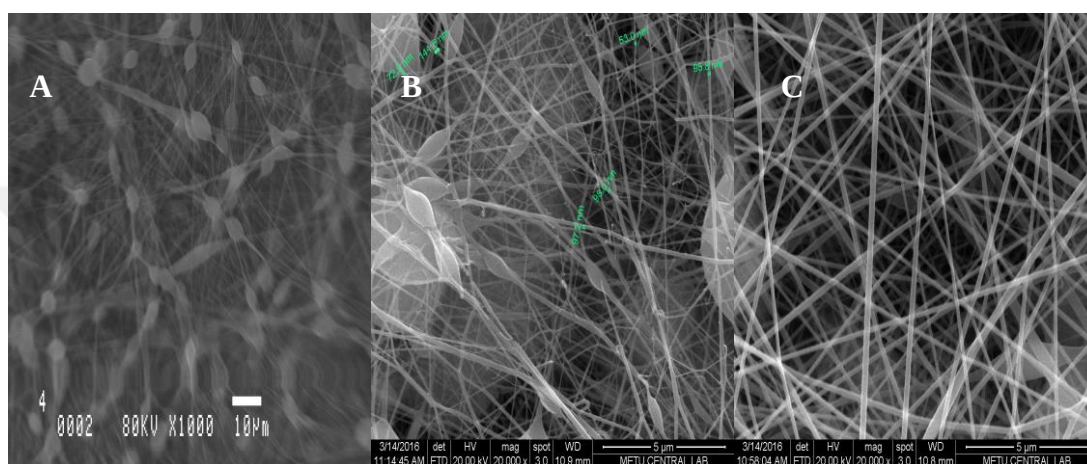


Figure 4.13. SEM image PLGA nanofiber: A) PLGA (15wt%); B) PLGA (20wt%); C) PLGA (25wt%).

In the electrospinning system, it is common to utilize some co-solvents to tune the solvent polarity which is lead to improving electrospinnability of polymer solution. The results have demonstrated that the surface morphology and diameter of the resultant fibers, could be altered easily by the different co-solvent ratio.

The surface morphology of the resultant fibers changed from bead full morphology (Figure 4.14.A) to relatively smooth surface (Figure 4.14.B), when DMF ration was decreased to 35%. Nanofiber with elongated beads and non-uniform diameter was formed, when Chloroform: DMF (60:40) was utilized (Figure 4.14.C).

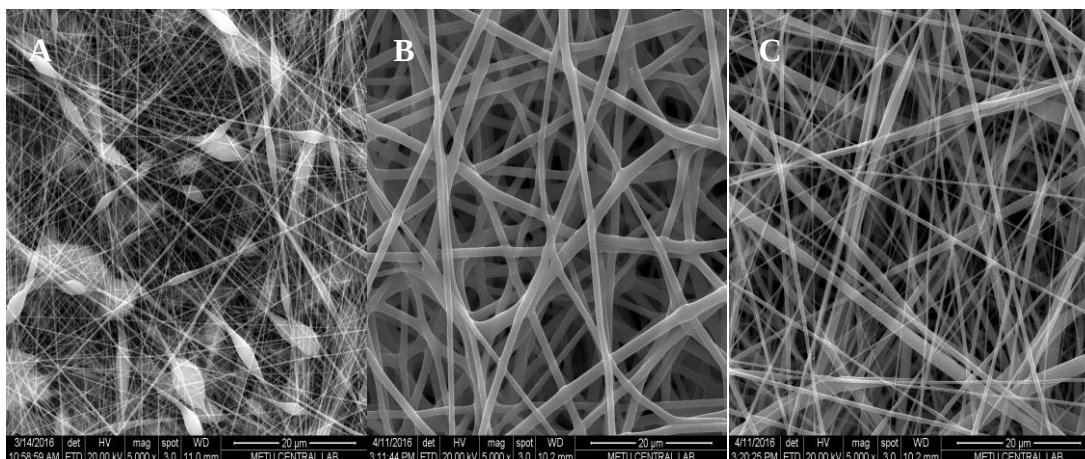


Figure 4.14. SEM image PLGA nanofiber: A) DMF: THF (65:35); B) THF : DMF (65:35); C) Chloroform : DMF (60:40). DMF= dimethylformamide, THF= tetrahydrofuran.

As the applied voltage increases more material is drawn into the electrical field leading to a larger jet diameter. The effect of applied voltage on the diameter of the resulting fiber is shown in (Figure 4.16) with an increase in applied voltage, fiber diameter was also increased.

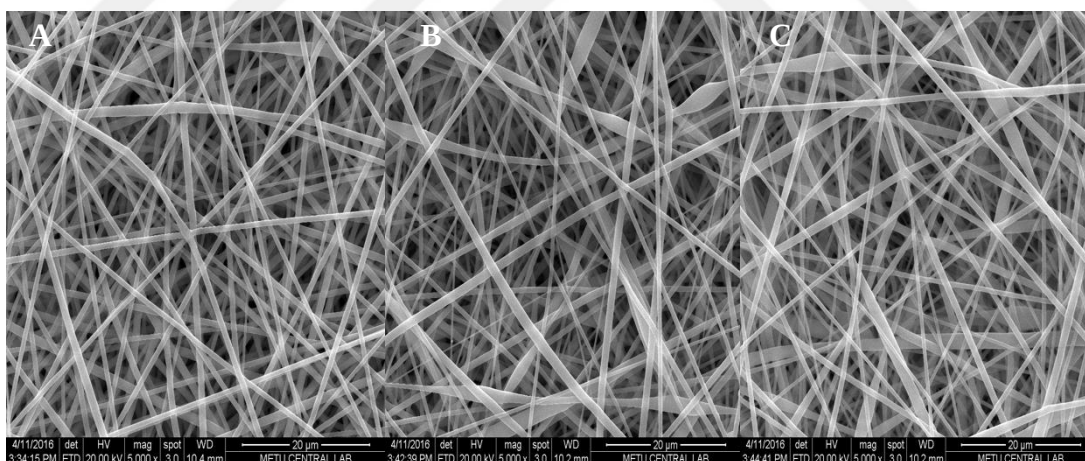


Figure 4.15. SEM image PLGA nanofiber: A) Applied voltage 19kv; B) Applied voltage 21kv; C) Applied voltage 22kv.

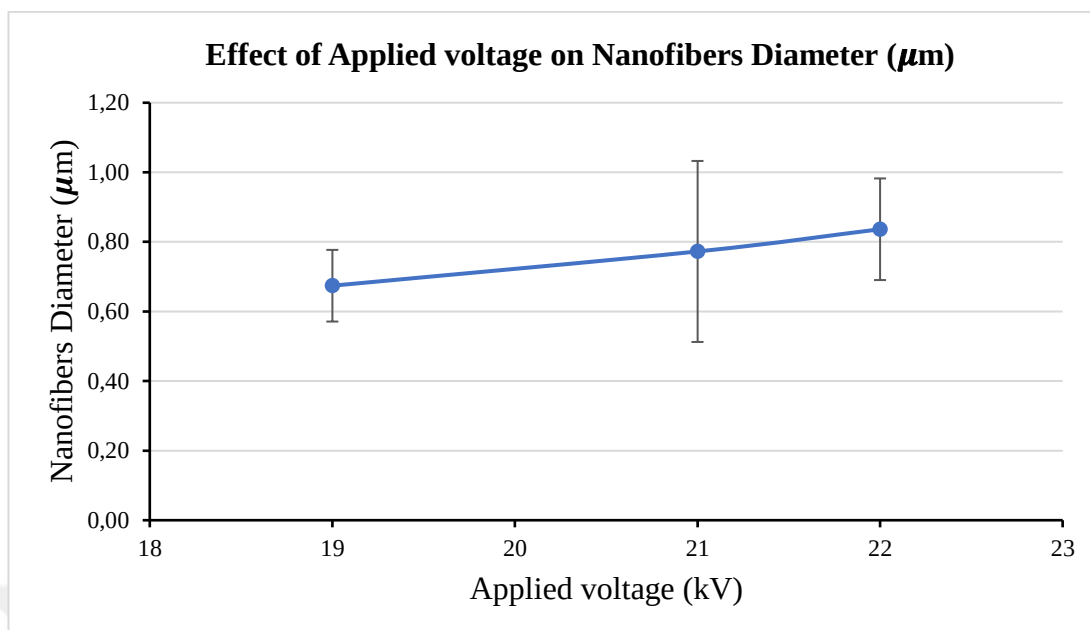


Figure 4.16. The effect of applied voltage on the diameter of the resulting fiber (mean $\pm$ SD, n=100).

As the distance between nozzle and collector was increased, the jet became more stretched, thereby reduced the jet diameter. As a result, the mean fiber diameter was also reduced (Figure 4.18).

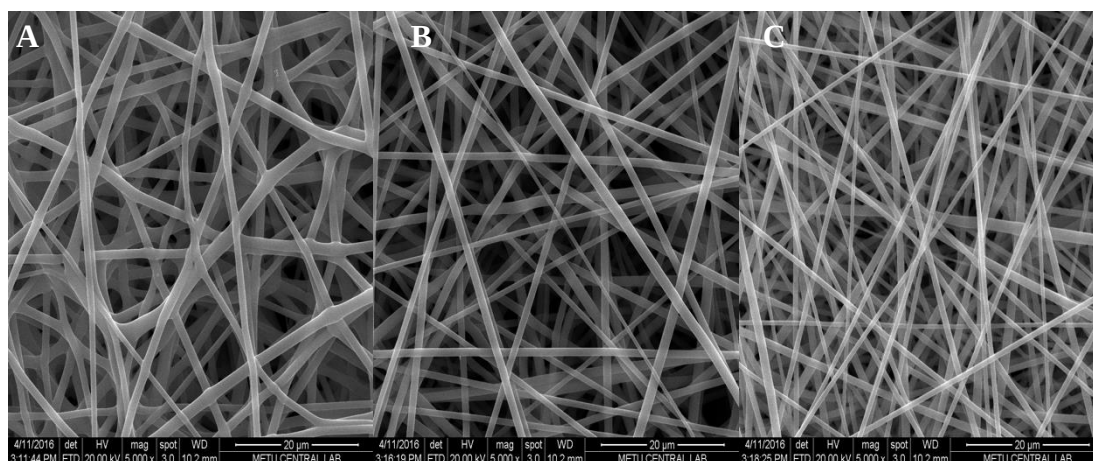


Figure 4.17. SEM image PLGA nanofiber: A) Nozzle to collector distance 12cm; B) Nozzle to collector distance 16cm; C) Nozzle to collector distance 18cm.

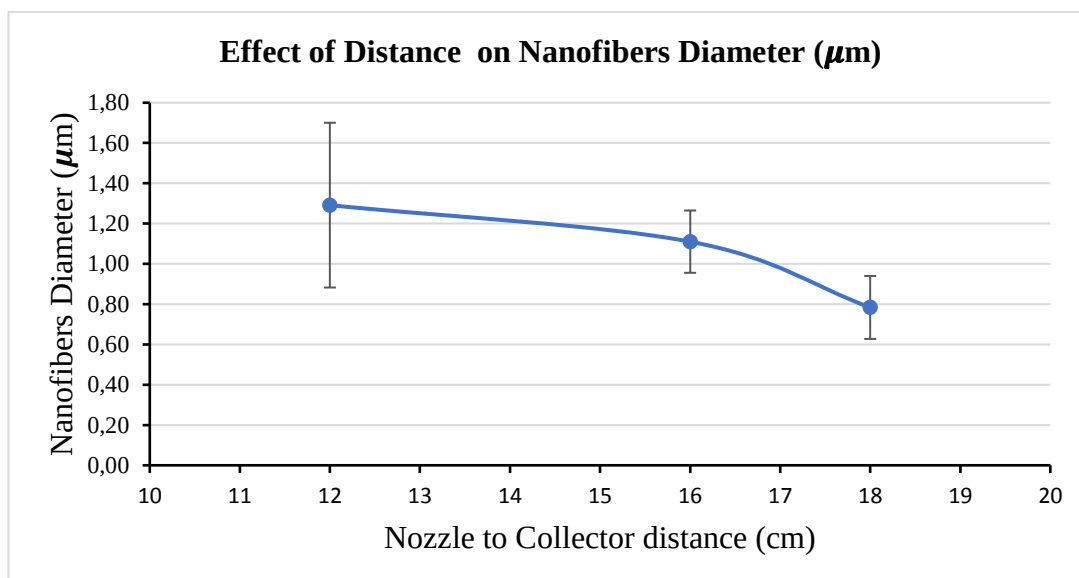


Figure 4.18. The effect of nozzle to collector distance on the diameter of the resulting fiber (mean $\pm$ SD, n=100).

When the polymer solution flow rate was increased, the fiber diameter also increased similarly (Figure 4.19).

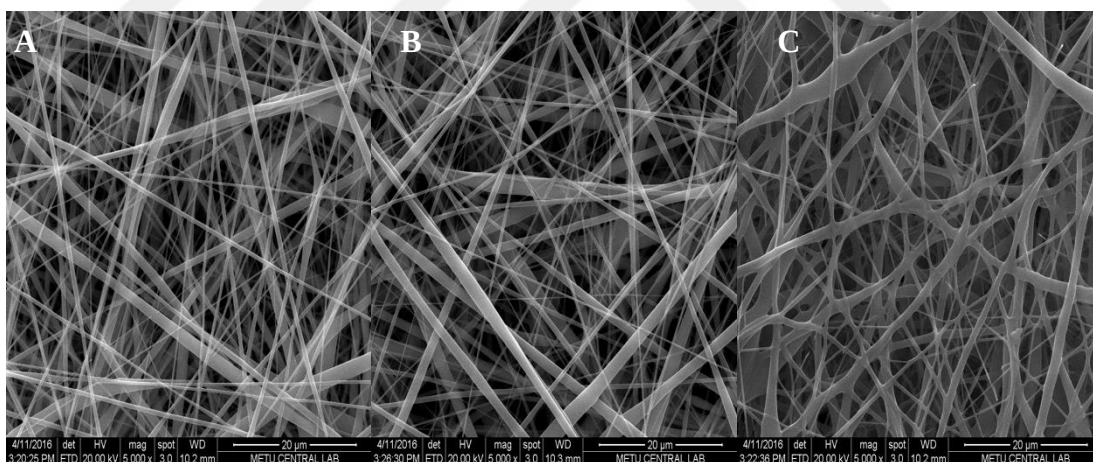


Figure 4.19. SEM image PLGA nanofiber: A) Flow rate 1mL/h; B) Flow rate 1.5mL/h; C) Flow rate 2mL/h.

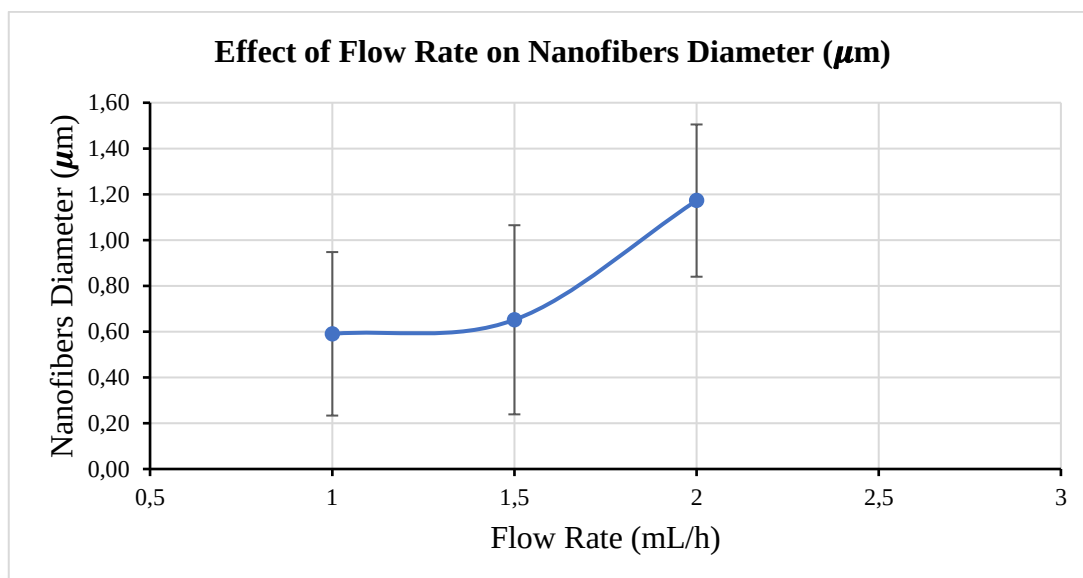


Figure 4.20. Effect of flow fate on nanofibers diameter (μm) (mean $\pm$ SD, $n=100$).

4.4. Characterization of PLGA/CH Electrospun Nanocomposite

4.4.1. Morphology

Smooth nanofibers with mean diameter of $340 \pm 69 \text{ nm}$ were produced from PLGA solution containing suspended CH nanoparticles by electrospinning method as shown in (Figure 4.21). The SEM images proved that electrospun composite nanofibers were prepared from the mixture of PLGA and ALA blend solution that contained ATR loaded CH nanoparticles where the core-sheath structure was made with CH nanoparticle as a core, and PLGA made the sheath of the nanocomposite structure. The CH nanoparticles were completely covered by PLGA nanofiber. Even nanoparticles bigger than nanofiber diameter, did not expose on the surface of nanofibers. Furthermore, the dispersion and encapsulation of the CH nanoparticles clearly observable in TEM images (Figure 4.22).

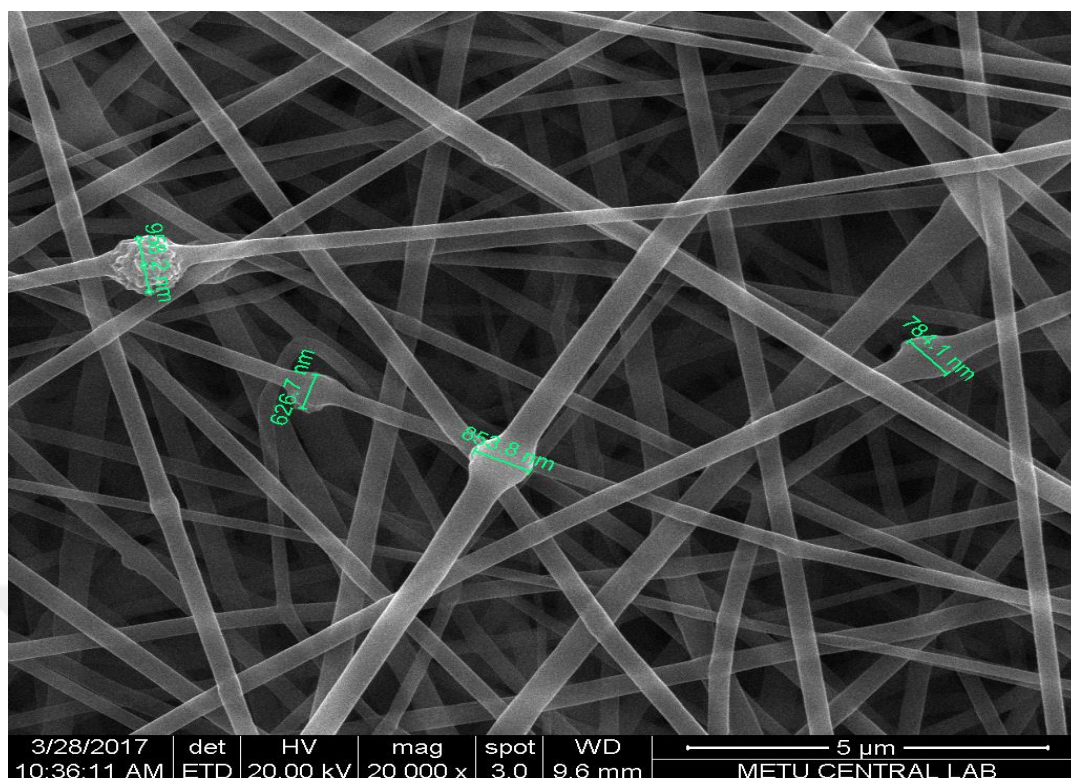


Figure 4.21. SEM image of the CH Nanoparticles-Containing PLGA Composite Nanofibers.

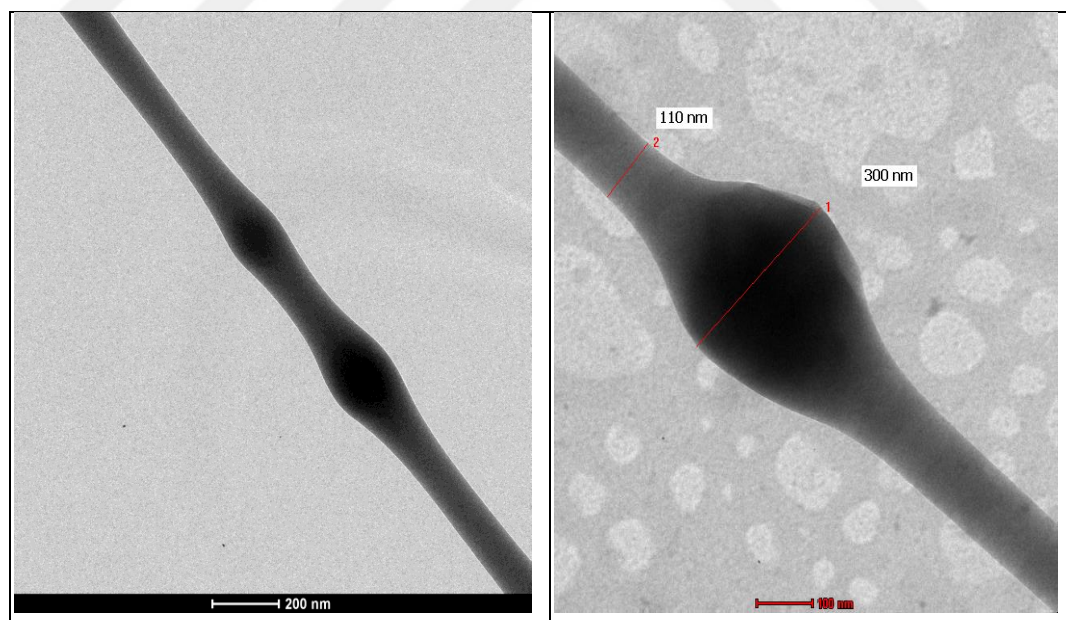


Figure 4.22. TEM image of the CH Nanoparticles-Containing PLGA Composite Nanofibers (Formulation A).

4.4.2. Encapsulation Efficiency

Encapsulation Efficiency (EE) was estimated and repeated three times. EE of ALA was found to be $92.76 \pm 3.53\%$, which was showed that formulation had a fairly good EE. In addition, EE for ATR found $89.27 \pm 5.053\%$. The result revealed that relatively high EE of active ingredients has been achieved.

4.4.3. Differential Scanning Calorimetry Analysis-DSC

DSC thermograms of the empty and loaded CH/PLGA electrospun nanocomposite formulations and components are shown in (Figures 4.13). In the DSC thermogram of ALA (Figure 4.23 A), a sharp endothermic peak at $62.55\text{ }^{\circ}\text{C}$ was observed. Similarly, three endothermic peaks at $97.20\text{ }^{\circ}\text{C}$, $156.05\text{ }^{\circ}\text{C}$, and $227.14\text{ }^{\circ}\text{C}$ were seen in the DSC thermogram of ATR (Figure 4.23.B). The DSC thermogram of loaded CH/PLGA electrospun nanocomposite formulation revealed that the endothermic peaks of ALA and ATR, which are the only active substances, were disappeared in the loaded electrospun nanocomposite formulation (Figure 4.23.D). It indicated the successful encapsulation of the active ingredients in the formulation.

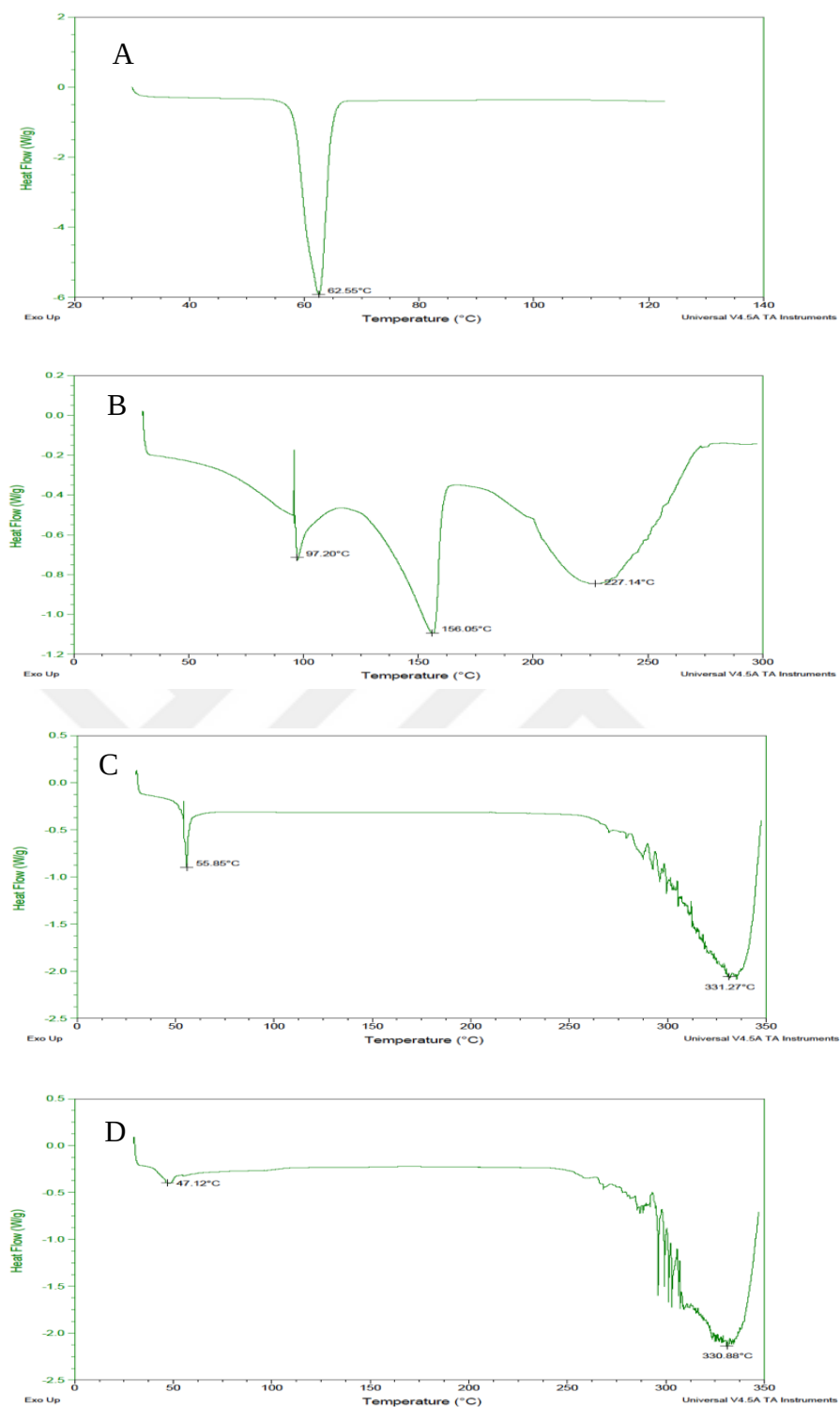


Figure 4.23. A) DSC thermogram of pure ALA; B) ATR; C) Empty CH/PLGA electrospun nanocomposite formulation; and D) loaded CH/PLGA electrospun nanofibers formulation.

4.4.4. X-Ray Analysis

The results of X-ray analysis demonstrated that ALA and ATR were in the crystal form (Figure 4.24). Empty nanofibers prepared with PLGA exhibit amorphous nature. And after loading the active ingredients (ALA and ATR) some peaks that possibly belong to ALA crystals were observed in diffractograms, which indicated the possible surface localization of ALA.

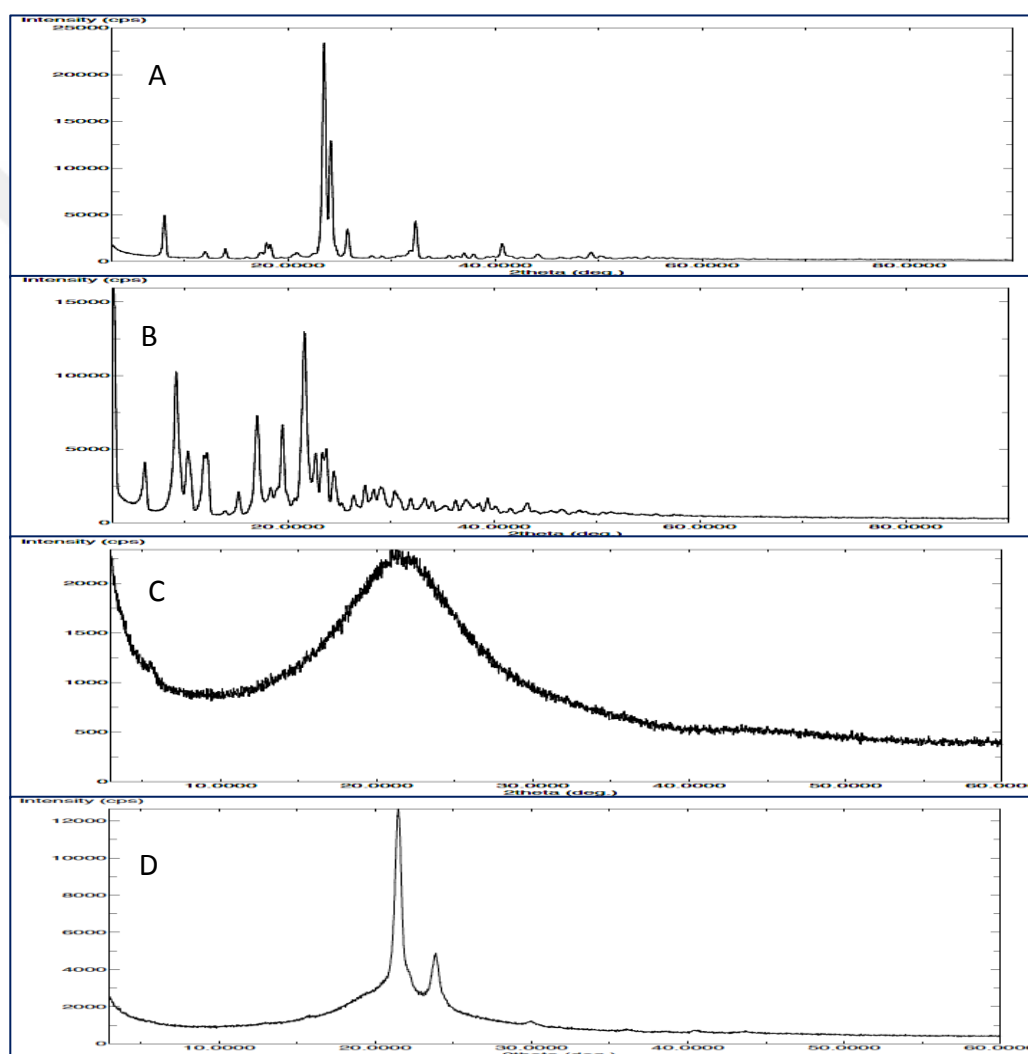


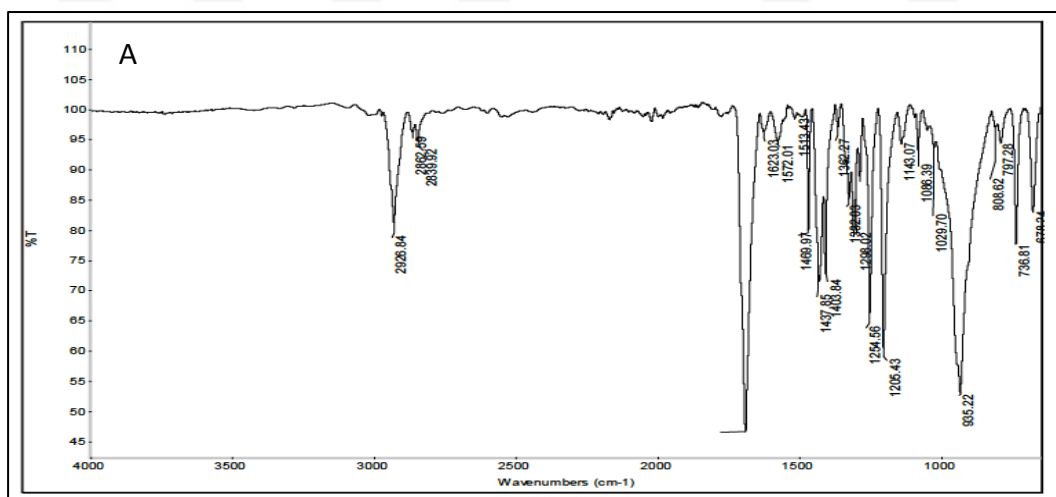
Figure 4.24. A) XRD diffractogram of pure ALA; B) ATR; C) Empty CH/PLGA electrospun nanocomposite formulation and D) loaded CH/PLGA electrospun nanofibers formulation.

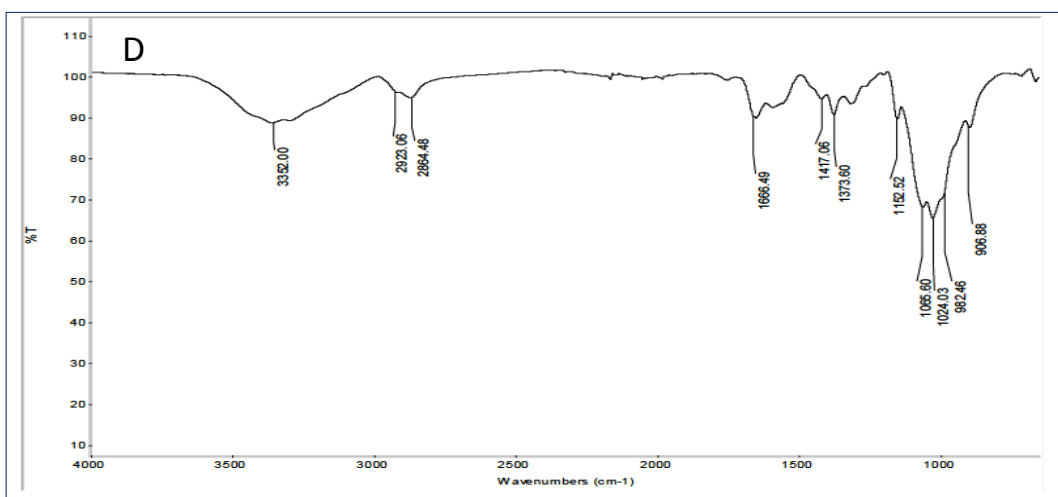
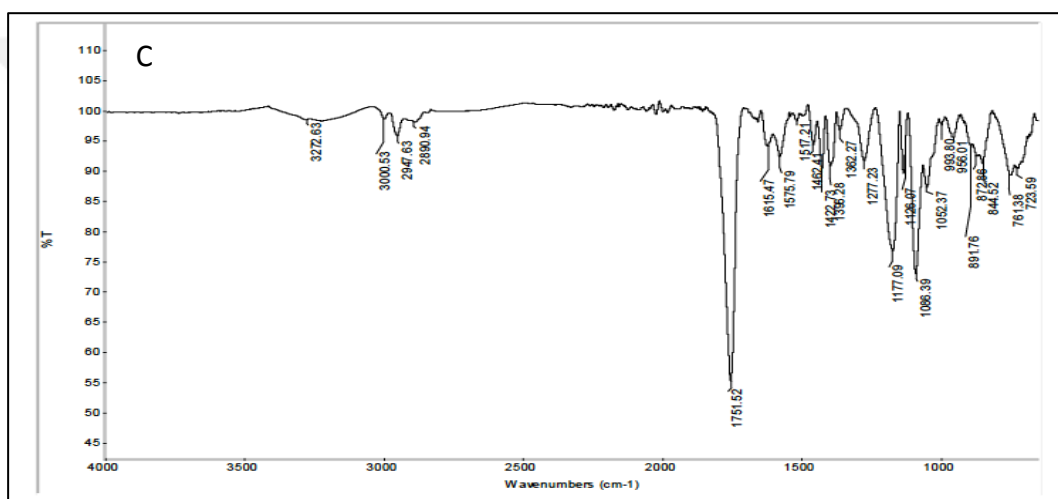
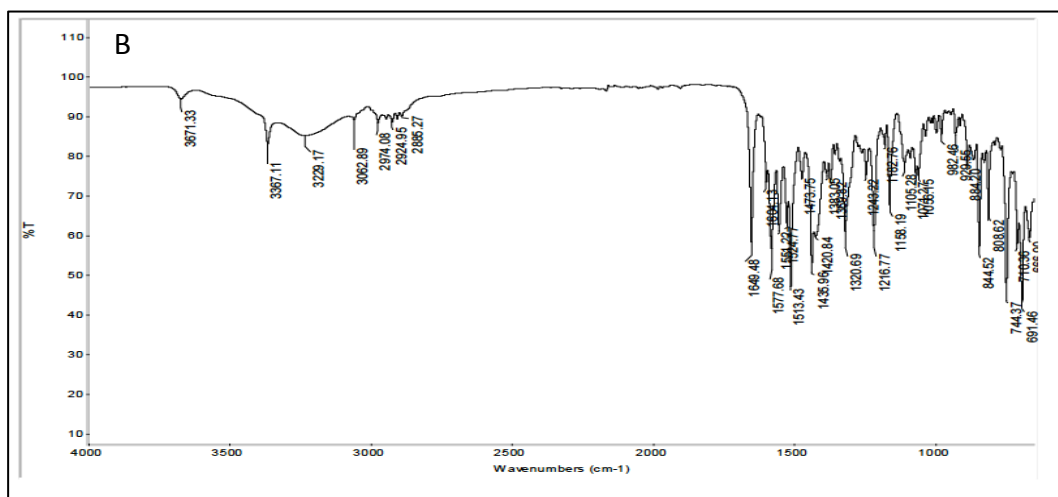
4.4.5. FT-IR

Figure 4.25, represents the FTIR spectra of pure ALA, pure ATR, pure CH, pure PLGA, empty CH/PLGA electrospun nanocomposite formulation, and drug loaded CH/PGLA electrospun nanocomposite formulation. When the FTIR spectra of ALA was examined, the C = O stretching pike at 1700 cm^{-1} and the C-H stretching pike at 1469 cm^{-1} are specific for ALA. ATR another active substance of formulation, showed peaks at 1649cm^{-1} , 1320cm^{-1} , and 1216cm^{-1} which are attributed to the stretching of the C-O, C-N, and C-F bonds respectively.

The disappearance of peaks at 3671 cm^{-1} and 3367 cm^{-1} of ATR's FI-TR spectra, in the encapsulation of ATR with PLGA suggests that ATR was successfully encapsulated. Comparison of empty formulation and ALA loaded formulation, the shoulder at 1760 cm^{-1} indicates that ALA was localized to the nanofiber surface.

It has been shown that both active substances were successfully encapsulated based on the fact that the loaded nanofiber, empty nanofiber and PLGA spectra are similar, and that the above-mentioned specific peaks do not appear on these spectra.





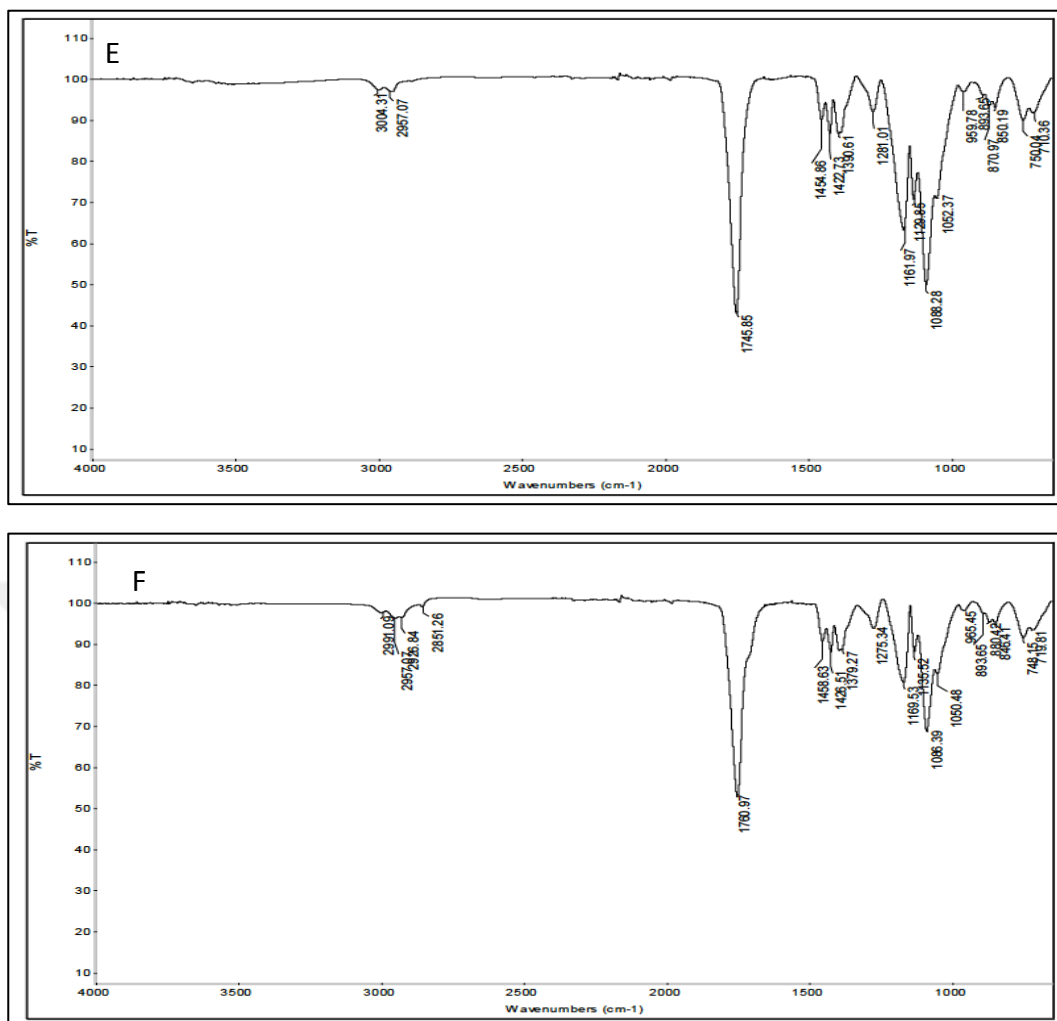


Figure 4.25. A) FTIR spectra of pure ALA; B) Pure ATR; C) Pure PLGA; D) Pure CH; E) empty CH/PLGA electrospun nanocomposite formulation; F) ATR and ALA loaded CH/PLGA electrospun nanocomposite formulation.

4.4.6. Porosity Measurements

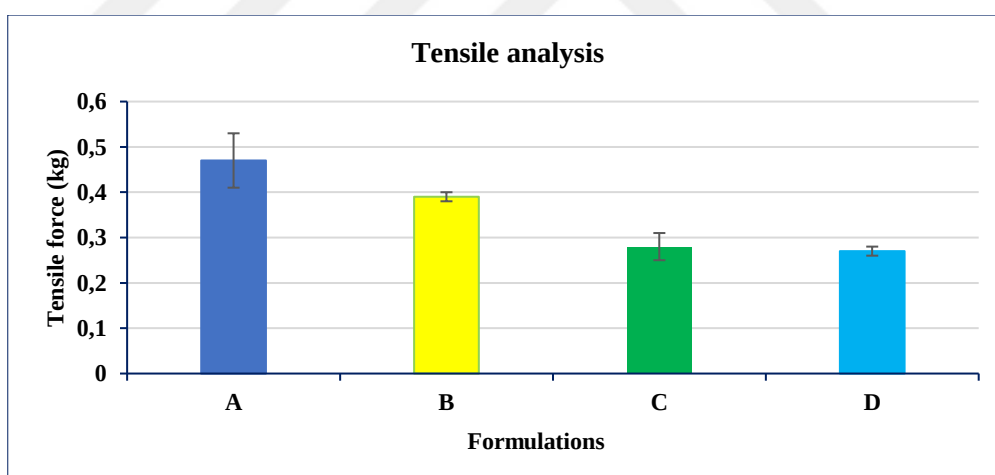
In accordance with these results (Table 4.12), it was determined that the porosity ratio (%) increased and the pore diameters increased in parallel with the increase of density of the electrospinning solution due to the addition of the active substance in the prepared nanofiber formulations. The increase in both parameters was found statistically significant ($p < 0.05$; ANOVA test).

Table 4.12. Porosity measurement data of nanofiber formulation.

Name of the samples	Porosity (%)	Pore Volume (mL)
Empty CH/PLGA composite nanofiber formulation	16.945±0.157	0.481±0.013
ALA and ATR loaded CH/PLGA composite nanofiber formulation	20.627±0.102	1.085±0.026

4.4.7. Texture Analysis

The mechanical properties of the formulations were evaluated using a texture analyser and the results were obtained for blank and drug incorporated formulations. It was observed that the mechanical strength of the nanofiber formulations decreased statistically in the presence of ALA and ATR. The structures showed less mechanical resistance ($p < 0.05$; Mann-Whitney U test). It is believed that this decrease in mechanical strength was caused by the addition of the active substance to nanofiber formulations. The results of tensile strength is represented in (Figure 4. 26).

**Figure 4.26** Mechanical strength of formulation A, B, C and D (mean $\pm$ SD, $n=3$).

Formulation A: PLGA/CH electrospun composite nanofiber formulation containing ALA and ATR.

Formulation B: PLGA/CH electrospun composite nanofiber formulation containing ALA without ATR.

Formulation C: PLGA/CH electrospun composite nanofiber formulation containing ATR without ALA

Formulation D: PLGA/CH electrospun composite nanofiber formulation without ALA and ATR.

4.5. In-Vitro Release Studies Of ALA And ATR From Composite Nanofiber Formulation

In-vitro release studies were performed in pH 6.8 PBS ($37 \pm 0.5^\circ\text{C}$ and 100 rpm) in a water bath equipped with a horizontal shaker in 3 glass vials. The in-vitro release test was continued up to 225h and at each predetermined time points, the sample was withdrawn and for maintenance of sink conditions, fresh medium was added immediately.

As expected, the CH/PLGA composite nanofiber formulation was demonstrated a typical dual release profile of ALA and ATR as shown in (Figure 4.27). ALA and ATR have different release characteristics in which ALA revealed an immediate release percentage of 83.90% within the first hour and sustained up to 8h. On the other hand, ATR exhibited a three-stage release profile which starts with a relatively lower initial release (22.07%) in comparison to ALA and followed with an increasing release (82.439%) up to 150hh. After 150h the release of ATR was in a sustained fashion.

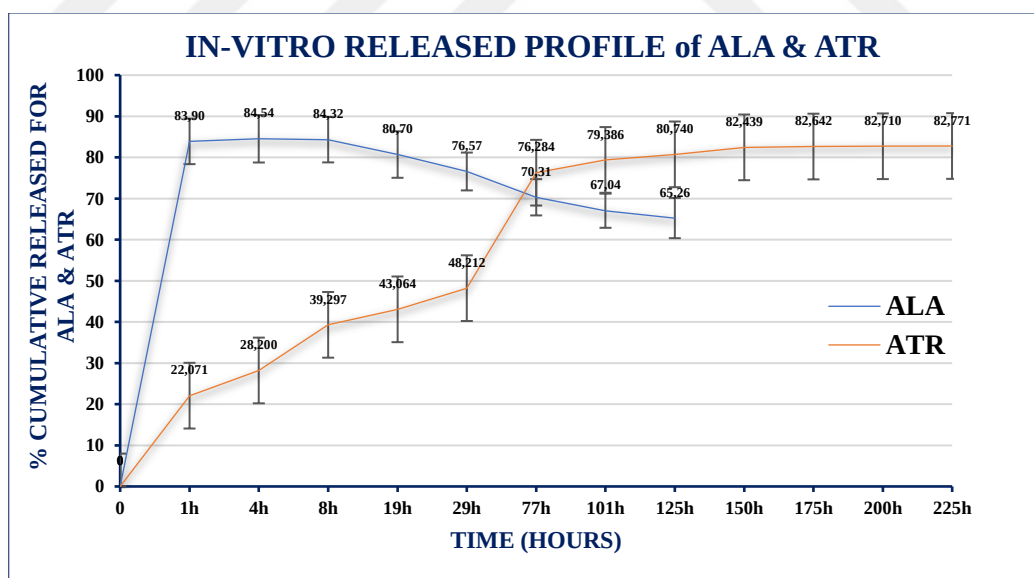


Figure 4.27. In-vitro release profile of ALA and ATR in PBS medium pH=6.8 (mean $\pm$ SD; n=3)

4.6. Cell Viability Of Electrospun Nanocomposites Formulations

The biocompatibility of newly-developed PLGA/ATR loaded CH electrospun nanocomposite was assessed using both fibroblast (L929) and neuroblastoma (B35) cell lines to figure out the potential as a drug delivery system in vitro, and suitable doses for in vivo studies were evaluated.

According to cell viability results, blank and loaded formulations were found to have no cytotoxic effect on both L-929 and B35 cell lines up to 48h. For L-929 cell line, viability was between 97.11 % to 119.070% and between 90.22% to 110.36% after 24 and 48h, respectively. A slight difference in cell viability was observed for formulation A in comparison with ATR solution ($p=0.0382$). In addition, cell viability was increased for formulation B when compared to both ATR solution and control group ($p<0.05$ and $p<0.01$, respectively) after 24h of incubation. Similarly, formulation B showed slight difference when compared to formulation C after 48h ($p=0.0335$).

The viability of B35 cells were observed between 92.11% -103.50 % and 98.18% -122.33% after 24 and 48 h respectively; showing the biocompatible nature of the formulations. The data which was obtained from Tukey's multiple comparisons test indicated that there is no significant difference between groups in terms of cell viability after 24h, while a slight difference was observed when formulation A was compared to formulation D, ATR solution, and control group ($p<0.05$). Formulation B was also showed slight difference in comparison with control group after 48 h of incubation ($p<0.05$).

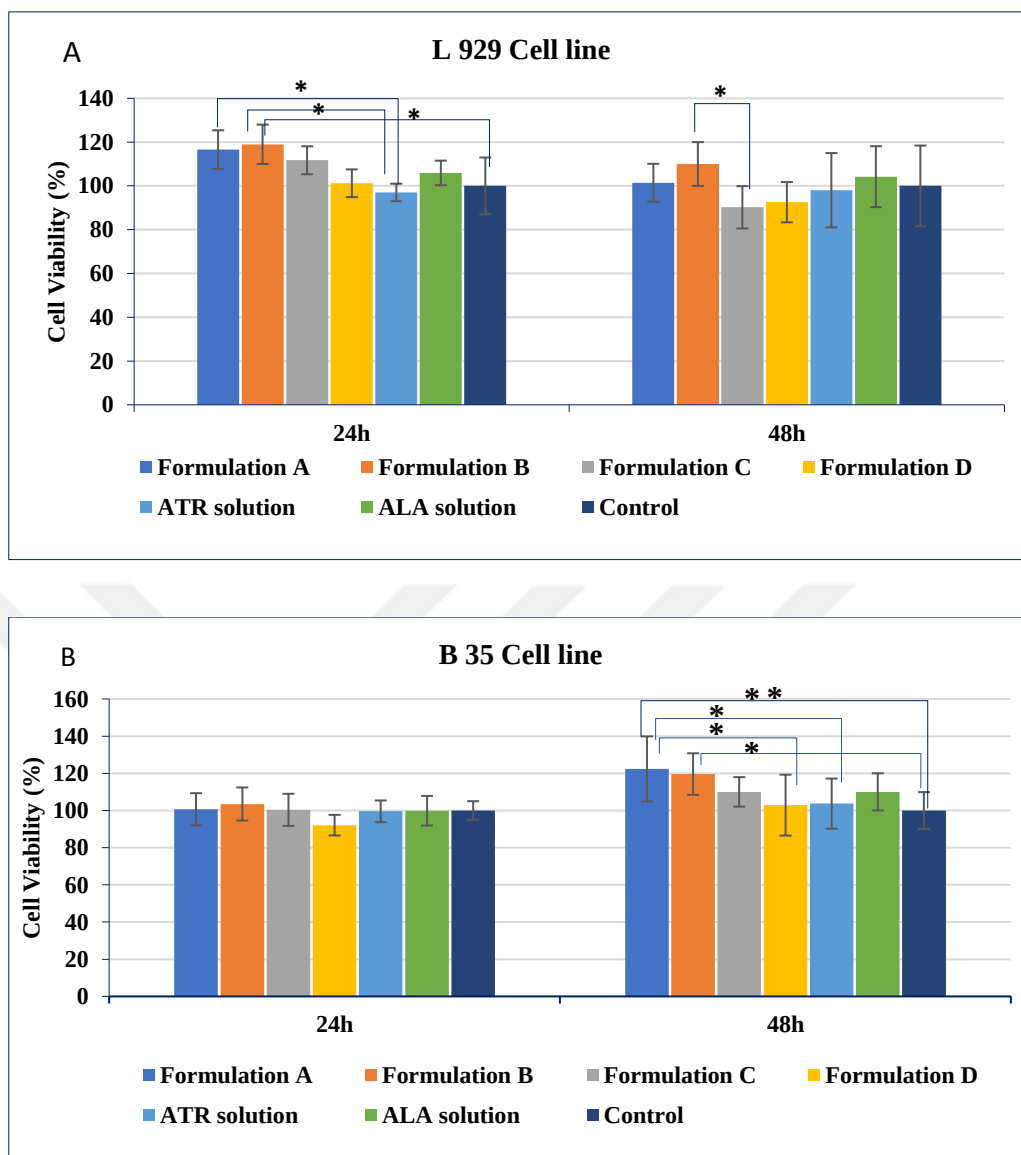


Figure 4.28. MTT assay for formulation A, B, C, D, ATR sol, ALA sol and control on L929 and B35 cell line after incubated for 24h and 48h (n=3, $\pm$ SD), *p<0.05; ** p<0.001.

4.7. Stability Test

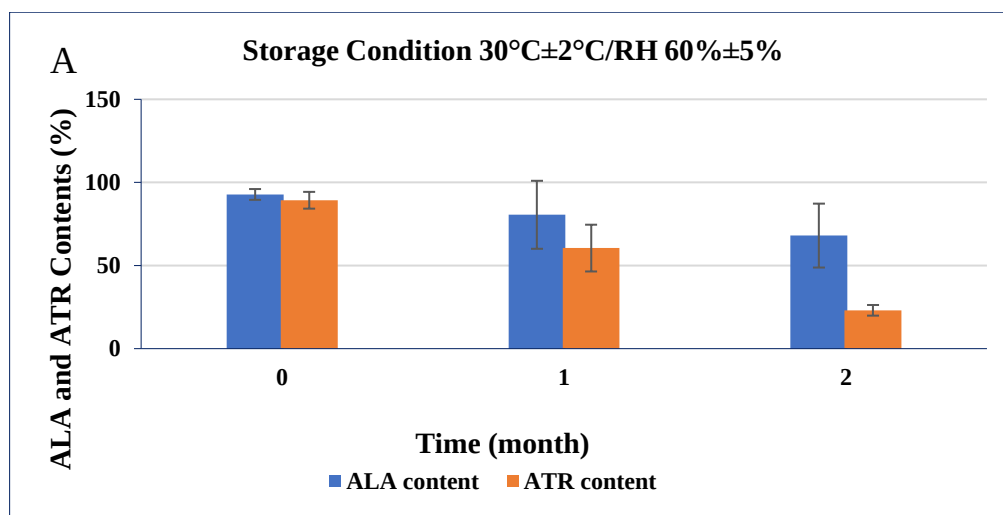
The stability study was achieved at three different conditions (A- $30^{\circ}\text{C} \pm 2^{\circ}\text{C}/65\% \text{ RH} \pm 5\% \text{ RH}$), (B- $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$) and (C- $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH}$) for six months using climate chamber. The samples were evaluated for ALA and ATR contents in the

formulation after maintenance under mentioned conditions. The results were presented as the mean of six replicates observations $\pm$ SD.

The samples which were stored under the condition A, was melted after two months and estimating the amount of ALA and ATR in the samples were impossible. As shown in (Figure 4.29.A) the content of ALA and ATR reduced rapidly in comparison to time zero. ($p < 0.0074$ $p < 0.0001$)

No significant difference was observed in the contents of ALA and ATR in the samples which were stored in the condition B (Figure 4.29.B) and no significant change was observed in the appearance feature of nanocomposite sheet.

During storage of formulation under the condition C, the contents of ALA and ATR were decreased significantly over each time point. And the physical appearance of sheet was also changed. The "Dunnett's multiple comparisons" test was conducted to compare each month with time zero and the differences are given respectively for 1st, 2nd, 3th, 4th, 5th and 6 months ($p < 0.0023$, $p < 0.0072$, $p < 0.0045$, $p < 0.0006$, $p < 0.0002$, $p < 0.0003$). In the case of ATR the differences for each month was $p < 0.0001$ (Figure 4.29. C)



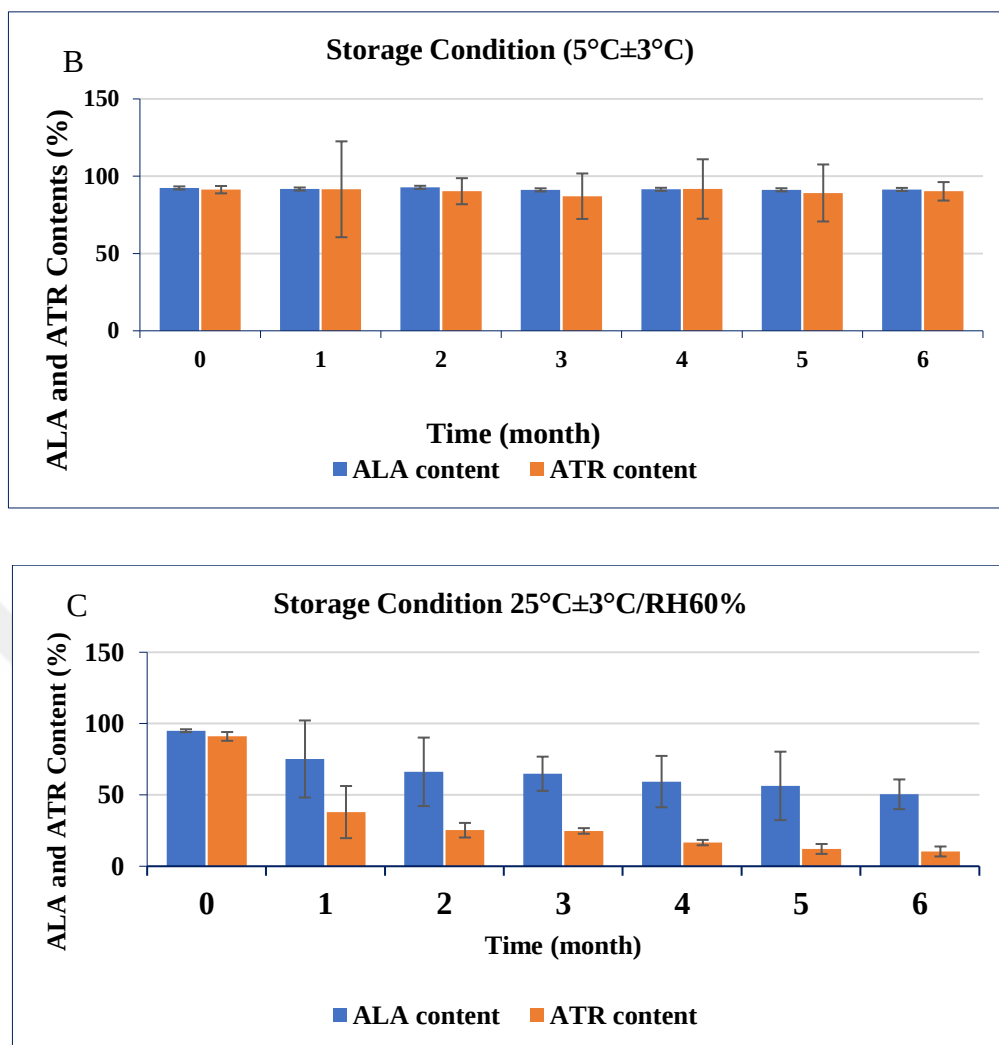


Figure 4.29. The contents of ALA and ATR in the composite nanofiber formulation A in each month: A) Storage Condition 30°C±2°C/RH 60%±5%; B) Storage Condition (5°C±3°C) and C) Storage Condition 25°C±3°C/RH60% (mean ± SD, n=6).

4.8. *In-vivo* Experiments

4.8.1. Sciatic Functional Index (SFI)

The SFI was measured for all groups at the 1-,3-,5-,7-,15- and 30days time points after formulations implantation and the results were summarized in (Figure 4.30 and 4.31). The SFI value around 0 represents normal nerve function, and closed to 100 represents the total loss of function. At first day after surgery, the waking track

measurements of all animals in each group showed a decline in SFI score, which is revealed dysfunction due to the nerve crush.

An overall comparison between groups showed significantly better functional recovery in group 2 ($p < 0.0005$), group 3 ($p < 0.002$), group 4 ($p < 0.05$) and group 5 ($p < 0.05$) when compared with the result of group 1. In addition to that, significant difference was found between group 2 and group 3 ($P < 0.0377$), group 2 and group 4 ($P < 0.0073$); but no significant difference was observed between group 3 and 4 ($p = 0.973$). (Figure 4.20).

Significant differences were seen in the (SFI) between group 1 and group 2 after day 5 ($p < 0.05$); and after day 7 SFI was improved significantly for group 3 and 4 when compared with the results of group 1 ($p < 0.05$, $p < 0.005$) (Figure 4.21).

Significant progressive improvement was observed in the SFI between time point assessment in group 2 and 3 after day 7 and group 4 after day 15 ($p < 0.05$, $p < 0.05$, $p < 0.0020$). On the other hand, in group 1 and 5 no improvement in motor function recovery was observed until day 30. The improvement ratio in the SFI for all experimental groups was not statistically significant on the last day of the examination.

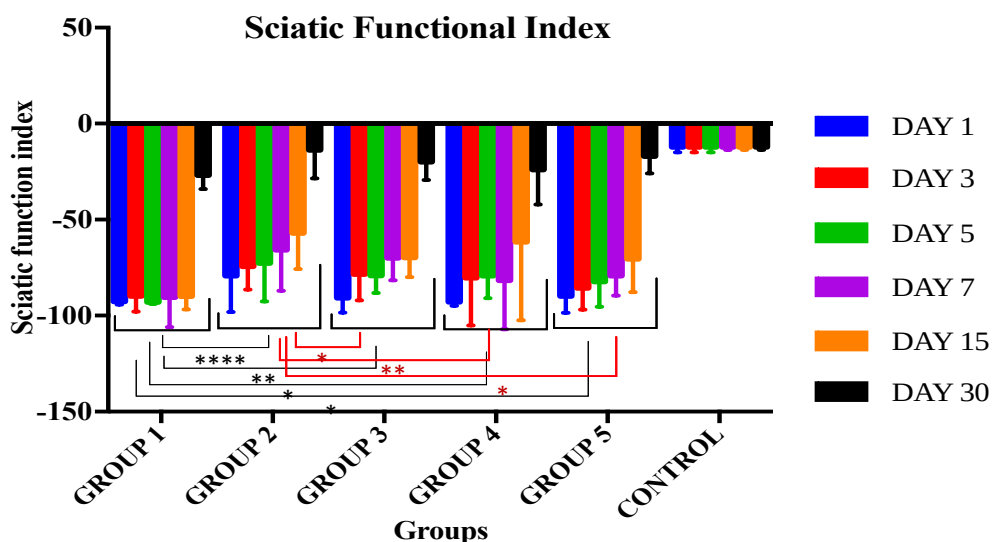


Figure 4.30. Sciatic functional index. An overall comparison of group 2, 3, 4 and 5 with group 1: (**** $p < 0.0005$), (** $p < 0.002$), (* $p < 0.05$).

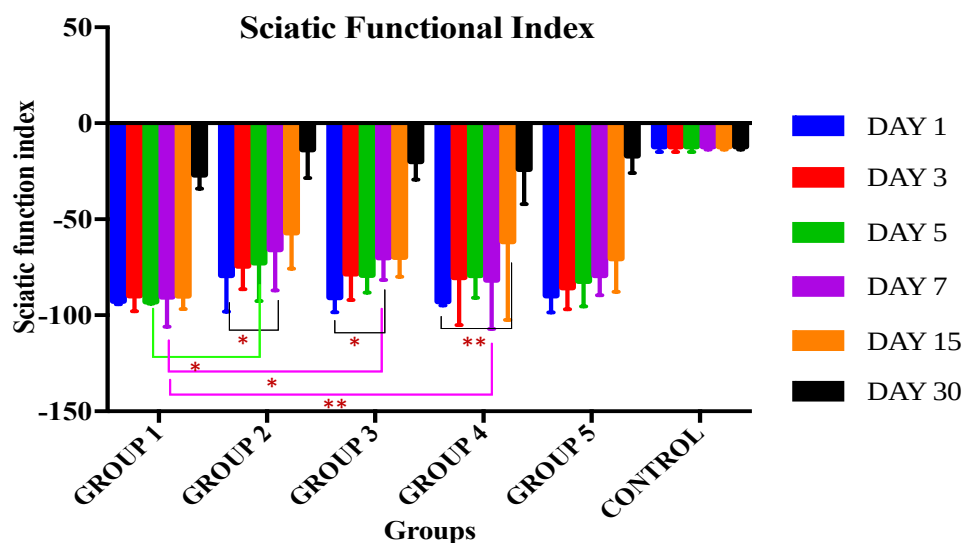


Figure 4.31. Sciatic functional index: comparison within time point (* $p<0.05$); (** $p<0.005$).

4.8.2. Behavioral Testing (BBB)

Regardless of the groups, all animals which underwent sciatic nerve crush exhibited significant reduction in motor function in comparison with the control group at first-day post-surgery ($p>0.0001$) and the highest degree of functional deficit was observed in group 1 and 5 (Figure 4.32).

In overall comparison between groups, significant progress was observed in BBB score in group 2, 3 and 4 in comparison with group 1 ($p=0.0001$). In addition significant difference was observed between group 3 and 4 when compare with group 2 ($p<0.0002$) (Figure 4.32).

During the 30 days of monitoring of all rats, the "Dunnett's multiple comparisons" test has shown that the BBB scores for group 2 and 3 improved significantly ($p=0.0001$) compared with group 1. On the other hand, group 4 and 5 did not show significant improvement in comparison to group 1.

After the third day, a progressive improvement in BBB score was observed in group 1 ($p=0.0256$), group 2 ($p=0.0163$) and group 5 ($p=0.0005$); but in group 3 no significant progressive was observed in BBB score statistically until 5 days. After day

5 the BBB score was increased significantly compare with initial days for group 3 ($p=0.0053$). The animals in group 4 did not show significant improvement in BBB score within the 30-day assessment.

The "Dunnett's multiple comparisons test" test has shown that the improvement was significant between all groups. The rats with a crushed-sciatic nerve in group 2 and 3 exhibited the highest BBB score, but did not achieve the level of recovery equal to control group.

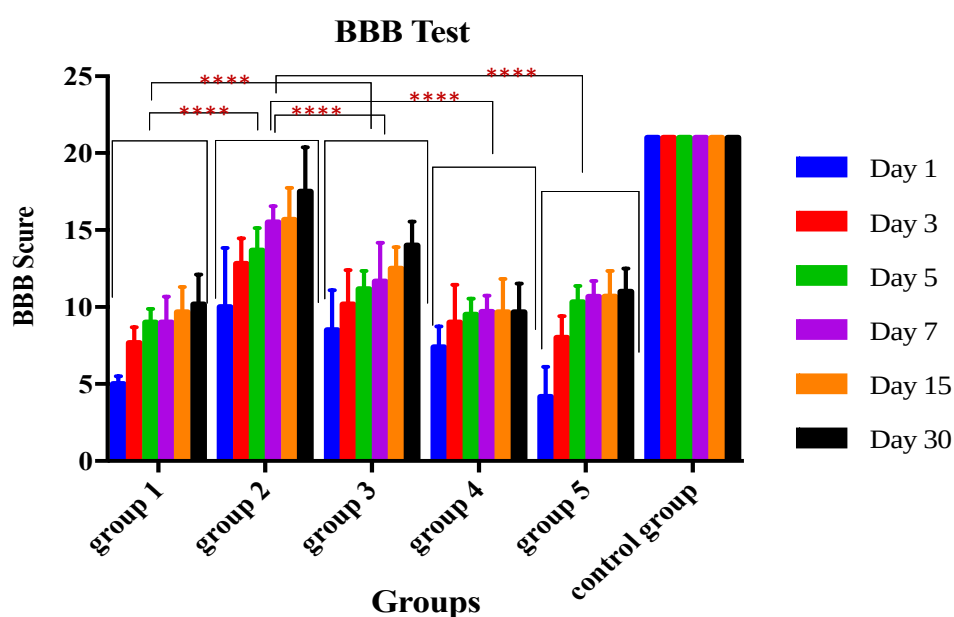


Figure 4.32. BBB Score for all groups: overall comparison between groups (**** $p<0.0002$).

4.8.3. Extensor Postural Thrust Test (EPT)

In first and third days post-surgery, the extensor postural trust was almost similar and high for all groups (ranging from 82.046% to 93.85%) (Figure 4.33)

During the recovery period of one month, The EPT response was also improved and the progress was significantly high in group 2, 3 and 5 in comparison to group 1 ($p<0.0002$, $p<0.0002$, $p<0.05$).

A significant better recovery of motor deficit was seen in group 2 ($P < 0.0005$) when compared with group 4, but no significant difference was seen between group 2 and 3 ($p = 0.1247$), group 3 and 4 ($p = 0.1671$). In group 2 motor deficit starts decreasing significantly after day 5 ($p < 0.05$), but in other groups, a significant difference was observed after day 15.

That EPT of animals in group 2 recovered significantly faster at the time point 7, when compared with initial time point ($p < 0.05$); while the animals in other groups did not show significant differences at mentioned timepoint. The p values for group 1, 3, 4, 5 were respectively ($p = 0.9331$; $p = 0.1656$; $p = 0.0774$; $p = 0.0528$).

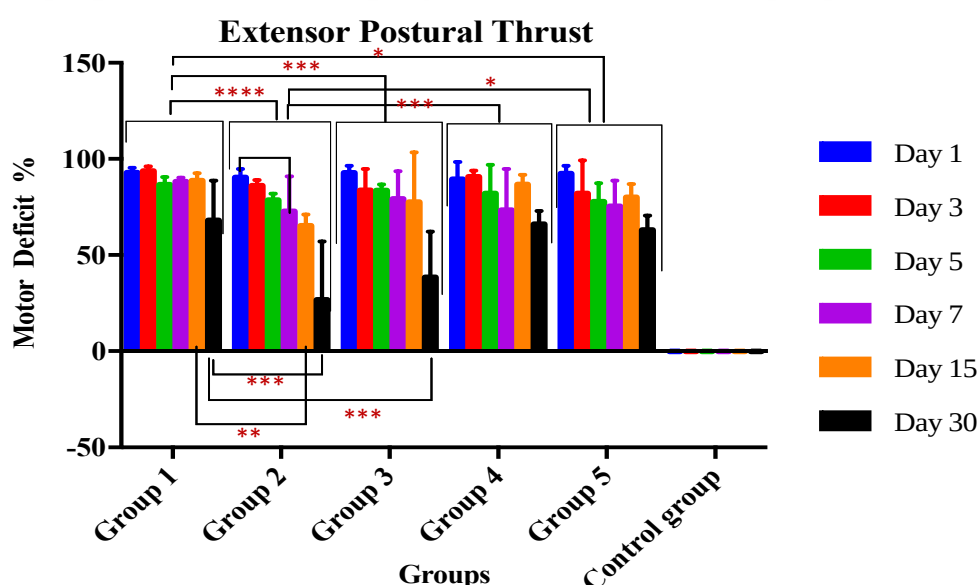


Figure 4.33. Extensor postural thrust comparison between groups and within groups in different time points. (**** $p < 0.0002$), (*** $p < 0.0005$), (** $p < 0.001$), (* $p < 0.05$).

4.8.4. Withdrawal Reflex Latency (WRL)

The WRL test was performed to quantify the nociception function latency. The average latency time of experimental hindlimb is shown in (Figure 4.34). The latency time was measured at each time point (1, 3, 5, 7, 15 and 30 days).

During follow-up period, nociception was recovered gradually in all groups and no significant difference was observed between groups at the last time point 30 day ($p = 0.999$).

A significant difference was observed in withdrawal response to the noxious thermal stimulus between groups. The latency times for group 2, 3, 4, 5 were decreased in compared to group 1 ($p < 0.0005$, $p < 0.0005$, $p < 0.0008$, $p < 0.0005$).

In addition, the withdrawal response to the noxious thermal stimulus become faster in group 2 than group 3 ($p < 0.008$) and group 4 ($p < 0.0002$); but there was no significant difference between group 3 and 4.

In terms of time, the comparison within time point for each group indicated a faster decline in the latency time (recovery of nociceptive function) in group 2 and 3 ($p < 0.05$, $p < 0.005$); while the improvement of latency time was not statistically significant in other groups ($p = 0.9194$).

In particular, an overall significance was found, between group 2 and other groups which indicate that WRL of animals in group 2 recovered faster and better at time point (1, 3, 5, 7 and 15) in comparison to the other groups.

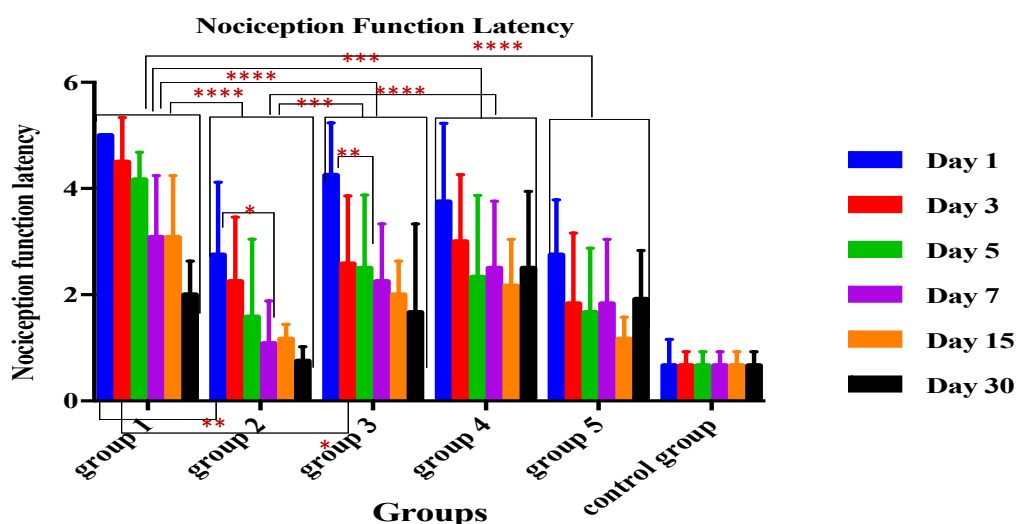


Figure 4.34. Withdrawal reflex latency (**** $p < 0.0002$), (** $p < 0.0005$), (* $p < 0.001$), (* $p < 0.05$).

4.8.5. Ultrastructural Examination

Figure 4.35, shows the transmission electron microscopic examination of the sciatic nerve samples of the group 1 to 5 at different time points.

In the TEM examination of the nerve samples of group 1, at time point (day 1, day 3, day 5 and day 7) ultrastructural pathological changes were observed both in all sizes of myelinated axons and Schwann cells (Figure 4.36. A, B and C). Additionally; vacuoles were present inside the cytoplasm of Schwann cells at timepoints (day 1, 3, 5 and 7); while at day 15 no vacuoles were detected and ultrastructural pathological findings were better than the previous timepoints. At day 30 of follow up; ultrastructural normal tissue was observed.

In the TEM examination of group 2 nearly the same ultrastructural pathological findings were observed at day 1, 3 and 5. However; beginning from day 7 the vacuoles could not be observed in the Schwann cells. At day 15 the axons were nearly normal in appearance and at day 30 ultrastructurally normal axons were observed. The healing process was most prominent in the small sized myelinated axons (Figure 4.36.A). It was followed by the medium and large sized myelinated axons. In group 2; mostly the ultrastructural pathological changes were observed in the medium and large sized myelinated axons (Figure 4.36. B and C).

After the control group; the least amount of ultrastructural pathology was observed in the second group. Starting from day 7, the ultrastructural normal myelinated axons in small size myelinated axons were increased faster in group 2. At day 15 significantly difference was observed between group 2 and group 1 ($p = 0.0282$), group 2 and group 5 ($p = 0.0138$), group 1 and 3 ($p = 0.0420$), group 3 and 5 ($p = 0.0208$), group 4 and 5 ($p = 0.0255$) (Figure 4.36.A).

On the other hand, separation in myelin configuration in small size axons was reduced on day 7 in group 2,3 and 4; while, until the day of 15 in the first and fifth groups remained unchanged. The decreasing rate was significant on day 15 for groups 2,3 and 4 in comparison with groups 1 and 5 (Figure 4.36. A).

Separation in myelin configuration, interruption in myelin configuration and honeycomb appearance were the main ultrastructural pathological changes observed in myelinated axons. Depending upon to the sizes of myelinated axons; the small sized myelinated axons showed the least ultrastructural pathological changes in all of the groups (Figure 4.36. A). The most severe ultrastructural pathological changes were detected in the large sized myelinated axons in all of the groups (Figure 4. 36. C).

Another common finding in all groups at day 30 the ultrastructures of the nerves were very similar with the control group meaning that in the rats; the pathology done by the nerve damage was complete healing in the 30th day of the trauma (Figure 4.36. A, B and C).

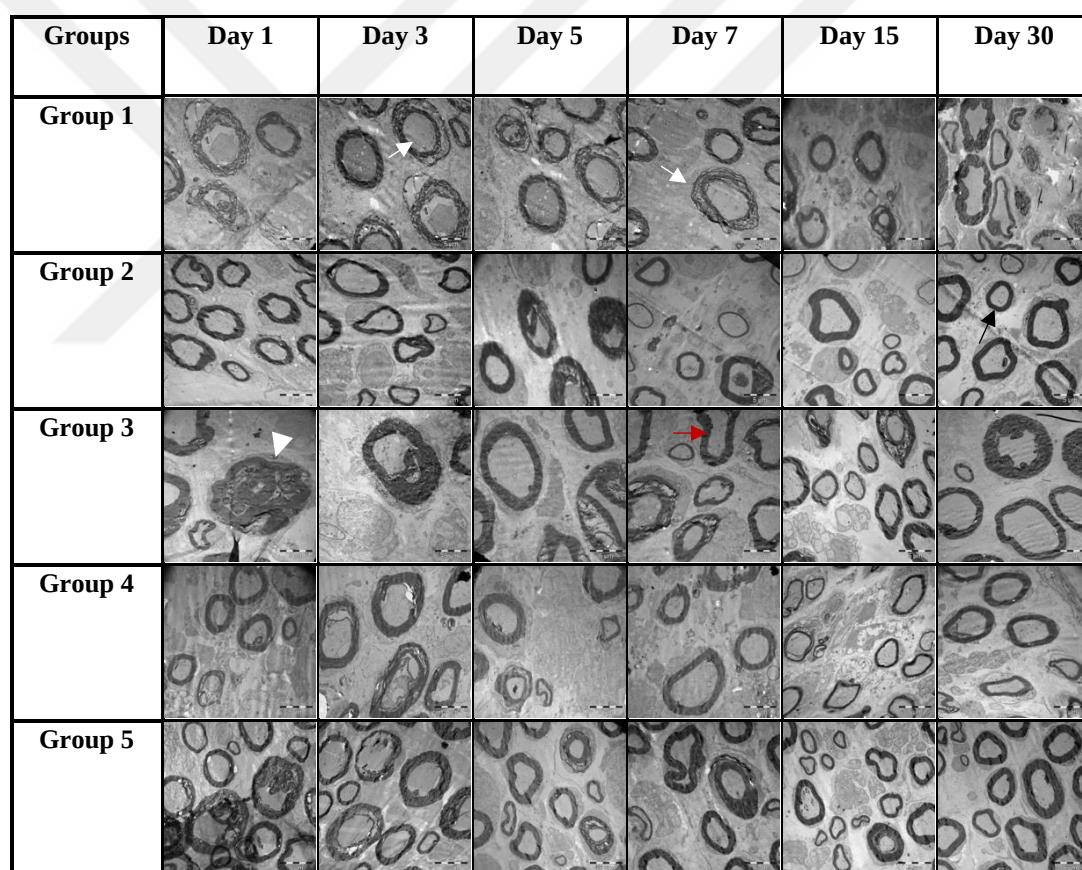


Figure 4.35. TEM images of sciatic nerve samples of group 1 to 5 at different time points. White arrow indicates separation of myelin. Black arrow indicates normal myelinated axon. Red arrow indicates interruption in configuration

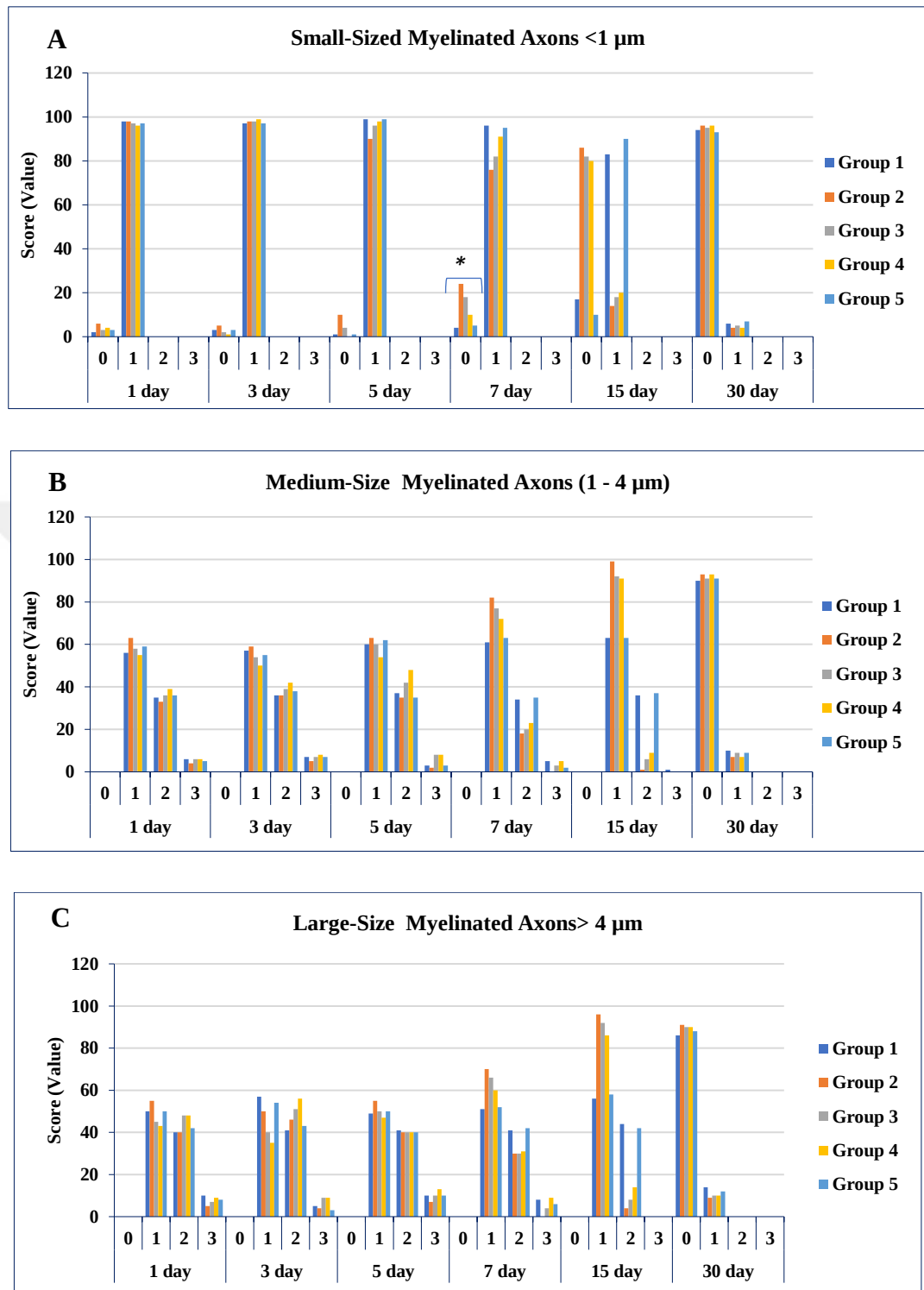


Figure 4.36. Quantitative analysis based on the scoring system: A) Small-Sized Myelinated Axons $<1\ \mu\text{m}$, B) Medium-Size Myelinated Axons (1-4 μm), C) Large-Size Myelinated Axons $>4\ \mu\text{m}$. *On day 7 ultrastructural myelinated axon increased significantly in group 2,3 and 4.

4.8.6. Determination of TNF- α , IL-1 β and IL-6 Levels

The TNF- α , IL- 1 β and IL-6 values of the groups were summarized in (Figure 4.37.A, B and C) respectively. The levels of TNF- α , IL- 1 β and IL-6 were determined at 1, 3, 5, 7, 15 and 30 days after trauma.

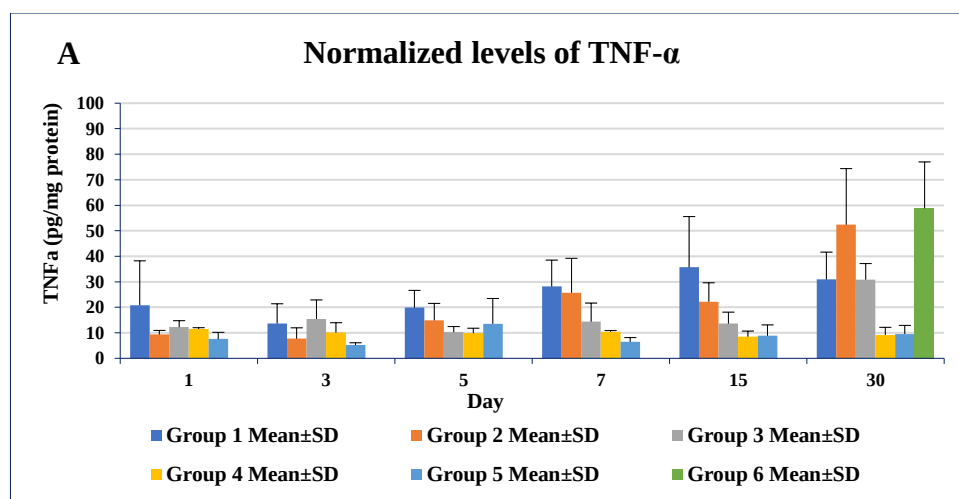
In general, when effect of the formulations between the groups was compared, the exact effect was observed on day 15 in all treatment groups.

A significantly lower level of TNF- α was observed in group 3 ($p < 0.0051$) and group 4 ($p < 0.0001$) in comparison with group 1.

The IL-1 β levels were lower in all groups as compared with the first group (trauma). This decrease was found to be statistically significant for all groups (2-4) ($p < 0.0103$, $p < 0.0003$, $p < 0.0001$).

In addition, the IL-6 level of group 2 and 4 were decreased significantly in comparison to group 1 ($p < 0.0365$, $p < 0.0343$).

The use of ATR alone in the neuroinflammatory process resulted in less cytokine activation compared to ALA administration ($P < 0.0001$).



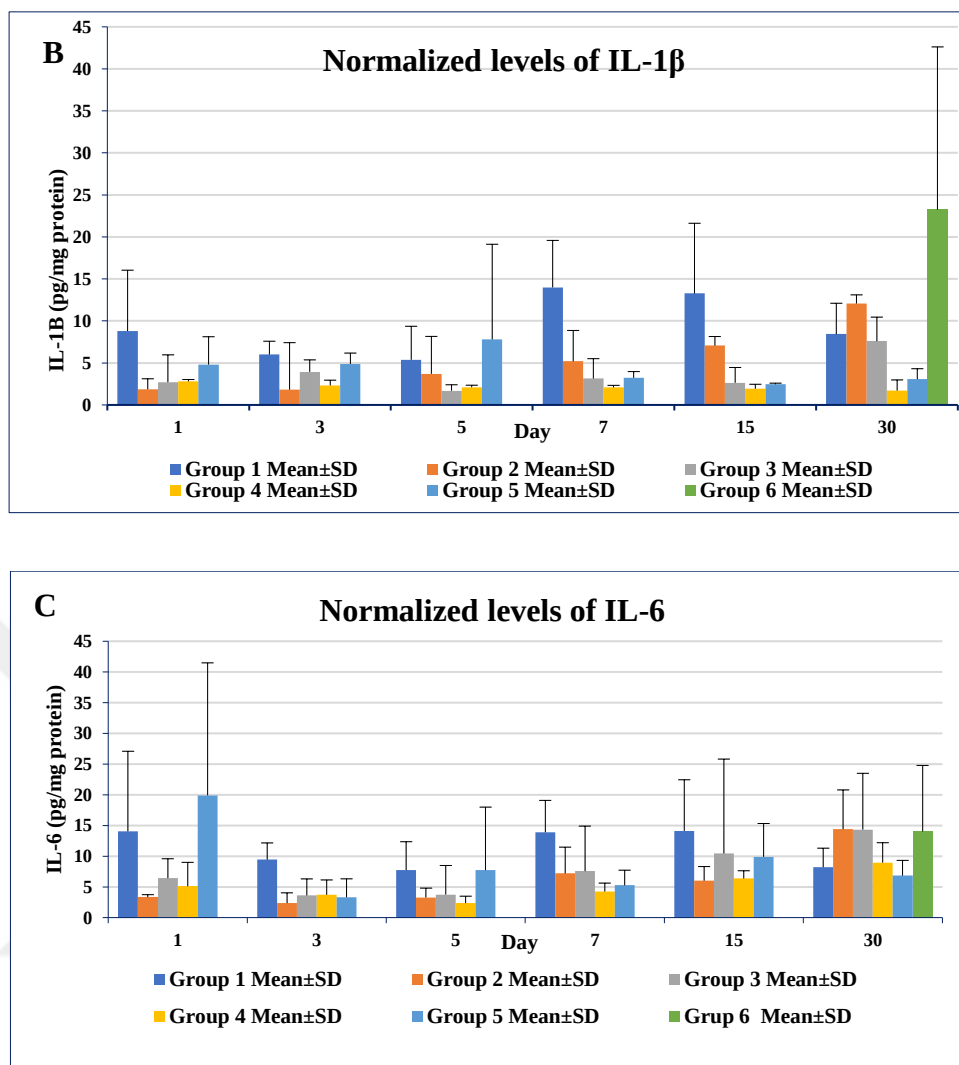


Figure 4.37. Values for TNF- α , IL-1 β and IL-6 for the experimental groups. The exact effect was observed on day 15 in comparison with group 1.

4.9. Ex Vivo Models of Neurotrauma/Organotypic Spinal Cord Culture.

As observed by the organotypic slice of spinal cord, the neuron loss in the trauma group was more severe in comparison to naive group. The loss of neuron in groups treated with the formulation containing 5% and 10% ALA were observed to be less than those in the trauma group as observed by Neun staining which is a neuronal marker. As a result, after trauma, the neurons were found to be better protected in 5% and 10% ALA groups (Figure 4.38.).

In comparison with the naive group, the cellular IL-1 β marking was not observed in the trauma group. The arrows indicate the cells, and the lack of cellular marking suggests that IL-1 β was released from cells and stimulates the inflammatory process after trauma. As a result, the formulation containing 5% ALA was considered as the most effective formulation for the suppression of the inflammation process (Figure 4.39).

Cellular IL-6 marking was not observed in the trauma group compared to naive tissue. Arrows point to cells with cellular marking. According to this, the tissues were treated with formulations containing 5 % and 20 % ALA represented a similar pattern to the naive tissues compared to the other groups (Figure 4.40).

Figure 4.41, shows the histopathological examination of spinal cord tissue sections with H & E. The result was indicated that after trauma, the tissues which was treated with formulations containing 5% and 20% ALA showed similar pattern to naive tissue. Therefore, the axon bundles were preserved in these two groups.

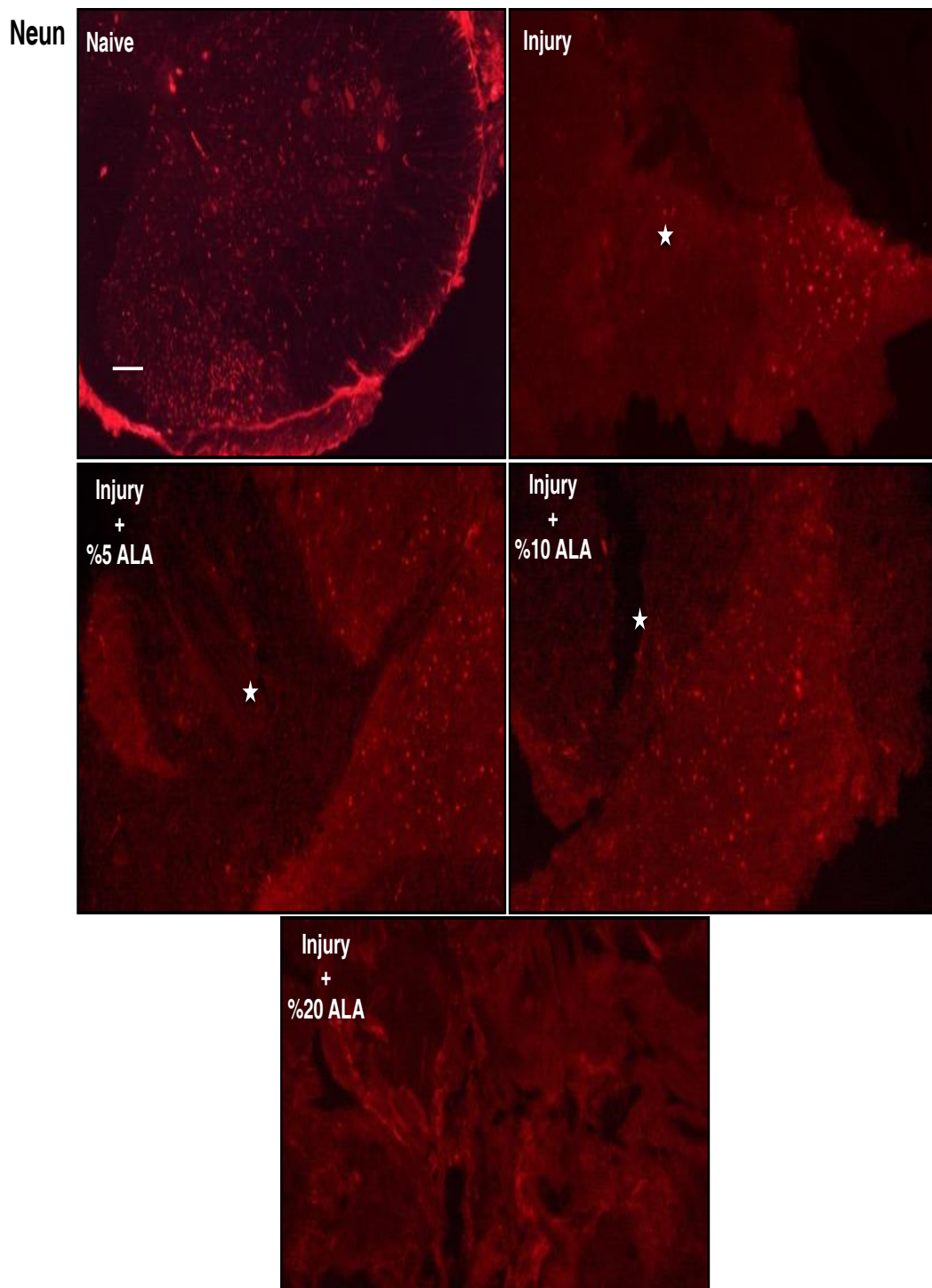


Figure 4.38. Marking of spinal cord tissue sections with neuronal cell marker Neun.

The stars indicate the neurons.* Neuronal Marker

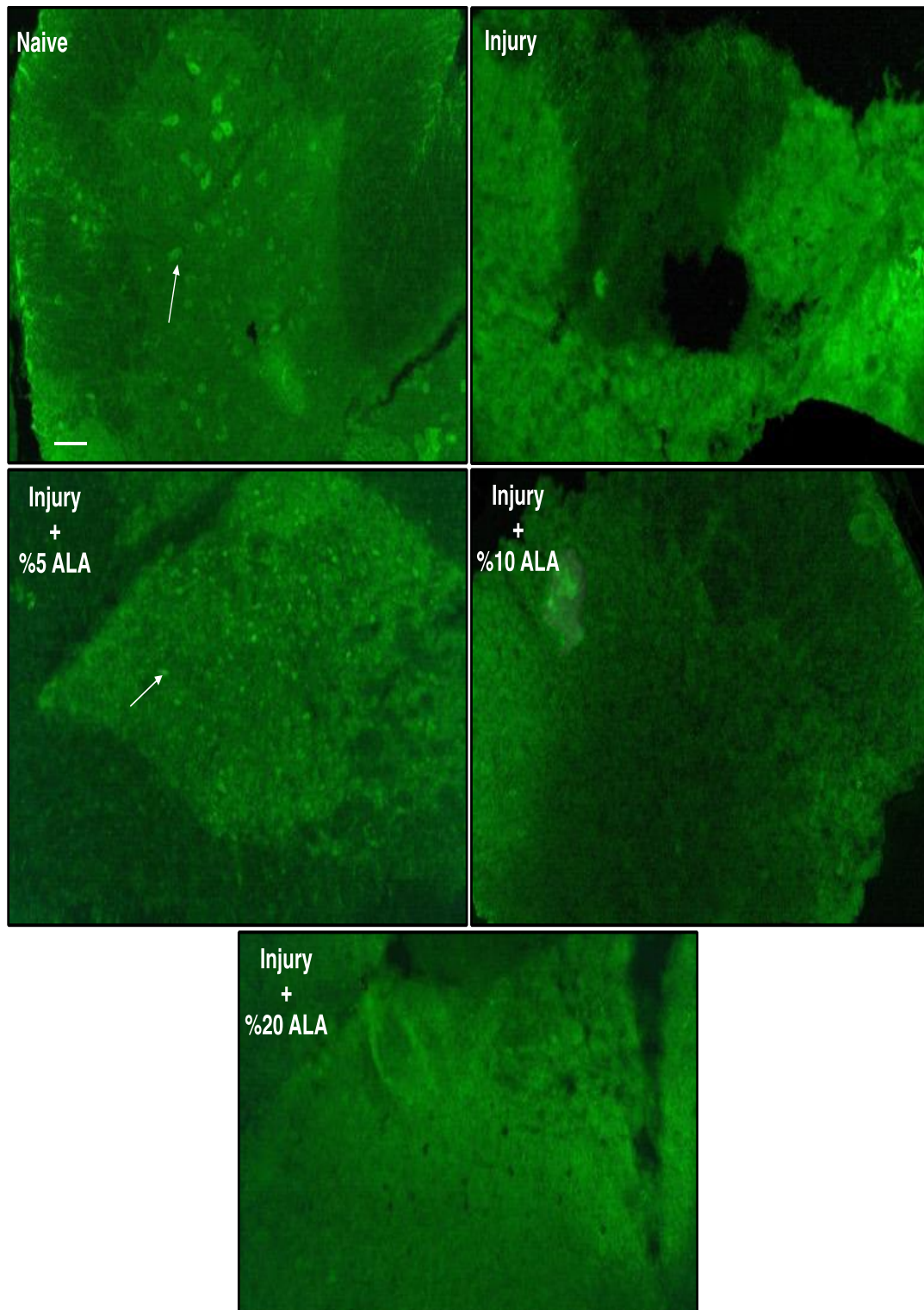


Figure 4.39. Immunofluorescence staining of spinal cord tissue section for interleukin IL-1 β . The arrows indicate the cells, and the lack of cellular marking suggests that IL-1 β was released from cells.

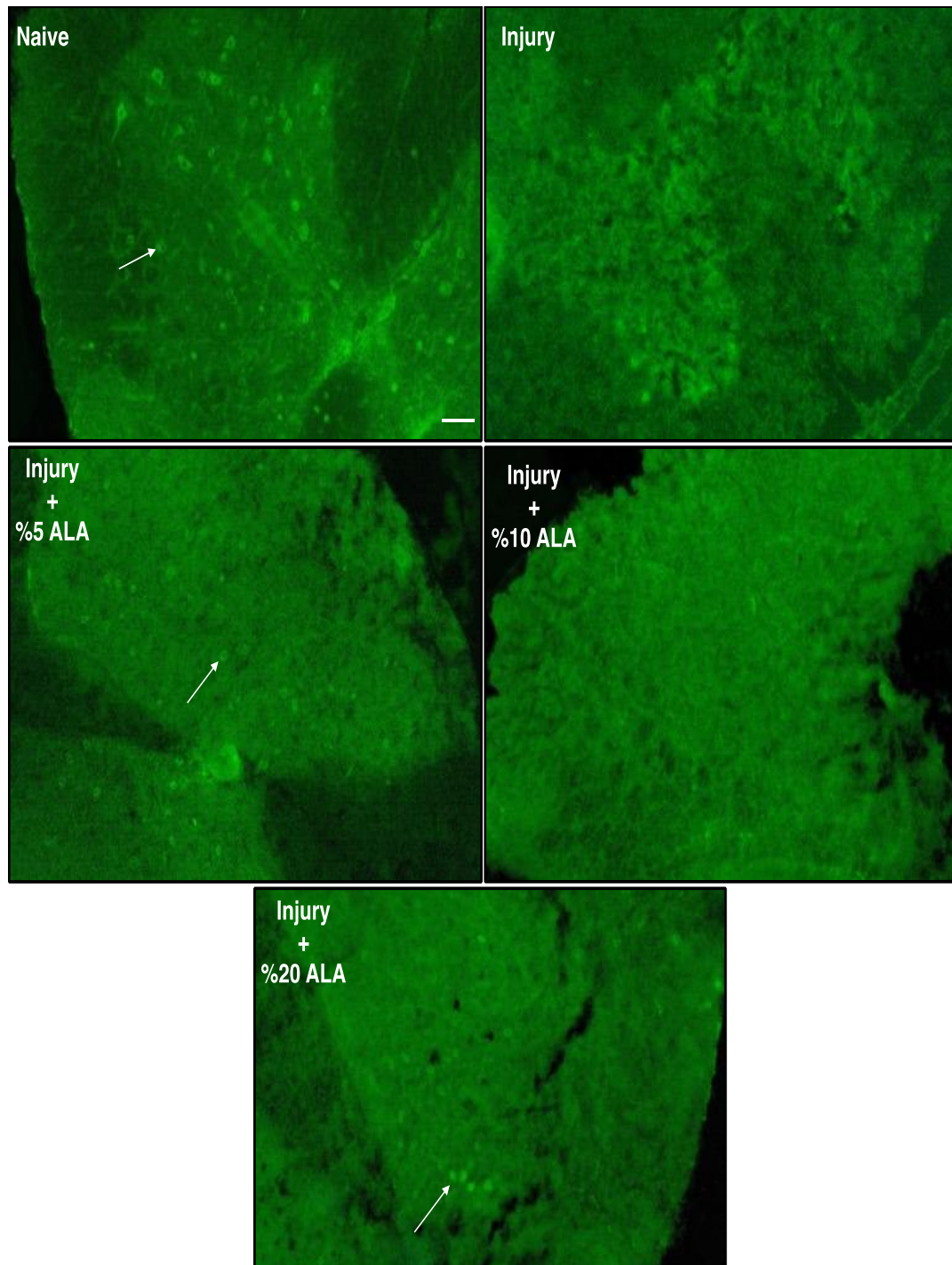


Figure 4.40 Immunofluorescence staining of spinal cord tissue section for interleukin IL-6. The arrows indicate the cells, and the lack of cellular marking suggests that IL-6 was released from cells.

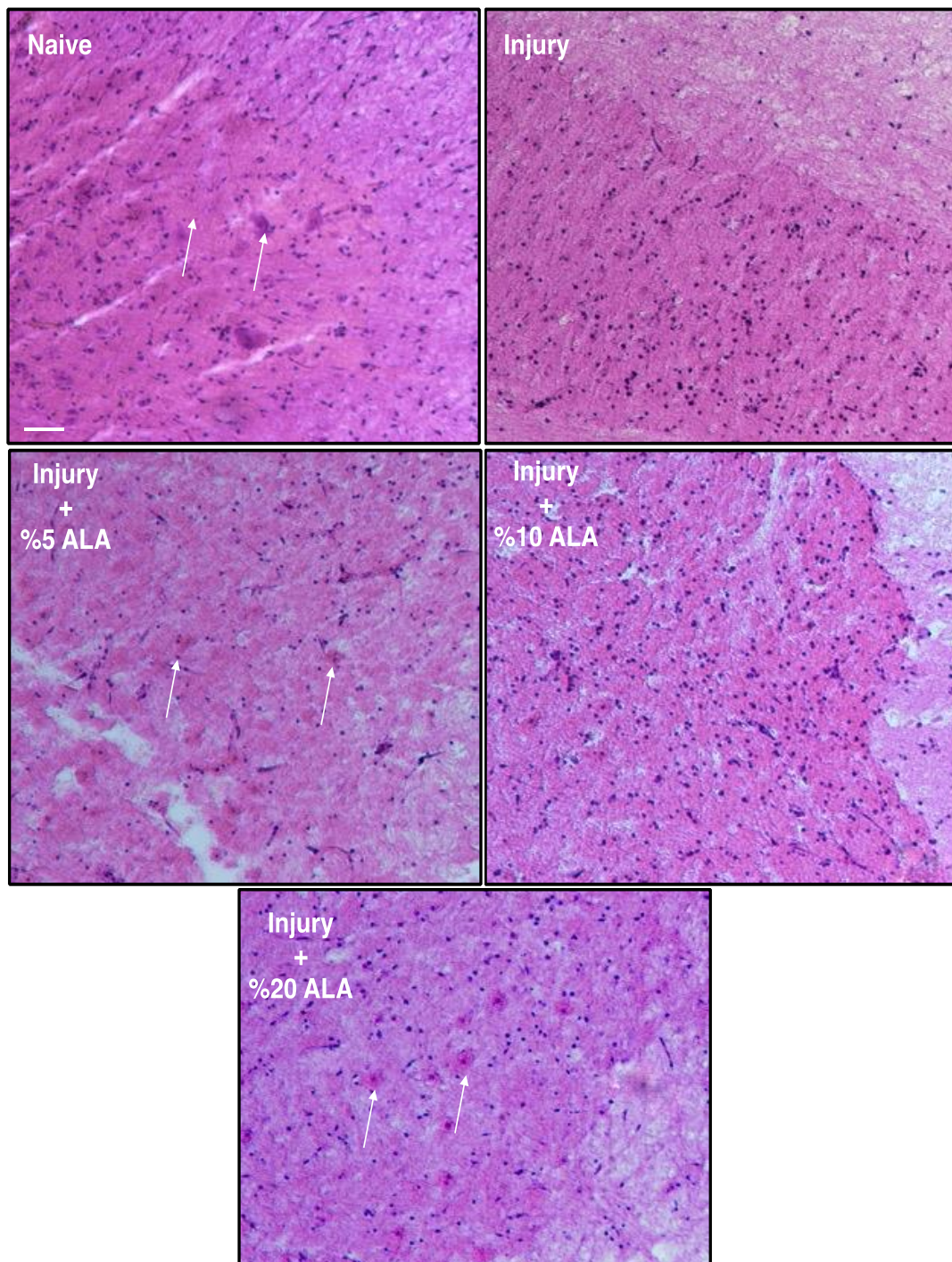


Figure 4.41. Histopathological examination of spinal cord tissue sections with H & E.

The white arrow shows the axon bundles.

5. DISCUSSION

In this chapter of thesis, analytical method validation, preparation and in-vitro characterization of ATR loaded CH nanoparticle, in-vitro cytotoxicity study of nanoparticle, preparation and in-vitro characterization of ALA loaded PLGA/ATR loaded CH electrospun nanocomposite and application of developed formulation on animal sciatic nerve crush model were evaluated.

5.1. Analytical Method Validation

Analytical method validation is defined as a whole set of operations performed to show that the properties of the developed analytical method are appropriate for the intended purposes.

According to the results, fully validated RP-HPLC method was considered as a suitable method for simultaneous quantitative determination of ALA and ATR.

The peak areas of the calibration standards were proportional to concentrations over the nominal concentrations range of 0.01-8.00 $\mu\text{g/mL}$. The calibration curves appeared linear across the concentrations range and the determination of coefficients (R^2) for ALA and ATR were found to be $R^2 = 0.9991$ and $R^2 = 0.999$ respectively, which shows the linearity of the method. Retention times of ALA and ATR were 6.2 min 9.3 min (Figure 4.1).

The detection limits of ALA and ATR were found to be 0.02 $\mu\text{g/mL}$ and 0.005 $\mu\text{g/mL}$ respectively, and the quantification limits were 0.08 $\mu\text{g/mL}$ and 0.016 $\mu\text{g/mL}$ respectively, which concluded that HPLC method has been found to be sensitive for our studies.

Representative chromatograms of unloaded PLGA/CH electrospun nanocomposite, standard solution of ALA, ATR and mobile phase revealed that, there was no any interference peak from the excipients and impurities observed at the retention time of ALA and ATR. It indicated that the developed method is very specific for ALA and ATR.

In a quantitative analysis, precession and accuracy are important for an assay to be reliable. The repeatability of the method was assessed over the 6-independent series of three different concentrations (1, 2 and 4 μ g/mL) within the same day. The intermediate precision values of the method were demonstrated by calculating RSD % of the data, which were obtained from analysis of the three mentioned concentrations over three consecutive days.

The intra-day, inter-day precision (RSD %) values were less than 2 % and intra-day, inter-day accuracy (Bias%) values were found below 2 % for ALA and ATR. All these data presented in (Table 4.5 and 4.6), indicate that the method is precise and accurate.

The results of robustness study, indicated that, the result of the method stay unaffected by small intentional change in pH (4.5 ± 0.2) and ratio of the ACN:Buffer ($\pm 2\%$). The RSD % values of the data obtained were below 2 %. On the other hand, small variations in flow rate ($1\text{mL/min} \pm 0.1$) and detection wavelength ($212 \pm 2\text{nm}$) had a remarkable effect on response. As evident the RSD% values were out of the range. Therefore, it is strongly recommended that special attention should be paid to these two parameters when using this method.

Stock solution stability was performed to check stability of ALA and ATR in stock solutions which was prepared in methanol and stored in refrigerator. The RSD % value of average of all time points (0, 4h, 24h and 48h) was less than 1 %. It indicates that stock solution was stable for 48 in refrigerator condition. Similarly, the stability of ALA and ATR in autosampler condition for 48h were investigated. The results revealed that both analytes were stable in autosampler during the analysis. The results obtained from all these stability studies are tabulated in (Table 4.9 and 4.10).

5.2. Formulation of Atorvastatin Loaded Chitosan Nanoparticles by Nano Spray Drying Method

A wide set of CH nanoparticle formulations were prepared by using an advanced Nano Spray dryer B-90 (Büchi), which provides maximum product yield,

with respect to conventional methods. In Nano Spray dryer technology, the characteristics of produced nanopowder directly depends on process and formulation variables.

Most recently, the results of researches about the effect of process parameters and formulation variables on final product properties have been published in the book: *Nanoencapsulation Technologies for the Food and Nutraceutical (189)*, the main process parameters and formulation variables and their influence on properties of the final product have been identified. It allows us to distinguish the most important parameters. Thereby reducing cost, material usage and producing nanoparticle with desired properties.

In the light of the above information, to produce nanopowder with specific features the influence of the most important input parameters (Table 3.1), were investigated on the characteristic of the final product.

5.2.1. Characterization of ATR loaded CH Nanoparticles

Morphology

Surface morphology of Nano Spray dried particles is one of the important characteristics, which plays a significant role on release profile of Nano Spray dried particles. Surface morphology characteristics of ATR chitosan nanoparticles were evaluated by SEM and TEM investigations. ATR nanoparticles were almost spherical with a wrinkled surface for all formulations (Figure 4.5). Nearly in all cases, small particles presented smoother surface with respect to bigger particles. It is not an unusual phenomenon in Nano Spray drying process, millions of droplets are produced by a vibrating mesh before particle formation. Droplet size and outlet temperature are the two major parameters, which directly affect the final particle surface morphology. Faster drying time and evaporation rate of small droplets prevent the movement of solids towards the center in contrast to bigger droplets. Accordingly, big and small particles with smooth surface could be produced in one batch. Furthermore, formulation parameters such as drug concentration induce some changes in surface

morphology of particles. It can be seen that, surface roughness of nanoparticles from F1 to F9 is also increased by increasing ATR concentration. The possible explanation for this phenomenon is that the drug crystallinity may decrease the surface smoothness of particles (156). TEM investigations also confirmed that the CH nanoparticles containing ATR were in spherical shape and in nano-diameter, which is in accordance with the particle size measurement results (Figure 4.6).

Product yield

The Nano Spray drying production yield was calculated based on comparing the amount of resulting powders weight with the total amount of initial solid contents in the formulations.

As shown in Table 4.11, product yield for all formulations varied within a range from 58.30% up to 74.10%. The highest product yield was obtained with formulations F12 and F11, which were prepared using spray cap (7 μ m and 5.5 μ m) and formulations with the highest amount of solid mass in initial solution (F7, F8, and F9). Product yield was increased with increase in the polymer concentration.

Changes in the product yield may be due to accumulation of particles around the spray cap or drying chamber. Manual collection of the powder is another reason for variation in the product yield (190).

Encapsulation efficiency

The EE describes the amount of intended ingredient that is loaded into the drug carrier. As shown in Table 4.11, the percentage of EE of the loaded CH nanoparticles were determined for ATR. ATR loading efficiency was decreased by decreasing the concentration of chitosan and ATR. For instance, the highest loading efficiency was obtained in the case of formulation F9 (53.61 $\pm$ 4.52 %) containing 0.04 %-w/v CH and 40 %-w/w ATR. The EE was higher in formulation F2 (48.95 $\pm$ 3.26 %) with respect to the formulation F4 (33.30 $\pm$ 4.01%). In spite of increasing CH concentration (0.02 % to 0.03 %) in the formulation F4, EE was reduced.

Particles Size Distribution

In Nano Spray dryer technology, the size of the solid particle depends on the spray mesh size and concentration of utilized polymers because spray mesh with smaller size produces small droplets and consequently small final solid powder (151).

Formulation F1, F11 and F12 were produced by using different spray mesh size (4 μ m, 5.5 μ m and 7 μ m) respectively. The particle size was increased by increasing spray mesh size (516.5 $\pm$ 59.48 nm, 602.3 $\pm$ 76.38 nm, 779.6 $\pm$ 24.39 nm). The same result was observed by Bürki et.al (191) for β -Galactosidase.

Physicochemical analysis of ATR loaded CH nanoparticle

In addition to surface morphology, size, release profile of nanoparticles, it is important to analyze some other physicochemical (thermal properties, formation of chemical bonds, crystalline or amorphous nature) properties of nanocarriers loaded with active ingredients.

DSC is a useful method for analyzing thermal properties (glass transition temperature, melting point, heat capacity) of nanoparticles. On the other hand, chemical interaction between components of a formulation can be identified by FITR.

The DSC thermogram of ATR (Figure 4.7), demonstrated three endothermic peaks at 97.54 °C, 256.39 °C and 228.84 °C. These endothermic peaks of ATR disappeared in the DSC curve of ATR loaded CH nanoparticles, which indicated successful encapsulation of ATR. Furthermore, the FTIR spectra of ATR loaded CH nanoparticle have supported the ATR encapsulation into the CH nanoparticles.

5.2.2. In-vitro Release

The type of polymer, its molecular weight and the size of particles are the major factors which influence the release of active ingredients. The exposed surface area of small particles are higher than larger particles, therefore small particles exhibits faster release of active ingredients (192).

Figure 4.9, shows the plot of cumulative percent release profile of ATR from CH Nano Spray dried formulations. Formulations F1, F2 and F3 (with smallest particle size 510.5 ± 22.33 nm, 566.1 ± 59.98 nm 580.4 ± 93.96 nm) exhibited the highest initial burst release (51.96, 51.49 and 56.85 %) during the first one hour compared to those with relatively bigger particles.

5.2.3. Cytotoxicity of CH Nanoparticles

Cell culture studies on L-929 and B-35 cell lines have proven safety results for the use of atorvastatin loaded chitosan nanoparticles for further animal studies without any cytotoxic effect. Since ATR has neuroprotective effects in addition to its cholesterol lowering effect, these nanoparticles could be promising nanosystems for the treatment of peripheral nerve injury.

5.3. Poly Lactic-co-Glycolic Acid/Chitosan Electrospun Nano Composite

5.3.1. Preparation and Morphological Characteristic

In this study, smooth and the randomly aligned ALA loaded PLGA/ ATR loaded CH electrospun nanocomposites were fabricated using electrospinning methods. Scaffolds were fabricated by dispersing Nano Spray dried CH containing ATR nano-powders in a PLGA solution and spun through a single nozzle electrospinning method.

Visual analysis of the SEM image (Figure 4.21) implied that there was no homogeneity in the diameter of the prepared electrospun nanocomposite. It is obvious that CH is a cationic polymer, therefore the presence of CH nano-powders in the PLGA solution may increase conductivity and surface charge densities, which reduced the whipping stability during the electrospinning (193). Hence in the high electrical field, the PLGA solution containing CH nanoparticles tolerated a whipping instability and form heterogeneous fibers in terms of diameter.

The preparation of electrospun nanocomposites has also been confirmed by TEM image (Figure 4.22). The uniform re-dispersion of nanoparticles into composite nanofibers is still a challenge in this novel drug delivery system. However, some cluster of particles were observed in SEM image, which may be solved through further extensive investigation in this area.

5.3.2. Encapsulation and In-Vitro Drug Release

As it was reported in Section 4.3.2 was reported, the encapsulation efficiency of ALA and ATR were $92.76 \pm 3.53\%$ and $89.27 \pm 5.053\%$ respectively. These results revealed relatively high EE of active ingredients.

The release behaviors of ALA and ATR from the composite nanofibers PLGA/CH were studied and shown in (Figure 4.27). It was observed that ALA was released much faster than ATR from PLGA/CH nanocomposite. At 8h, the cumulative drug-release rate reached to 84.32% for ALA, whereas ATR was reached to plateau (82.439%) after 150h.

The possible explanation of different release profile of ALA and ATR from nanocomposite, may be because of the higher polarity of ALA, which led to encapsulation of ALA near to the surface or on the surface of the PLGA nanofiber. In addition, ATR has more possible hydrogen binding sides, where it can form strong interactions with the CH polymer. On the other hand, CH containing ATR nanoparticles settled into the nanofibers rather than on the surfaces of the fiber. The obtained results complied with the results reported by Eroglu et al. (35).

ALA which was encapsulated directly by PLGA nanofibers could be released in the medium through desorption or diffusion mechanism from the PLGA nanofibers. However, ATR encapsulated in the CH nanoparticles sustain two barriers, the nanoparticle and nanofiber, lead to reducing the release rate.

5.3.3. Physicochemical Analysis of Electrospun PLGA/CH Nanocomposite

Differential scanning calorimetry analysis provides information about physical status of the drugs in the formulations. All pure components of formulation gave the peaks related to the phase transition temperatures. There were no peaks observed at the temperature range of 30-350°C for ALA and ATR in full formulation. The result indicates the successful encapsulation of ALA and ATR in electrospun nanocomposite formulation.

FT-IR spectra of pure ALA, pure ATR, pure PLGA, pure CH, empty PLGA/CH electrospun nanocomposite formulation, ALA and ATR loaded PLGA/CH electrospun nanocomposite are shown in Figure 4.25. The FTIR spectra of PLGA/CS indicated that ALA and ATR loaded CH nanoparticles were embedded within the nanocomposite sheet.

The crystalline structure of pure ALA and ATR before and after the loading into the electrospun PLGA/CH nanocomposite was characterized using XRD (Figure 4.24). After loading of ALA and ATR, some peaks that possibly belong to ALA crystals were observed in diffractograms, which indicated the possible surface localization of ALA.

5.3.4. Porosity Measurements

The obtained porosity values were similar to the nanofiber formulations prepared by PLGA in the literature (194, 195). However, it should be noted that the porosity values of the prepared formulations are not standard and may vary depending on the molecular weight of the PLGA, the solvent system and the percentage of polymer. According to these results, it was determined that the porosity value (%) and the pore diameters were increased in parallel with the addition of active substance in the prepared electrospun nanocomposite formulations and the increase in both parameters was statistically significant ($p < 0.05$).

5.3.5. Texture Analysis

Mechanical properties of formulation A, B, C, D were investigated. As shown in Figure 4.26, the tensile strength of formulations was decreased with respect to existence of ALA. The results suggested that the electrospun nanocomposite sheet has enough mechanical strength to withstand implantation process in experimental animals.

5.3.6. Cell Viability of Electrospun Nanocomposites Formulations

Fibroblasts are widely distributed in many types of human tissues, such as tendon, ligament and skin. Because of its excellent reproducibility of L929 cell line, it makes a useful screening model for in-vitro toxicity test. On the other hand it is recommended as standard cell line for in-vitro toxicity evaluation of polymeric systems for therapeutic use in humans by USP 28 (196). In addition, the developed formulations were implanted around sciatic nerve, therefore, the L929 and B35 cell line were preferred to use in this study.

In order to investigate the cytotoxicity of prepared formulations (Table 3.3), MTT assays were performed as explained in the section 4.4. (Figure 4.19) shows the viabilities of L929 and B35 cell line after incubated with extract of formulations for 24h and 48h incubation time. The viabilities for L-929 fibroblast cell line, were 116.586%, 119.070%, 111.700%, 101.200% after 24h and 101.420%, 110.366%, 90.226%, 92.548% after 48h with formulations A, B, C and D, respectively.

The viability of B35 cells at 24 h and 48 h were between 97% -116.586 % and 90.226% -110%, respectively. The results indicated that developed formulation displayed no cytotoxicity on L929 and B35. The results of cell viability suggested that the formulation can be used as drug delivery system.

5.3.7. Stability Test

From the pharmaceutical formulations point of view, the stability of polymeric formulation in the storage condition is remarkably important. In order to make ensure

that the particular drug dose intended for the biological study remain stable in the storage condition, the stability test is strongly required.

Stability study for formulation (A) under the ICH guideline was performed in the programmable environmental test chamber at different conditions temperature (A- $30^{\circ}\text{C} \pm 2^{\circ}\text{C}/65\% \text{ RH} \pm 5\% \text{ RH}$, B- $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and C- $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH}$) for 6 months. In every month the content of active ingredients in the formulation was tested.

The content of active ingredients decreased gradually in every month in the formulation that was stored in condition A and C; whereas no significant difference was observed in the formulation which was stored in condition B. The result suggested that condition B is the optimum storage condition for developed formulation.

PLGA is a heat and humidity sensitive polymer. At lower temperatures, the degradation of PLGA is directly related to time and temperature and significantly accelerates in the presence of impurities and humidity (197). During the degradation process, the number of carboxylic end groups, which are known as a autocatalyze ester hydrolysis, will increase and causes breaking of ester linkages.

The results of the stability test suggested that the storage temperature and humidity are critical factors to the stability of the ALA loaded PLGA nanofiber containing ATR loaded CH nanoparticle.

5.3.8. In-Vivo Study

5.3.9. Functional Assessments

Transmission electron microscope examination and functional recovery assessments were used to evaluate neural regeneration after sciatic nerve crush injury.

Walking track analysis is commonly performed for functional evaluation after PNI in the rat model and for the first time, it proposed by Medinaceli et al. (198). It is an easy, non-invasive and reliable method, and can be used frequently to measure functional recovery over several time points in the same animal.

After compression of the sciatic nerve by aneurysm clip, a loss of functional deficit was observed in all animals. Following that, the SFI increased and normal values were achieved in one month. These findings are in agreement with the results of several researchers, whose studies have also indicated normal fingerprint only one month after nerve crush as done by Gudemez et al. (199) and Oliveira et al. (200).

SFI in the group 2 and 3 demonstrated that functional outcomes were improved faster at every test point with respected to group 1. SFI results were revealed, a significant improvement on the process of nerve regeneration in group 2 was started on day 5, whereas in group 3 and 4 after day 7 significant improvement was observed in SFI.

The BBB test initially developed to evaluate the locomotion of the hindlimb in the spinal cord injury model. Since the spinal cord and sciatic nerve lesion have many similarities in hindlimb locomotion BBB can be used to evaluate locomotion in SNI. The study was conducted by Schiaveto de Souza et al. (201) was utilized the BBB scale to investigate the peripheral nerve injury. In order to the possible correlation, the results were contrasted with SFI. The study suggested that a high correlation exists between the SFI and the BBB test in assessing functional recovery after the sciatic nerve crush injury.

During the 30th day monitoring of all rats, the BBB scores for group 2 and 3 considerably improved ($p < 0.0001$) when compared with group 1. The rats with a crushed-sciatic nerve in group 2 and 3 exhibited the highest BBB score; but did not achieve the level of recovery equal to control group.

The EPT measures the strength in grams (g) which is produced by the hindlimb of the rat against the surface of the balance, and commonly utilized to assess the performance of the gastrocnemius-soleus muscles as it was described by Thalhamme et al. (185).

A significant better recovery of motor deficit was seen in group 2 and 3. In group 2 motor deficit starts decreasing significantly after day 5, but in other groups,

significant difference was observed after day 15. It indicated faster recovery of animals in group 2 and 3. In addition, the comparison within time point for each group indicated a faster decline in the latency time (recovery of nociceptive function) in group 2 and 3 while the improvement of latency time was not statistically significant in other groups.

The results obtained from functional assessment experiments representing indication of successful end organ reinnervation in the formulation A and B treated animals.

5.3.10. Transmission Electron Microscope Examination of Sciatic Nerve Samples

The investigation of the morphological feature of myelinated axons provides important information about the pathophysiology of sciatic nerve crush injury. When the ultrastructural investigation of myelinated nerve fibers was evaluated, pathological changes were observed in axons. Regardless of the size of the myelinated axon, ultrastructural pathological changes were observed in group 1 at time point (day 1, day 3, day 5 and day 7). In group 2 nearly the same ultrastructural pathological findings were observed at day 1, day 3 and day 5. At day 15 the axons were nearly normal in appearance and at day 30 ultrastructurally normal axons were observed. The results suggested that, the healing process was faster in group 2 than group 1.

The small sized myelinated axons showed the least ultrastructural pathological changes in all of the groups (Figure 4.36.A). The most severe ultrastructural pathological changes were detected in the large sized myelinated axons in all of the groups (Figure 4.36.C).

Comparative evaluation of the findings; in terms of the amount of ultrastructural pathological changes from the best to the worst; the following order was detected: Groups 6, 2, 3, 4, 5, 1. The unmyelinated axons were normal ultrastructurally in all of the groups.

As regards of the morphological results revealed, that application of ALA and ATR as active ingredients in the component of PLGA/CH electrospun nanocomposite, resulted in faster improvement functional recovery of the sciatic nerve during the study period.

5.3.11. Determination of TNF- α , IL-1 β and IL-6 Levels

TNF- α is mainly released by activated macrophages, but it can also produce by other cell types like T cell and lymphocytes. In the inflammation condition, TNF- α level is increased and it point out an inflammation process. TNF- α play significant role in both central and peripheral nervous system degeneration process.

In inflammation condition monocytes, macrophages, astrocytes and oligodendrocytes have the key role in production of pro-inflammatory cytokine such as IL-1 β . IL-6 is a multifunctional pro-inflammatory cytokine is also induced during the nerve injury associated with inflammation (202). The data which was obtained from the determination of TNF- α , IL-1 β and IL-6 Levels, revealed that the exact effect of formulation was observed on day 15 in all treatment groups. The level of TNF- α in groups 3 and 4 decreased in comparison with group 1. In the case IL- IL-1 β , significant decrease was observed in all groups which were treated with formulations containing ALA and ATR. The level of IL-6 shows decreasing in group 2 and 4.

The data suggested that the use of ATR alone in the neuroinflammatory process resulted in less cytokine activation compared to ALA administration. Whereas the combined application of ALA and ATR was the most effective and maximum effect was observed on day 15th. In the longer treatment window (30 days) there was no significant difference in the result of the treatment.

In the ex vivo models of neurotrauma/organotypic spinal cord culture study, the Neun staining observation suggested that after trauma, the neurons were found to be better protected in the groups which treated with formulations containing 5% and 10% ALA.

According to release of IL-1 β and IL-6 to the outside the neuron, it is believed that formulation with 5% ALA was considered as the most effective formulation for the suppression of the inflammation process.



6. CONCLUSION

The results of this thesis study are as follows:

The simultaneous quantitative analysis of ALA and ATR was successfully performed by the validated HPLC method. The validated HPLC method was found to be a suitable for the quantification of ALA and ATR.

ATR loaded CH nanoparticles were successfully fabricated by Nano Spray drying method. The process and material parameters were optimized over 12 formulations. The product yield for all formulations varied within a range from 58.30% up to 74.10%. The highest loading efficiency was obtained in the case of formulation F9 ($53.61 \pm 4.52\%$) containing 0.04%-w/v CH and 40%-w/w ATR. The mean particle size values for all formulations were found to be in submicron range ($510.5 \pm 22.33\text{nm}$ - $820.0 \pm 98.71\text{nm}$) with almost a narrow particle size distribution (PDI) value and having positively charged. All nanoparticles were found to be distinct, spherical with relatively smooth surface ATR shows an initial burst release which was followed by sustained release in 200h period. In cytotoxicity studies, CH nanoparticles exhibited no cytotoxic effect.

ALA loaded PLGA nanofibers containing ATR loaded CH nanoparticles were successfully prepared by electrospinning method and TEM image indicated the encapsulation of CH nanoparticles inside the nanofiber.

The diameter values of nanofibers were in submicron range with a smooth surface. ALA shown a faster release while ATR was release in a sustained manner. DSC and FT-IR analyses indicated the successful encapsulation of the active ingredients in the formulation. Diffractogram of formulation showed that, some ALA crystals were possibly localized on surface of nanofiber. Tensile analysis results indicated that the mechanical strength of the nanofiber formulations decreased statistically and the structures had less mechanical resistance in terms of presence of active ingredients.

The results obtained from functional and sensory assessment experiments representing indication of successful end organ reinnervation in the formulation A and B treated animals. As regards of the morphological results revealed, that application of ALA and ATR as active ingredients in the component of PLGA/CH electrospun nanocomposite, resulted in faster improvement functional recovery of the sciatic nerve during the study period.

The data which was obtained from the determination of TNF- α , IL-1 β and IL-6 Levels, revealed that the exact effect of formulation was observed on day 15 in all treatment groups.

As the conclusion of this study, we might state that ATR loaded chitosan nanoparticles that are embedded in ALA containing nanofibers could be promising drug delivery systems for neuroprotection after peripheral sciatic nerve injury, especially depending on the faster recovery within the first 15 days period after trauma.

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8. SUPPLEMENTS



9. CURRICULUM VITAE



Name	Mohammad Karim Haidar
Date of Birth	02/04/1975
Place of Birth	Kabul Afghanistan
Email	mk_haidar@hotmail.com

Education

1- B.Sc. Pharm "very good grade" Faculty of Pharmacy, Kabul University, Jamaal mina Kabul, Afghanistan, April 1991-1997

2- M.Sc. Drug Delivery Department, Faculty of Pharmaceutical Science, Kyoto University, Kyoto, Japan, October 2006-2009

Study on "Optimization of process parameters for controlled release microspheres of minocycline HCl based on the robust design method".

Thesis Supervisor: Associate Professor Fumiyoshi Yamashita

3- Ph.D. Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University September 2012-

Study on "Development of nano-formulations containing neuroprotective active ingredient and evaluation of efficiency in peripheral nerve injury model"

Thesis Supervisor: Professor. Dr. Hakan EROĞLU

Publications

1. Eroğlu H., **Haidar M.K.**, Nemutlu E., Öztürk Ş., Bayram C., Ulubayram K., et al. Dual release behavior of atorvastatin and alpha-lipoic acid from PLGA microspheres for the combination therapy in peripheral nerve injury. *Journal of Drug Delivery Science and Technology*. 2017; 39: 455-66 (**Science Citation Index-Q2-impact factor: 2.297**)
2. **Haidar M.K.**, Eroglu H. Nanofibers: New Insights for Drug Delivery and Tissue Engineering. *Current Topics in Medicinal Chemistry*. 2017; 17(13): 1564-79. (**Science Citation Index Expanded-Q2-impact factor: 3.374**)

Awards

1. **Best Poster Award** in 19th International Pharmaceutical Technology Symposium-IPTS 2018 with the poster presentation titled “Development of Novel Polymeric Nanofibrous Composite Containing Nanoparticles For Dual And Localized Neuroprotective Drug Delivery”, 17-19 September 2018, Antalya, Turkey.

Oral Presentation

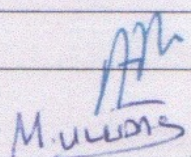
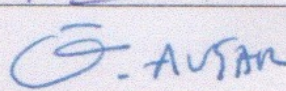
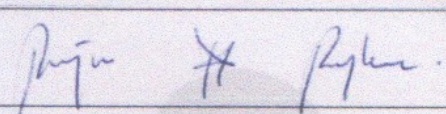
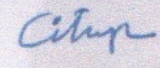
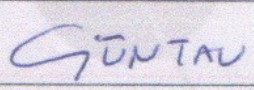
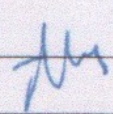
1. **Haidar M.K.**, Timur S.S, Gürsoy R.N, Nemutlu E., Demirbolat G.M., Türkoğlu Ö.F., Kazancı A., Sargon M.F., Gök M., Bodur E., Ulubayram K. Öner L, Eroğlu H. Application of A Novel Polymeric Electrospun Nanocomposite Incorporating Neuroprotective Drugs In Rat Sciatic Nerve Injury Model, 19th International Pharmaceutical Technology Symposium-IPTS 2018, 17-19 September 2018 Antalya, Turkey.

Poster Presentations

1. **Haidar M.K.**, Eroğlu H., Systematic formulation study for fabrication of poly lactic-co-glycolic acid and polycaprolactone nanofibers via electrospinning, 18th International Pharmaceutical Technology Symposium-IPTS 2016, September 18-21, 2016, Antalya, Turkey.
2. **Haidar M.K.**, Demirbolat G.M., Nemutlu E., Ulubayram K. Öner L., Eroğlu H., Formulation of Atorvastatin Calcium Loaded Chitosan Nanoparticles by Nano-spray Drying Method for Sustained Drug Delivery, 3rd International Congress of the Greek Local Chapter of the Controlled Release Society (CRS), 19-20 June 2017, Athens, Greece.
3. **Haidar M.K.**, Timur S.S., Gürsoy R.N., Nemutlu E., Demirbolat G.M., Ulubayram K., Öner L., Eroğlu H., In Vitro Cytotoxicity Study of Chitosan Nanoparticles Containing Atorvastatin Calcium For Treatment Of Peripheral Nerve Injury, 19th International Pharmaceutical Technology Symposium-IPTS 2018, 17-19 September 2018 Antalya, Turkey.
4. **Haidar M.K.**, Timur S.S., Gürsoy R.N., Nemutlu E., Demirbolat G.M., Ulubayram K., Öner L., Eroğlu H., Development of Novel Polymeric Nanofibrous Composite Containing Nanoparticles For Dual And Localized Neuroprotective Drug Delivery, 19th International Pharmaceutical Technology Symposium-IPTS 2018, 17-19 September 2018 Antalya, Turkey.

ETHICAL COMMITTEE APPROVAL

KOBAY DHL A.Ş. YEREL ETİK KURULU BAŞVURU ONAYI

BAŞVURU BİLGİLERİ	Protokol Numarası	127
	Protokol Adı	Nöroprotektif etkin madde içeren nano-formülasyonların geliştirilmesi ve periferik sinir hasarı modelinde etkinliklerinin değerlendirilmesi
	Başvuru Tarihi	20/01/2015
	Sorumlu Araştırmacı Adı-Unvanı	Doç. Dr. Hakan EROĞLU
	Sorumlu Araştırmacı Çalıştığı Kurum	Hacettepe Üniversitesi Eczacılık Fakültesi
	Onay Numarası	127
KARAR BİLGİLERİ	Onay Tarihi	22.01.2015
	Onaylanan Hayvan Türü ve Sayısı	Sprague Dawley Sıçan(90 Ad.) – CD1 Fare (6 Ad.)
	Onay Bilgileri	Proje amaç, gerekçe, yaklaşım ve yöntem yönünden incelenmiş ve çalışmanın gerçekleştirilmesinde Etik sakınca bulunmadığına karar verilmiştir.
	Etik Kurul Başkanı Veteriner Hekim A. Begüm BUĞDAYCI AÇIKKOL	
KOBAY DHL A.Ş. YEREL ETİK KURUL ÜYELERİ	Etik Kurul Üyesi Doç.Dr M.Orhan ULUDAĞ	
	Etik Kurul Üyesi Veteriner Sağlık Teknikeri Özge AVŞAR	
	Etik Kurul Üyesi Dr Buğra Adil BUYRUKÇU	
	Etik Kurul Üyesi Doç. Dr Cihangir ÇAKICI	
	Etik Kurul Üyesi Dilara GÜNTAV	
	Etik Kurul Üyesi Fatih ALTINSOY	

26.06.2018

İlgili Makama ;

20.01.2015 tarih ve 127 sayılı karar ile onay verilen ve 'Nöroprotektif etkin madde içeren nanoformülasyonların geliştirilmesi ve periferik sinir hasarı modelinde etkinliklerin değerlendirilmesi' çalışması ile ilgili olarak tarafımıza 25.06.2018 tarihinde tarafımıza hayvan sayısının düzenlenmesi hakkında dilekçe ile başvurmuş bulunmaktasınız.

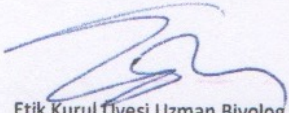
Dilekçenizde ilgili gerekçeler değerlendirilmiş olup söz konusu hayvan sayılarının 144 adet Sprague Dawley erkek ve 6 adet CD1 fare kullanımı oy birliği ile kabul edilmiştir

Saygılarımızla...

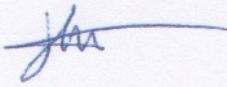
Etik Kurul Başkanı Veteriner Hekim
A. Begüm BUĞDAYCI AÇIKKOL

Ali

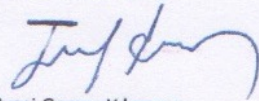
Etik Kurul Üyesi Prof..Dr Güneş Esendağlı



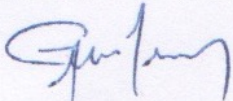
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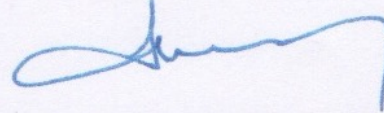
Etik Kurul Üyesi Turgut ALTUN



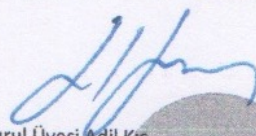
Etik Kurul Üyesi Gamze Yılmaz



Etik Kurul Üyesi Veteriner Hekim Salih Salar



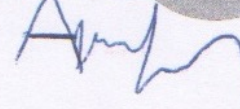
Etik Kurul Üyesi Uzman Biyolog Uygur Saçık



Etik Kurul Üyesi Adil Kış



Etik Kurul Üyesi Ali Varol



Ekler : 1)Etik Kurul Onay Formu Fotokopisi

2)Prof.Dr.Hakan Eroğlu Dilekçe Fotokopisi



PH.D. THESIS

ORIJINALLIK RAPORU

% **17**

BENZERLIK ENDEKSI

% **12**

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KAYNAKLARI

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YAYINLAR

% **5**

ÖĞRENCİ ÖDEVLERİ

BİRİNCİL KAYNAKLAR

1

Hakan Eroğlu, Mohammad Karim Haidar, Emirhan Nemutlu, Şükrü Öztürk, Cem Bayram, Kezban Ulubayram, Levent Öner. "Dual release behavior of atorvastatin and alpha-lipoic acid from PLGA microspheres for the combination therapy in peripheral nerve injury", Journal of Drug Delivery Science and Technology, 2017
Yayın

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Eroglu, Hakan, Emirhan Nemutlu, Omer Faruk Turkoglu, Osman Nacar, Ebru Bodur, Mustafa Fevzi Sargon, Etem Beskonakli, and Levent Oner. "A Quadruped Study on Chitosan Microspheres Containing Atorvastatin Calcium: Preparation, Characterization, Quantification and in-Vivo Application", CHEMICAL & PHARMACEUTICAL BULLETIN, 2010.
Yayın

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İnternet Kaynağı

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www.turkishneurosurgery.org.tr

