MARMARA UNIVERSITY

INSTITUTE OF SCIENCES

SYNTHESIS AND CHARACTERIZATION OF HYALURONIC ACID (HA) BASED NANOPARTICLES FOR USE IN DRUG DELIVERY SYSTEM

NUR GÜLER

MASTER THESIS

Department of Chemical Engineering

Thesis Supervisor

Prof. Dr. MEHMET SAYIP EROĞLU

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April, 2023 **Nur GÜLER**

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İLAÇ SALIM SİSTEMİNDE KULLANIM İÇİN HYALÜRONİK ASİT BAZLI NANOPARTİKÜLLERİN SENTEZİ VE KARAKTERİZASYONU

Hastalıklarda tedaviler genellikle kişiye ve hastalığının derecesine özel olduğu halde, her yöntemin farklı yan etkileri bulunmaktadır. Bu nedenle, ilaçla tedavinin yan etkilerini minimize etmek ve hatta yok etmek amacıyla birçok farklı yöntem geliştirilmektedir. Bu yöntemlerden biri, kontrollü ilaç salımı yapabilen nanopartiküller içine hapsedilmiş ilaç etken maddelerinin kullanımıdır.

Metabolik sistemlerde polisakkaritler, hücrelerin ana enerji kaynağı olarak önemli bir işleve sahiptir ve kanser hücreleri sağlıklı hücrelerden daha fazla enerjiye ihtiyaç duyar. Bu nedenle polisakkaritler tümör hücreleri tarafından daha çok tercih edilmektedir ve yüksek hücre adezyonuna sahiptir. Bu da, kanser hücrelerinde mukozal bariyerin aşılmasıyla sonuçlanan daha etkili reseptör-aracılı endositoz sağlar.

Tez çalışmasında ana bileşik olarak kıkırdak ve sinovyal sıvılar dahil birçok doku ve sıvıda bulunan Hyaluronik asit (HA) kullanılmıştır. HA, biyouyumlu ve biyobozunur olması, ağız yoluyla verilebilmesi, kanser hücrelerini hedefleyebilmesi ve bağ dokularına afinitesi nedenleriyle ilaç salım sistemlerinde önemli bir konuma sahiptir. Ayrıca, direk karıştırma sonucu hidrofobik-hidrofilik etkileşim ile misel, hidrojel ve elektrostatik kompleks oluşturma sağlayan özgün fiziksel özellikleri bulunmaktadır.

Tez çalışmasında HA'nın özgün metabolik özellikleri dikkate alınarak, HA-bazlı amfifilik kemoterapik ilaç taşıyıcısı hazırlanmıştır. HA'ya amfifilik özellik oluşturabilmek için hidrofobik özellikteki hekzanoik anhidrit ile tepkimeye sokularak hyaluronan-hexanoic acid (HA-HEX) kopolimeri hazırlanmıştır.

Sentezlenen HA-HEX kopolimeri Nükleer Manyetik Rezonans (NMR) ve Fourier Dönüşümlü Kızılötesi Spektroskopisi (FTIR) ile tanımlanmıştır. Hazırlanan nano/mikro partiküllerin tanımlanması ise Diferensiyal Tarama Kalorimetresi (DSC) ve Taramalı Elektron Mikroskopu (SEM) kullanılarak yapılmıştır.

Ayrıca, fizyolojik sıvı olarak PBS (Phosphate Buffered Saline) çözeltisi içinde, model ilaç olarak 5-amino salisilik asitin (5-ASA) 37ºC'de salım çalışmaları yapılarak zamana karşı salınım profili elde edilmiştir.

ABSTRACT

SYNTHESIS AND CHARACTERIZATION OF HYALURONIC ACID (HA) BASED NANOPARTICLES FOR USE IN DRUG DELIVERY SYSTEM

Treatments are mostly unique to patients and stage of illness, however, each still has side-effects. There are many researches on methods to minimize and even eliminate the side effects. One of the methods is ensuring release of drug in controlled manner.

In metabolic processes, polysaccharides are the main energy source, and cancer cells need more energy than healthy cells. Therefore, polysaccharides are more preferred by tumor cells, and have high cellular adhesion providing more effective receptor-mediated endocytosis at cancer cell membranes resulted overcoming the mucosal barrier.

In our project, Hyaluronic acid (HA) was used as basis. HA is found in tissues and fluids, including articular cartilage and synovial fluids. It is a promising material in preparation of drug delivery systems due to biocompatibility, biodegradability, orally administrability, targeting property on cancer cells and affinity for connective tissues. It has unique physical properties that form micelles and hydrogels, through hydrophobic-hydrophilic interactions and electrostatic complex-formation reactions.

Considering its unique metabolic properties, HA-based amphiphilic chemotherapeutic drug carrier was prepared. To give amphiphilic nature, hydrophilic HA was reacted with hydrophobic hexanoic anhydride and hyaluronan-hexanoic acid (HA-HEX) was synthesized.

Characterization of synthesized HA-HEX was performed using NMR and FTIR techniques. Physicochemical characterization of the nano/microparticles prepared from HA-HEX for use as drug carriers, was performed using DSC and SEM techniques.

In drug release studies, Mesalazine (5-ASA) was used as model drug. Release profile from the nano/microparticles was determined in phosphate buffer saline (PBS, pH 7.4) at 37ºC, and statistical analyses of drug release behavior was performed.

CLAIM FOR ORIGINALITY

SYNTHESIS AND CHARACTERIZATION OF HYALURONIC ACID (HA) BASED NANOPARTICLES FOR USE IN DRUG DELIVERY SYSTEM

There are many studies focused on increasing the efficiency of drug delivery systems by designing new systems utilizing different natural biodegradable polymers. However, the use of Hyaluronic acid, a linear polysaccharide with a high potential for targeted drug delivery, is still limited, and very few studies have been reported on polymersome/hollow nanoparticles formation from HA-based diblock copolymers [1]. Moreover, to best of our knowledge, there is no research on the synthesis of HA-based nanoparticles using hexanoic anhydride without any prior modification, for drug delivery applications. As the frequently used intermediate in synthesis, Hexanoic anhydride, rapidly hydrolyses to the naturally available fatty acid (hexanoic acid), it is expected to be biodegradable and biocompatible as HA.

In this thesis, the HA-HEX nanoparticles were prepared, and self-assembled into micelles by efficiently capturing drug. The drug release pattern is promising as well. Therefore, they are expected to be a highly efficient, non-toxic, and low-cost solution for materials for drug delivery systems.

This study is supposed to lead on drugs that is expected to be utilized for treatments even of neurological manifestations, given HA ability to target these cells and to easily be modified due to its functional groups. Additionally, the use of natural biodegradable polymers (including HA), is a desirable, inexpensive, advantageous, and health-wise more conformable method than the use of synthetic polymers for obtaining novel structural materials[2].

SYMBOLS

^{o}C : Celsius degree

% : Percentage

(abs) : absorption value measured with UV Spectrophotometer

cm^{-1} : reciprocal centimeter (or wavenumber)

C_t : concentration at time "t"

g/L : Gram/Liter

g/L aq.sol : Gram/Liter aqueous solution

g/cm^3 : Gram/centimeter cube

M : Molar

m : Weight

mg : milligram

mL : milliliter

mmHg : millimeter Mercury (hydrargyrum)

nm : nanometer

pH : Power of Hydrogen (acidity or basicity of an aqueous solution)

ppm : Parts Per Million

R^2 : Standard deviation

V : Volume

µm : micrometer

ABBREVIATIONS

5-ASA **:** 5-Aminosalicylic Acid (Mesalazine)

DMF **:** Dimethylformamide

MW **:** Molecular weight

GAG **:** Glycosaminoglycan

HA **:** Hyaluronic acid (Hyaluronan)

HAS **:** Hyaluronan synthase

HMW **:** Higher Molecular Weight

HEX **:** Hexanoic anhydride

LMW **:** Lower Molecular Weight

NMR **:** Nuclear Magnetic Resonance

PBS **:** Phosphate Buffered Saline

st **:** Stretching

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1. INTRODUCTION

1.1 Aim of the study

This thesis aimed at the synthesis and characterization of hexanoyl hyaluronic acid (HA-HEX) to prepare nano/microparticles for controlled drug delivery of 5-aminosalicylic (5-ASA).

1.2 Drug Delivery Systems

Drug Delivery System (DDS) is defined as a formulation or a device that enables the carrying of a therapeutic substance into the body and improves its efficacy and safety by controlling the rate, time, and place of release of drugs in the body [3].

The use of DDS decreases or even eliminates the side effects depending on the aim or way of use. It can be utilized to deliver the drug to the specified cells such as the cancer cells, or support the immune system by enabling the delivery of the immunotherapeutic agents through the biological barriers that are blocking the immunotherapy, as well as for targeting a pathogen or a chemical released from it for diagnosis.

Examples of drug delivery methods include encapsulation of the drug within hydrogel [4], as drug-drug conjugates [1] or on quantum dots [5], polymersomes [6], and other nanomaterials [7]. From the listed methods, nanomaterials (typically in size from 1-100 nm up to 1000 nm on nanopolymers) have the advantages of better drug encapsulation and cell targeting with longer blood circulation and retention, and higher surface area, stability and permeability due to their nanoscale size. The cell targeting property of the nanomaterials minimizes the impact on healthy cells. The cytotoxicity challenge of polymeric nanomaterials is mostly overcome using biocompatible and biodegradable polymers. However, this challenge remains valid as it is difficult to predict the change of nanomaterial properties *in vivo*. Modifications on those polymers would improve their properties, drug delivery, and targeting functions. Figure ***1.1*** shows different types of nanomaterials [7].

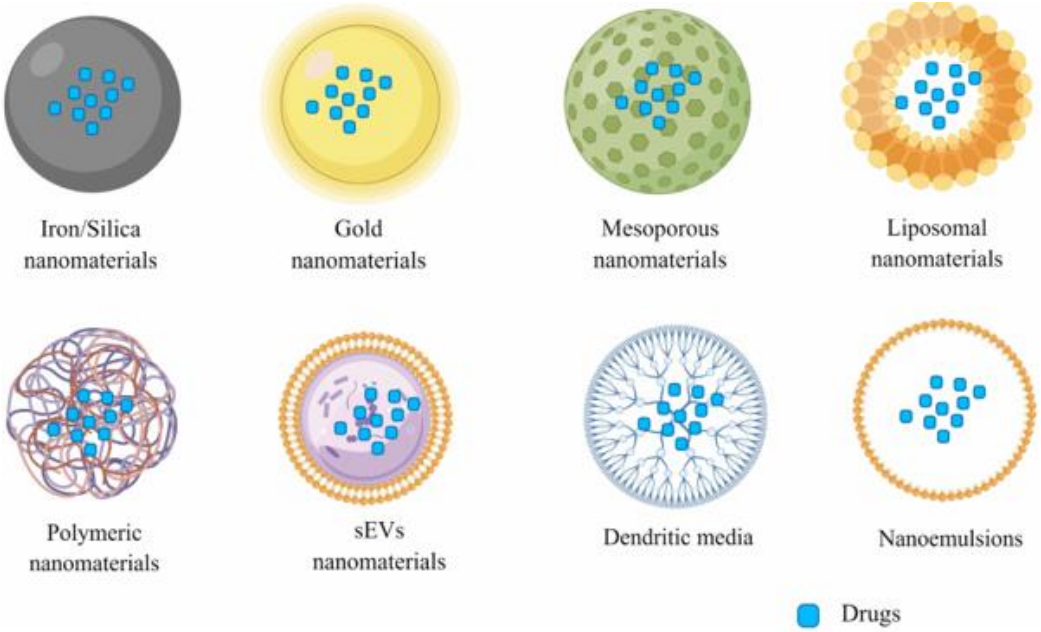

Figure 1.1 The structural maps of different types of nanomaterials [7]

1.3 Hyaluronic Acid

Hyaluronic acid (HA), also called hyaluronan, is a type of polymeric carbohydrates (mucopolysaccharide) that is available in extracellular matrix of living organisms and has important role in physiological processes.

Due to its versatile and bioactive properties, it has found many application areas. Additionally, it is nontoxic, biocompatible and biodegradable as it is naturally available, can easily form supramolecular networks such as hydrogels and nanoparticles, and biologically active.

1.3.1 History of Hyaluronic Acid

HA was first discovered in 1934 by Karl Meyer and John Palmer. They have called the material as combination of “hyaloid” referring to the vitreous body as the source, and “uronic acid” referring to one of the two sugar molecules that form HA[8].

HA was first considered for commercial use in 1942 by Endre Balazs, claiming that it could be used instead of egg whites in the bakery on his patent submission. Endre Balazs named it as hyaluronan in 1986, as it acts more as a salt *in vivo*. Later in the 1950s, HA started to be used in eye surgery replacement of the vitreous[8].

1.3.2 Applications of Hyaluronic Acid

Hyaluronic acid was used for the first time as an ophthalmic hydrogel. That is followed by areas from cosmetics and wound healing to biomedical applications such as carbohydrate scaffolds and osteoarthritis, as well as drug delivery systems for different treatments such as cancer or periodontics. Besides delivering the drug or gene plasmids to targeted cells for treatment, it can be used to deliver agents for the identification of cancer cells through screening. HA is also used in surgery as an alternative antiadhesion barrier to prevent peritoneal adhesions which are pathological bonds.

HA has a wide range of uses due to its biocompatibility, biodegradability, and nontoxicity, as well as the fact that it is highly hydrophilic and can easily be modified. The most attractive physicochemical property of HA is its high water solubility, being the attraction point in cosmetics for lubrication and hydration. When used for drug delivery systems, HA can provide drug targeting, controlled drug release, and sensitivity to the microenvironment.

The molecular weight (MW) of HA has an important impact on its use area, due to reasons like its ability to pass through the biological barriers (e.g. as epithelial, enzymatic, or mucosal barriers); i.e. while low molecular weight (LMW) HA can easily pass, high molecular weight (HMW) HA has challenges to pass through.

Applications depending on the MW can be seen in Figure ***1.2***[8].

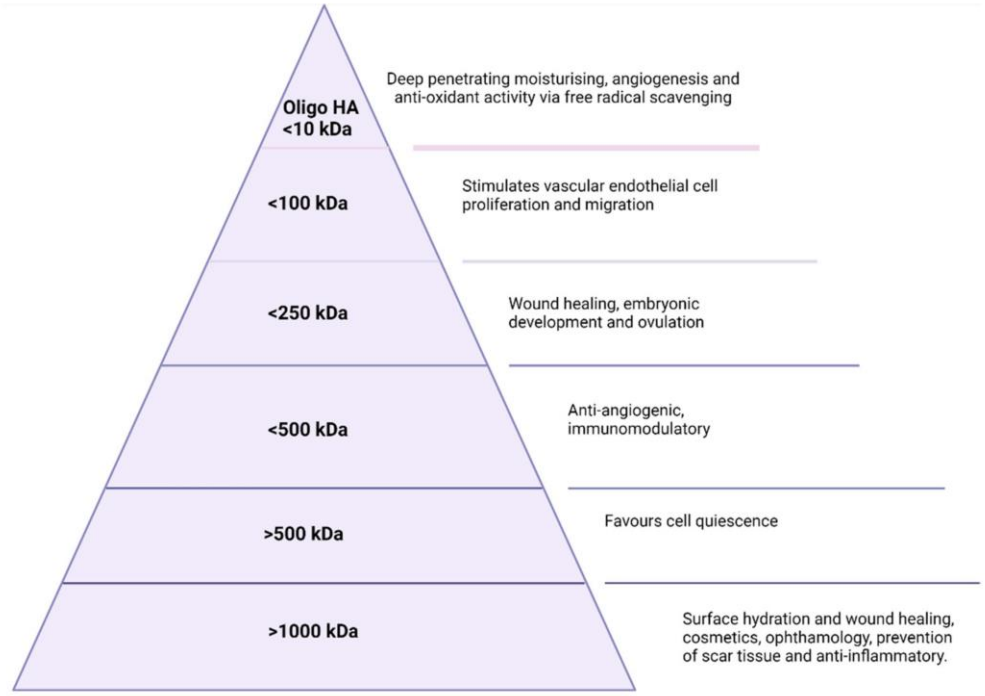

Figure 1.2 Molecular weight-dependent applications of HA.[8]

1.3.3 Chemical and Physicochemical Properties of Hyaluronic acid

Hyaluronic acid (HA) is a glycosaminoglycan (GAG), which is a group of carbohydrate ($(C_xH_2O)_y$) formed of 1,4, D-glucuronic acid and 1,3, D-N-acetyl glucosamine, linked by glycosidic bonds. HA has a carboxyl group, four hydroxyl groups and an amide group on each unit. The chemical structure is as shown in Figure ***1.3***.

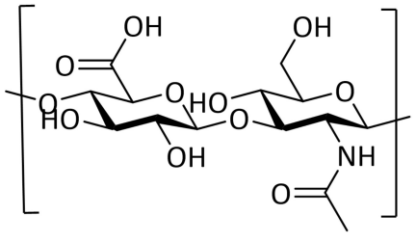

Figure 1.3 Chemical structure of HA[3]

Due to its anionic nature, it interacts with cationic polymers, surfactants, and lipids. It has a high viscoelasticity due to the hydrogen bonds. It is also highly hydrophilic because of its hydroxyl and carboxyl groups that provide the negative charge, which leads to easy formation of nanoparticles, and protects drugs from early absorption at the blood vessel, leading to increased blood circulation. HA can be deacetylated by its

acetamide group, as well as easily modified from its hydroxyl and carboxyl groups, to provide functionalization. By modification, the solubility, stability, degradation and responsiveness can be improved and tailored for the use.

HA can be obtained from animal or specific microorganisms, as well as using the biotechnological solutions. It is the only monosaccharides that is produced on plasma membrane, not in the Golgi apparatus. It is synthesized by 3 different enzymes (HAS 1-3) which effects the MW of synthesized HA, varying from 5 to 20 000 kDa, and can be divided to smaller MWs *in vivo*. Although there are only few investigations done on the MW impact, it is seen that while the LMW HA could cause inflammation as well as act as antioxidant, HMW HA has anti-inflammatory activity[3, 8].

HA is a bioactive molecule, which has different functions such as cellular interactions that impact the change and increase of the cell number, recognition through receptors, as well as hydration and formation of the extracellular matrix structure.

The receptor that is mainly interacting with HA is CD44, enabling the cellular intake of HA by endocytosis. CD44 is one of the overexpressed receptors in tumor cells, mostly in the form of CD44v, while having low availability on normal cells. HA also has lower affinity to the standard CD44 (CD44s) than the overexpressed form on tumors (CD44v). That makes HA a very efficient drug carrier for cancer treatments on targeting cancer cells.

Other most known receptors interacting with HA are the receptor for hyaluronan-mediated motility (RHAMM), Neurocan, GHAP (glial HA binding protein), Aggrecan, and TSG6 (TNF-stimulated gene 6)[8].

1.4 Hexanoic Anhydride

Hexanoic Anhydride (HEX), also called Caproic anhydride, is a fatty acid anhydride which is easily hydrolyzed to hexanoic acid. Some physico-chemical properties are given in Table ***1.1***.

Table 1.1 The physico-chemical properties of Hexanoyl anhydride (HEX)[9-11]

Molecular formula	C_{12} H_{22} O_3
Molecular Weight	214.3
pH (72 g/L aq.sol)	4.8
Physical state	Liquid
Appearance	Clear, Light yellow
Odor	Odorless
Melting point/range	-40ºC
Boiling point/range (@760mmHg)	246-248ºC
Vapor density	7.39
Density@20ºC [g/cm3]	0.928
Water solubility of Hexanoic acid*	10.82 g/L

* HEX hydrolyses rapidly in contact with water under the formation of hexanoic acid. Therefore, no water solubility can be determined for the parent substance[11].

As a fatty acid, it attacks to –OH groups of hydrophilic polymers, enable the stability of new structure in aqueous solutions and thus potentially used for targeted delivery of drugs.

Being available within renewable sources makes HEX a good option to be used for modification and functionalization of drug delivery materials[12].

1.5 HA-based nanoparticles

As summarized in section *1.2*, nanoparticles are a type of drug delivery systems that have received high attention due to their advantages of high stability, selectivity, and slow release leading to improved bioavailability. They provide high concentrations of chemotherapeutic drugs to be carried to tumor cells while protecting healthy cells. They also have improved penetrability due to their small size.

In nanotechnology the efficient drug delivery that could overcome all barriers on the process is suggested to have effective stability, surface and size, which is referred to as the “3S” transition[3]. HA-based nanomaterials are from those exceptional drug delivery biomaterials meeting the “3S” transition criteria. Producing from hyaluronic

acid, which is a naturally available polymer, they are biocompatible and biodegradable, as well.

HA efficiency as drug delivery polymer, such as absorption, blood circulation time (impacting the administration frequency), drug release profile and cell targeting, can be improved by modification of the polymer with active molecules. The modification of HA can be performed through its functional groups: Hydroxyl Groups, Carboxyl Group (Amidation or Esterification), or Amide Group. The advantage of modifying hyaluronic acid via hydroxyl groups is not as complicated as modification via the amide groups since the hydroxymethyl groups on the main chain are sterically more available. However, the nanoparticles would still be recognized by the degradative enzymes through its Carboxyl groups.

HA-based nanoparticle used for drug delivery can be in different forms including nanoparticles, micelles, hollow particles, drug conjugated polymers, and nanogels (Figure ***1.4***).

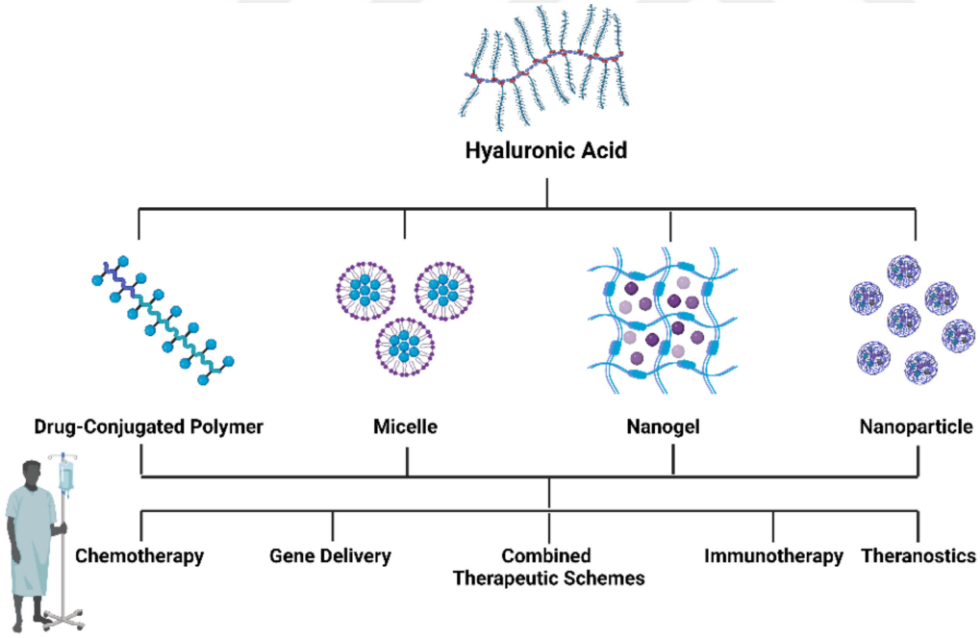

Figure 1.4 Example forms of HA-based nanomaterials and applications[3]

The micelle form of HA is formed by modifying the hydrophilic HA with hydrophobic substances to have an amphiphilic character through functional groups, thus forming nanoparticles with a hydrophilic shell with a high water solubility and a hydrophobic core. On the other hand, although hollow nanoparticles are also formed as a result of

amphiphilic property, they have the same inner and outer sides, which can be both hydrophilic and hydrophobic (liposomal nanomaterial) depending on the nature of the polymer, as shown in Figure ***1.5***. Thus, in addition to holding hydrophilic drugs, they can also carry hydrophobic drugs.

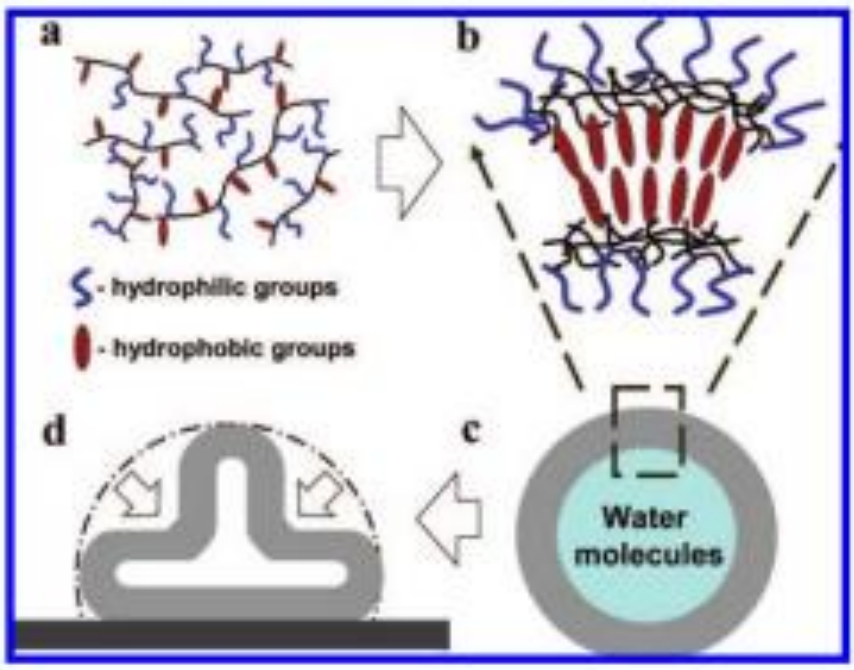

Figure 1.5 Schematic illustration of formation process of hallow nanocapsules[13]

Each form has its own characteristics and similar advantages. Overall, they all aim to protect the drug from fast release by providing greater surface area, better adhesion on targeted cells, improved cellular permeability, and increased stability against macrophages. They also provide a higher drug concentration on tumor cells, which means less damage to healthy cells and more cytotoxicity to cancer cells. However, for example, the nano gels provide particularly increased drug solubility and encapsulation thanks to their matrix form, while the micelles can bind and hold hydrophobic drugs very well, and the hollow nanoparticles have both hydrophilic and hydrophobic bilayers that protect the drug from the fast release. Despite the advantages listed above, these forms lack sufficient research for human use.

2. MATERIAL AND METHOD

2.1 Materials

Hyaluronic acid (HA, 59 kDa) was supplied from Sangherb, China. Hexanoic Anhydride (HEX), Phosphate-buffered Saline tablet (PBS), Methanol and Dimethylformamide (DMF) were purchased from Sigma-Aldrich /Merck, Germany. 5-Aminosalicylic acid (5-(ASA)) was supplied from Alfa Aeser. All solvents had more than 98% purity.

2.2 Equipments

2.2.1 Magnetic Stirrer

The synthesis was done using Heidolph MR 3001K magnetic stirrer.

2.2.2 Rotary Evaporator

Heidolph Laborota 4000 Rotary evaporator was used for solvent evaporation.

2.2.3 Vacuum Drying Oven

The synthesized samples were completely dried using MMM MedCenter Vacucell 22 Vacuum drying oven.

2.2.4 Freeze Dryer

CHRIST Alpha 2-3 LSCbasic was used to dry the drug containing nano/micro particles. The Freeze Dryer is using the vacuum brand of Vacuubrand RZ 2.5.

2.2.5 NMR (Nuclear Magnetic Resonance)

The chemical characterization of the copolymers was done by ^{1}H NMR technique using Varian 600 MHz NMR instrument.

2.2.6 Homogenizer

DAIHAN Scientific HG-15D Homogenizer was used for the formation of nanoparticles.

2.2.7 Shaking Incubator

The drug release studies were performed using N-BIOTEK NB-205 Shaking Incubator at 37^{o}C and 150 rpm.

2.2.8 UV Spectrophotometer

The drug concentration and release profile were determined using Agilent Cary 60 UV-VIS spectrophotometer with a Cary WinUV software.

2.2.9 SEM (Scanning Electron Microscopy)

SEM images of the drug loaded and empty particles were recorded using Hitachi SU5000 FEG SEM instrument at different magnifications.

2.2.10 FTIR (Fourier Transform Infrared Spectroscopy)

Thermo Fisher Scientific NICOLET 6700 instrument was used to record the FTIR spectra of the copolymer. FTIR spectra were recorded between 400-4000 cm^{-1}.

2.2.11 DSC (Differential Scanning Calorimetry)

DSC analysis was performed using PerkinElmer Jade DSC instrument. The instrument was run before measurements with empty pan and then the thermograms were recorded at a heating rate of 10ºC/min under N_2 atmosphere between 25ºC and 290ºC.

2.3 Synthesis of Hexanoyl Hyaluronic Acid (HA-HEX)

2 g of HA was weighed on a reaction flask. 500 mL of water:methanol (v/v) was added on HA to dissolve it. After HA was dissolved completely, 1 mL of Hexanoic anhydride was added. Reaction was achieved in 72 hours and under room temperature. After the reaction was terminated, the polymer was precipitated in acetone. The precipitated polymer was dialyzed (MW 12-14 kDA cut off) for 3 days. After the dialysis, it was dried with Freeze Dryer and weighed (0.26 g).

The reaction mechanism is shown on

Figure **2.1.**

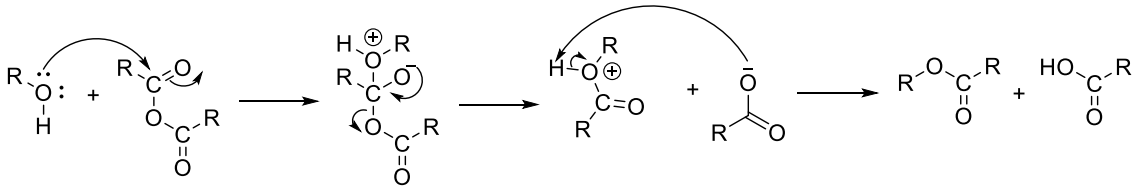

Figure 2.1 Synthesis mechanism of amphiphilic HA-Hex

2.4 Preparation of HA-HEX micelles containing drug

8 mg of HA-HEX copolymer was dissolved in 15 mL of 0.1 M PBS. 2 mg of 5-ASA was dissolved in 5 mL of 0.1 M PBS and added on the prepared solution containing the HA-HEX copolymer. The final solution was mixed approximately 15 minutes with Homogenizer for the formation of micelles.

2.5 Drug-release analysis

5 mL of micelle solution was placed on the membrane cap, while 45 mL of 0.1 M PBS was added on the tube beneath. The tube was placed on the Shaking Incubator at 37ºC and 150 rpm.

3 mL samples were drawn from the tube, analyzed at UV Spectrophotometer and reloaded to the tube. The analyses was done at every 30 min for 4 h, followed by each hour until the 12th hour, every 12 h until the 72nd hour and completed on 144th hour by one analysis a day.

3. RESULTS AND DISCUSSION

3.1 Characterization Studies of Hexanoyl Hyaluronic Acid (HA-HEX)

3.1.1 FTIR Analysis

Figure ***3.1*** shows the FTIR spectrum of hexanoyl hyaluronic acid. The stretching vibration absorbance peaks of –OH and –NH bonds were overlapped and observed at between 3200 cm^{-1} and 3600 cm^{-1}. The N-H bonds of acetamide groups belonging to glucoseamine unit were observed at 1490 cm^{-1}. The strong absorption peak at 1596 cm^{-1} was due to the C=O stretching of carbonyl groups of hyaluronic acid and the hexanoyl group. The another strong absorption peaks at between 950 cm^{-1} and 1100 cm^{-1} were assigned to the two different glucosidic C-O-C bonds of hyaluronic acid. The different C-H bending peaks of both hyaluronic acid and hexanoyl groups were observed at 1390cm^{-1} and 1400 cm^{-1}, while the C-H strecthing peaks at 2915 cm^{-1} [17].

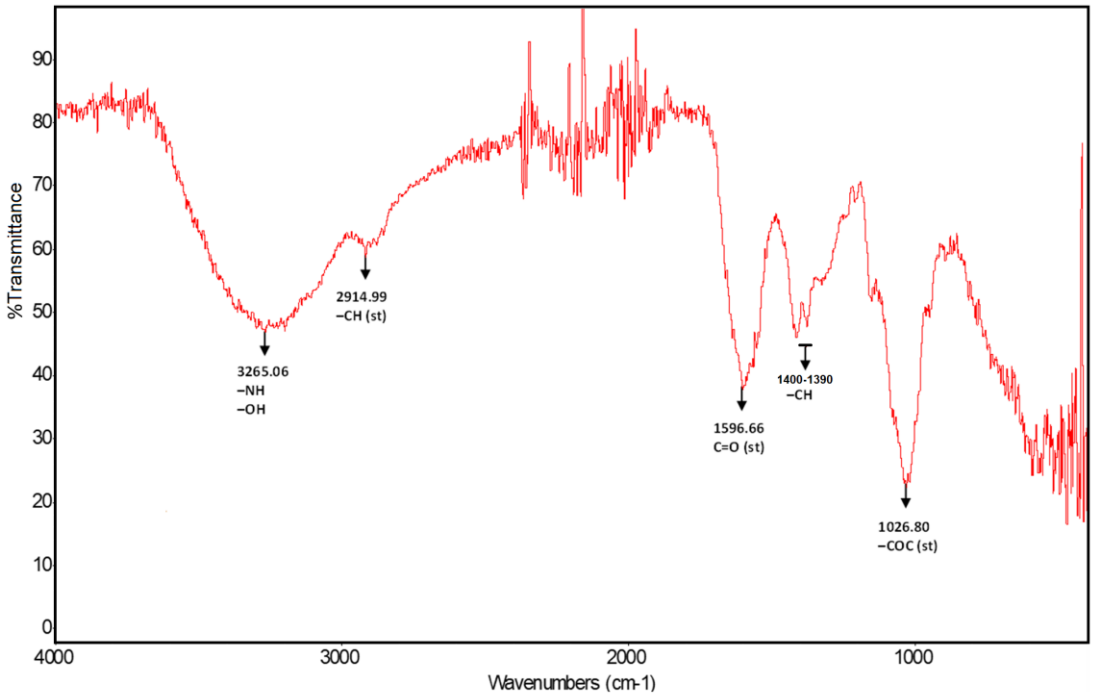

Figure 3.1 FTIR spectrum of HA-HEX nanoparticle

3.1.2 ^{1}H NMR Analysis

In order to better understand the synthesis of HA-HEX, the comparative structural characterization of HA and the synthesized HA-HEX were performed using ^{1}H NMR

spectroscopy. D_2O was used as solvent.

Figure ***3.2*** shows the ^1H NMR spectrum of HA. The characteristic proton peaks were observed at between 3.21 ppm and 3.71 ppm (-C-H of glucuronic acid and acetyl glucosamine units of hyaluronic acid), at 4.32 ppm and 4.43 ppm (two different signals of the anomeric protons of glucuronic acid and acetyl glucosamine units, respectively), and at 1.89 ppm (CH_3 protons of glycose amine units).

Figure ***3.3*** shows the ^1H NMR spectrum of the synthesized hexanoyl hyaluronic acid (HA-HEX). In this spectrum, in addition to the characteristic peaks of hyaluronic acid, the proton peaks of hexanoyl group at 0.9 ppm ($-CH_3$), at between 1.3 ppm and 1.5 ppm (different $-CH_2$ protons), and at 2.1 ppm ($-CH_2$-CO- protons) were observed. The appearance of these C-H proton signals identified the conversion of hyaluronic acid to HA-HEX[18, 19].

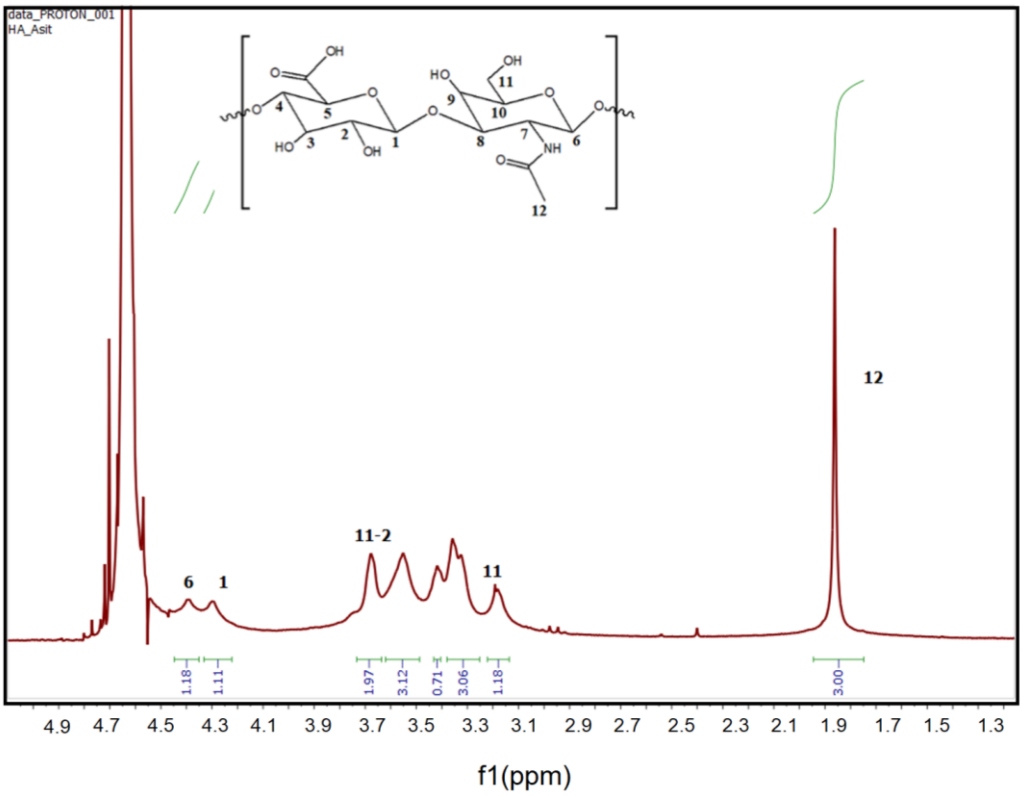

Figure 3.2 ^1H NMR spectrum of HA

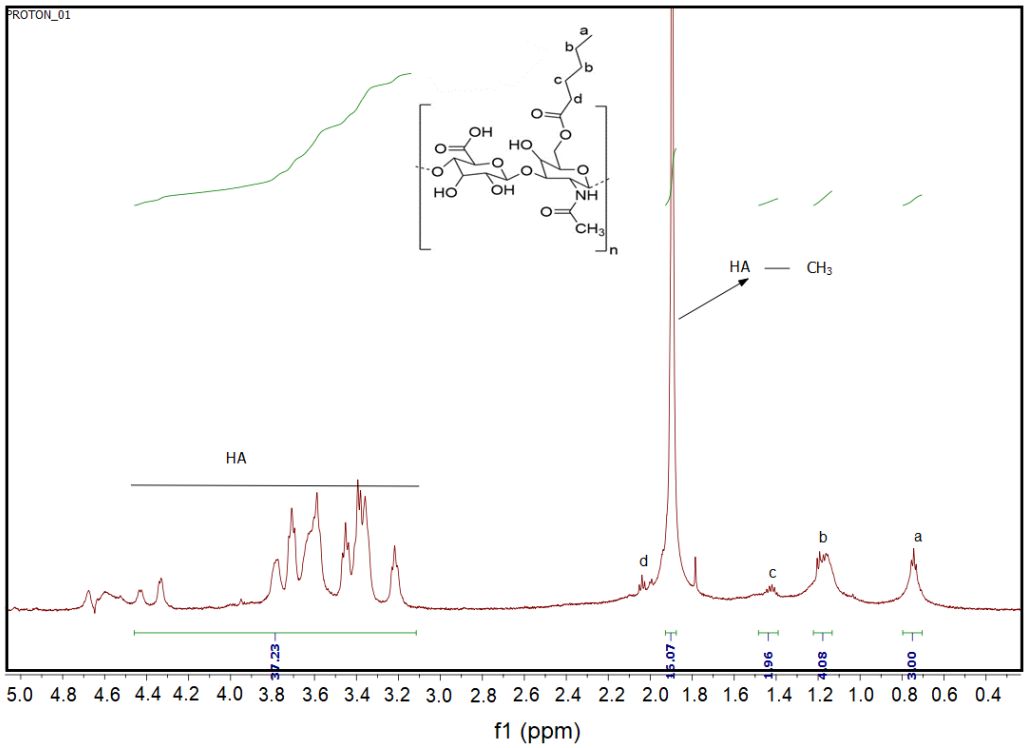

Figure 3.3 ^{1}H NMR spectrum of HA-HEX

3.1.3 DSC Analysis

Differential scanning calorimetry (DSC) is a useful technique to determine any physical change of materials accompanied by heat release or absorption at any temperature. Therefore considering that hyaluronic acid does not show any melting behavior, the formation of HA-HEX was thermally proved by determining the melting temperature of hexanoyl groups of the HA-HEX.

Figure 3.4 shows the DSC thermogram of HA-HEX. While hyaluronic acid does not have a melting endotherm at less than 100 ºC, the endothermic peak observed at nearly 50 ºC was due to the melting of hexanoyl groups, which consist of six carbon and they make weak crystalline structure resulted in showing relatively low melting temperature. The endotherm over this temperature was due to the elimination of free water present in hyaluronic acid structure. This was reported as temperature range between 80 ºC and 108 ºC [19, 20]. On the other hand, as reported in the literature the endothermic decomposition peak at 240 ºC was assigned to the thermal decomposition of hyaluronic acid [20].

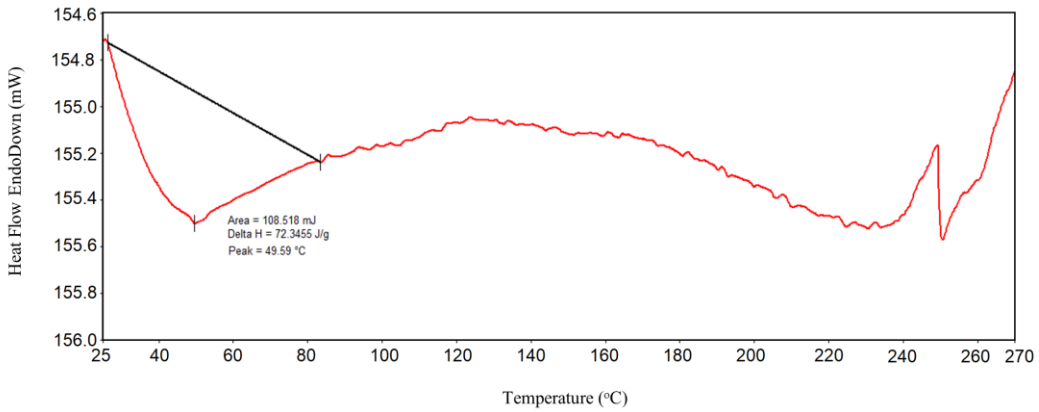

Figure 3.4 DSC thermogram of HA-HEX copolymer

3.1.4 SEM

Scanning electron microscopy (SEM) is a useful technique to perform shape and size analysis of nanoparticles. The SEM images of the nanoparticles before and after drug loading are given in Figure ***3.5***. Before SEM analysis, the particles were isolated from the dispersion using centrifugation and following lyophilization under vacuum at -60ºC. The isolated creamy samples were applied on the carbon sheet and their SEM images were recorded. As seen in Figure ***3.5***.a, the unloaded particles were spherical in shape but partially embedded into the HA-HEX matrix. They had a smooth surface with nearly uniform particle distribution and an average size of 400 nm. It has been reported in a previous study [21], that it is possible to obtain a more uniform particle distribution with ultracentrifugation. As seen from Figure ***3.5***.b., after drug loading, the HA-HEX particles had an uneven and deformed particle shape with larger particle sizes, indicating the successful drug loading.

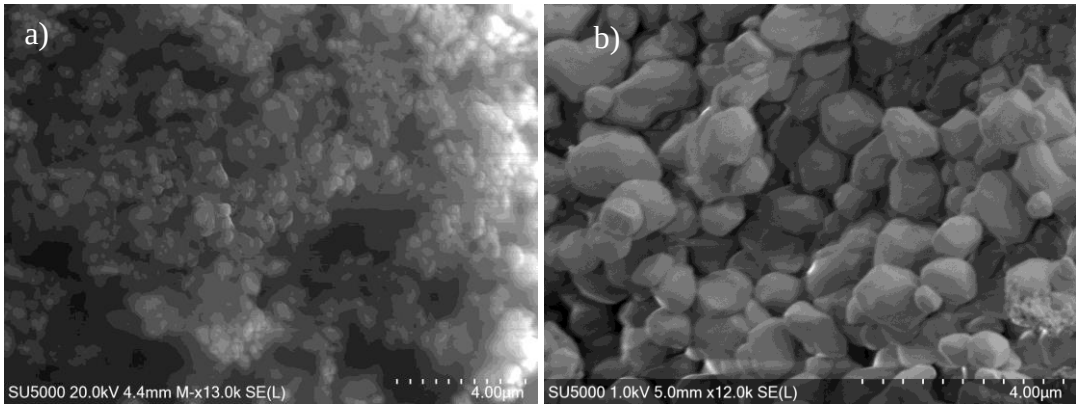

Figure 3.5 SEM image of drug unloaded and loaded HA-HEX particles taken at different acceleration voltage and magnification. a) unloaded particles at 13 K magnification and 20 KV. b) drug loaded particles at 12 K magnification and 5 KV.

3.1.5 Determination of Drug Release Profile of HA-HEX nanoparticles

Drug release characteristics of 5-ASA from the nano/micro particles was determined in PBS (pH 7.4) at 37ºC using UV-VIS spectroscopy. The amount drug released from the micelles was calculated using the previously prepared calibration curve, which was expressed according to the following equations:

$$C_t = 21.02 \times (abs) - 0.0042 \qquad R^2 = 0{,}9999 \qquad (3.1)$$

$$Drug\ release\ \% = \frac{C_t \times V}{m_i} \times 100 \qquad (3.2)$$

In these equations, (abs) being the absorption value measured with UV Spectrophotometer, C_t the respective concentration at the time of measurement, V the volume of the PBS solution in the tube (45 mL) and m_i the initial weight of drug within the micelles.

Due to the losses caused by the analyses, the final volume of the solution in the tube was measured as 43 mL. Thus, the volume was adjusted on the calculations, accordingly. (Figure ***3.6***)

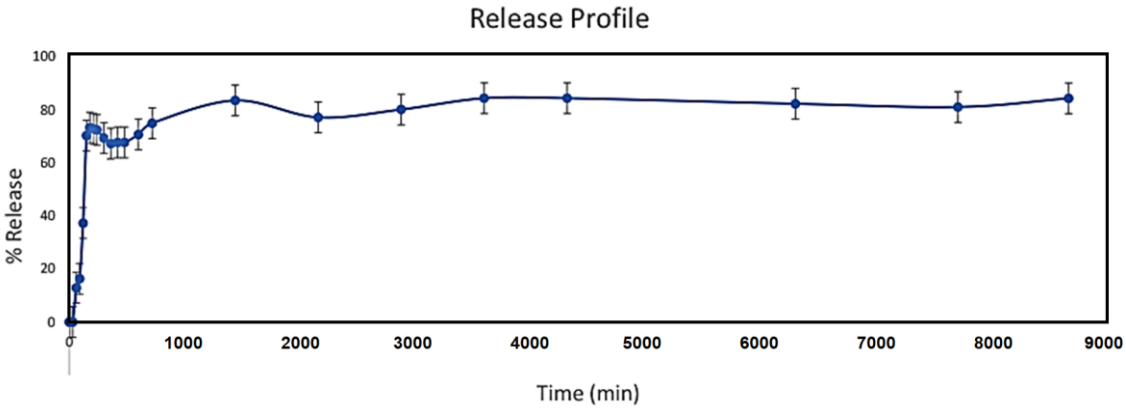

Figure 3.6 Drug release profile

As seen in Figure ***3.6***, while within 3 hours, nearly 84% of 5-ASA was released from the micelles, 16% remained. The standard deviation was calculated automatically using excel. Four different mathematical modelling was implemented and the best model for the release profile was found to be "First Order" with an R^2=0.3831 (Figure 3.7).

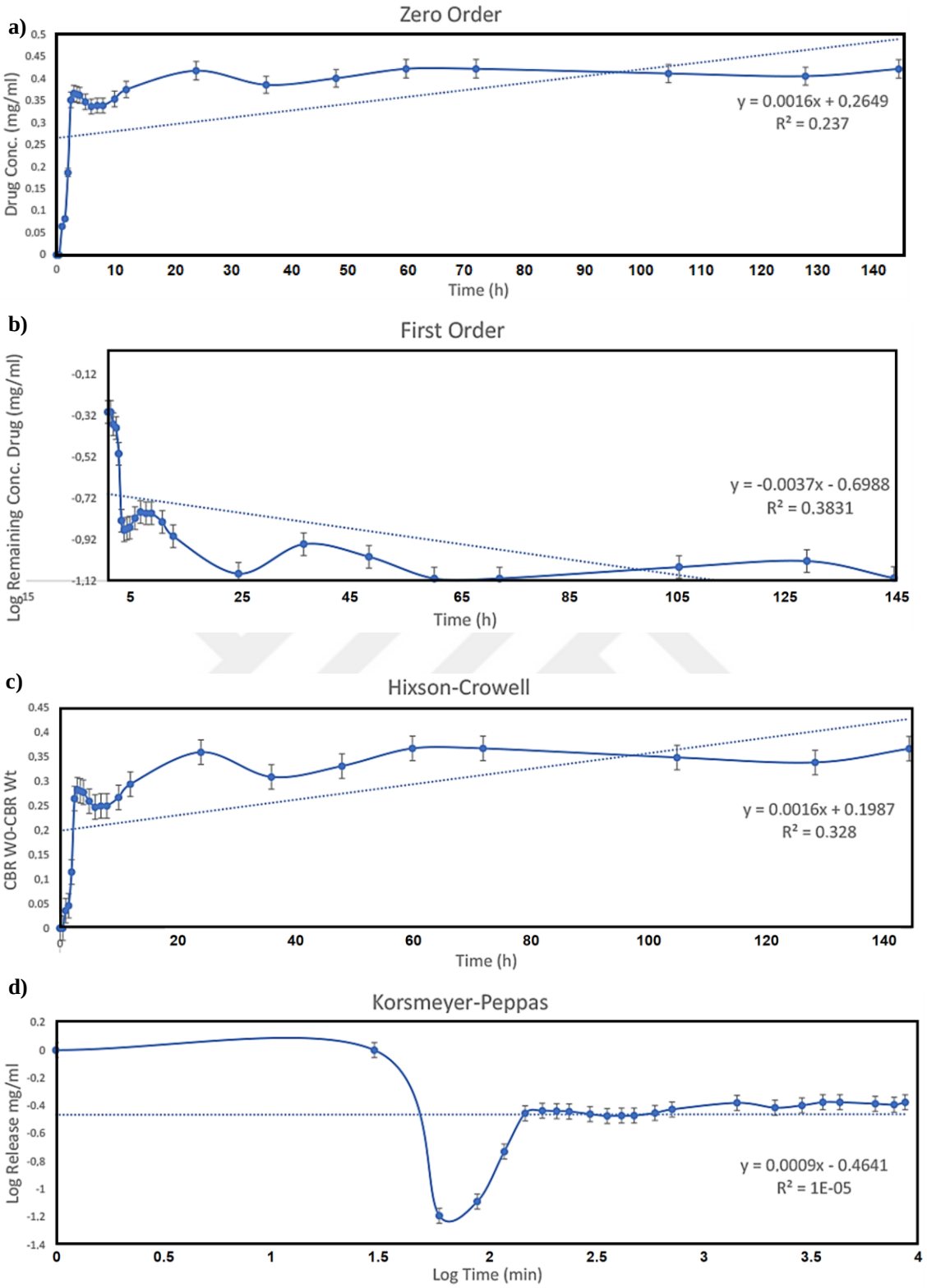

Figure 3.7 Mathematical modeling of drug release profile a) Zero order, R^2=0.237 b) First order, R^2=0.3831 c) Hixson-Crowell, R^2=0.328 d) Korsmeyer-Peppas, R^2=10^{-5}

At the end of the drug-release process, the micelle solution was measured as 3.98 mL. The missing 1.02 mL (from 5 mL) is believed to be resulting from both slight evaporation and PBS solution passing through the membrane into the tube.

The drug amount remaining in the micelles was analysed using UV Spectrophotometer by dissolving the micelles in DMF. For this, 2 mL of DMF was added in 1.5mL of the micelle solution from the membrane cap. The calibration curve equation of 5-ASA in 1.5:2 PBS:DMF (3.3 – 3.5) was used for the calculation.

$$C = 0.0668 \times (abs) + 0.0011 \qquad R^2 = 0.9998 \qquad (3.3)$$

$$m = C \times v \qquad (3.4)$$

$$Remaining\ drug\ \% = \frac{m}{m_i} \times 100 \qquad (3.5)$$

Where (abs) is the measured absorbance, C is the calculated concentration of remaining drug, v is the volume of micelle solution (5 mL), m is the weight of the measured drug, and m_i the initial weight of drug within the micelles.

The remaining drug percentage was found to be 16 %, which was conforming the released percentage seen on the drug release profile.

4. CONCLUSIONS

The reaction between hyaluronic acid and hexanoic anhydride successfully synthesized the hexanoyl hyaluronic acid (HA-HEX). The spectroscopic and thermal characterization of the synthesized amphiphilic molecule was performed. Micro/nanoparticle dispersion was prepared from synthesized HA-HEX, and the controlled release of 5-amino salicylic acid (5-ASA) from them was studied. 5-ASA is essential for treating mild to moderately severe inflammatory bowel disease, including ulcerative colitis and Crohn's disease. The drug release studies were performed in PBS at 37 ºC using UV-VIS spectroscopy. The amount of drug released from the particles was calculated using the previously prepared concentration-absorbance calibration curve. The average size of the drug-loaded particles was higher than that of the unloaded micelles, as observed from the SEM images. From the drug release profile, it was observed that 84% of 5-ASA was released within 3 hours, and 16 % of it remained. The amount of the remained 5-ASA was determined by bursting the micelles in PBS/DMF mixture followed by UV-VIS measurements. The synthesized novel amphiphilic hexanoyl hyaluronic acid and the prepared nano/micro micelles are expected to be highly efficient and non-toxic solutions with a low-cost solution on drug delivery systems. The stability, in-vivo compatibility, and biodegradation kinetics should also be studied to enable their use for actual treatment. Additionally, that should be followed with the scale-up study to overcome challenges such as high hydrophilicity and high viscosity.

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