

Ain Shams University
Faculty of Pharmacy
Department of Pharmaceutics and Industrial Pharmacy

Lipid Polymer Hybrid Nanoparticles as an Efficient Hepatic Drug Delivery System

Thesis Submitted in Partial Fulfilment of the Requirements for the Master Degree in Pharmaceutical Sciences

(Pharmaceutical Technology)

By

Mohamed Hamdi Antar Amira

Bachelor of Pharmaceutical Sciences, 2008, Al-Azhar University

Under the supervision of

Prof. Gehanne Abdel Samie Awad

Professor of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Ain Shams University

Dr Enas Mostafa Elmowafy

Dr Hend Mohamed Abdel-Bar

Associate Professor of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Ain Shams University Associate Professor of Pharmaceutics, Faculty of Pharmacy, University of Sadat City

Cairo

2019



جامعة عين شمس كلية الصيدلة قسم الصيدلانيات و الصيدلة الصناعية

جسیمات نانویه بولیمریه دهنیه هجینه کنظام توصیل دوائی فعال للکبد

رسالة مقدمة من الصيدلي / محمد حمدي عنتر عميره

مقدمة للحصول على درجة الملچيستير في العلوم الصيدلية (التكنولوجيا الصيدلية)

تحت إشراف

أرد/ چيهان عبد السميع عوض استاذ الصيدلانيات و الصيدله الصناعيه ، كلية الصيدلة ، جامعة عين شمس

د/ هند محمد عبد البر

أستاذ مساعد بقسم الصيدلانيات ، كلية الصيدلة ، جامعة مدينة السادات د/ ايناس مصطفى محمد الموافى

أستاذ مساعد بقسم الصيدلانيات و الصيدله الصناعيه، كلية الصيدلة ، جامعة عين شمس

 $(Y \cdot 19)$

رسم الله الرحمن الرحيم (وَكَذَٰلِكَ مَكُنّا لِيُوسَهُ فِي الْأَرْضِ وَلِنْعَلِّمَهُ مِن تَأْوِيلِ الْأَمَا حِيثِ وَاللَّهُ عَالِبَةً عَلَىٰ أَمْرِهِ وَلَكِنَّ أَكْثَرَ النَّاسِ لا يَعْلَمُونَ) سورة يوسف (21)

Acknowledgment

Praise is to **ALLAH**, the most merciful and most gracious, by the help of whom this work has been completed.

I would first like to thank my thesis supervisor **Prof. Dr Gehanne Abdel Samie Awad**, Professor of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Ain Shams University. The door to **Prof. Dr Gehanne** office was always open whenever I ran into a trouble spot or had a question about my research or writing. She consistently allowed this paper to be my own work, but steered me in the right direction whenever she thought I needed it.

I have to record my thankfulness to **Dr Enas Elmwafy**, Associate Professor of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Ain Shams University for her great efforts, keen supervision, valuable guidance, caring, patience, warm advices, continuous enforcement and generous support.

I would like to express my sincere gratitude to my advisor **Dr Hend Mohamed Abdel-Bar**, Associate Professor of Pharmaceutics and Industrial
Pharmacy, Faculty of Pharmacy, University of Sadat City for the continuous
support of my master study and research, for her patience, motivation,
enthusiasm, and immense knowledge. Her guidance helped me in all the time of
research and writing of this thesis. I could not have imagined having a better
advisor and mentor for this study.

I am also grateful to **Prof. Dr Khuloud Al-Jamal,** Professor in Drug Delivery and Nanomedicine, Institute of Pharmaceutical Science, Department of Pharmacy & Forensic Science, King's College London, UK for her kind cooperation in conducting cellular uptake, In vitro cytotoxicity and In vivo imaging and organ biodistribution investigations incorporated in this study.

Finally, I must express my very profound gratitude to my beloved father, my brother **Emad** and my sisters for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

THANK YOU.

MOHAMED HAMDI EMERA
2019

Dedicated To The Soul of my Mother, and my Sister Nehal.



List of Contents

Title	Page
List of Tables	iv
List of Figures	vii
List of Abbreviations	xi
Abstract	xv
General Introduction	1
Scope of Work	12
Chapter I: Preparation and Characterization of Entecavir Lipid Polyme	r Hybrid
Nanoparticles.	
Introduction	15
Methodology	22
Materials and Equipment	22
HPLC assay of entecavir	24
Preparation of optimized ELPH (OELPH)	25
In vitro characterization of the prepared OELPH	30
Statistical analysis	33
Results and Discussion	34
Analytical assay of E	34
Preparation of OELPH	39
Complete characterization of the selected OELPH	53
Effect of different lipids and lipid combinations on OELPH	56
Effect of Vitamin E coat on E GMS LPH	65
Characterization of the selected EELPH	70
Conclusions	75



Introduction 77 Methodology 83 Materials and Equipment 83 Synthesis of E hyaluronate conjugate (E-HA) 84 Characterization of E-HA conjugate Preparation and optimization of vitamin E coated E-HA LPH (EE-HA LPH) 85 In vitro characterization of the prepared EE-HA LPH 88 Statistical Analysis 89 Results and Discussion 90 Synthesis of E hyaluronate (E-HA) conjugate 90 Characterization of E-HA conjugate 92 Preparation of EE-HALPH 99 Physicochemical characterization of the optimized EE-HA LPH 108 Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology 124 Materials and Equipment 124 Preparation of florescence labelled E LPH 126 Statistical Analysis 129	Chapter II: Preparation and Characterization of Entecavir-Hyalurone	ate conjugate
Methodology 83 Materials and Equipment 83 Synthesis of E hyaluronate conjugate (E-HA) 84 Characterization of E-HA conjugate Preparation and optimization of vitamin E coated E-HA LPH (EE-HA LPH) 86 In vitro characterization of the prepared EE-HA LPH 88 Statistical Analysis 89 Results and Discussion 90 Synthesis of E hyaluronate (E-HA) conjugate 90 Characterization of E-HA conjugate 92 Preparation of EE-HALPH 99 Physicochemical characterization of the optimized EE-HA LPH 108 Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology 124 Materials and Equipment 124 Preparation of florescence labelled E LPH 126 Statistical Analysis 129	Lipid Polymer Hybrid Nanoparticles	
Materials and Equipment Synthesis of E hyaluronate conjugate (E-HA) Characterization of E-HA conjugate Preparation and optimization of vitamin E coated E-HA LPH (EE-HA LPH) In vitro characterization of the prepared EE-HA LPH Statistical Analysis Results and Discussion Synthesis of E hyaluronate (E-HA) conjugate Characterization of E-HA conjugate Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology Materials and Equipment Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs Statistical Analysis 129	Introduction	77
Synthesis of E hyaluronate conjugate (E-HA) Characterization of E-HA conjugate Preparation and optimization of vitamin E coated E-HA LPH (EE-HA LPH) In vitro characterization of the prepared EE-HA LPH Statistical Analysis Results and Discussion Synthesis of E hyaluronate (E-HA) conjugate Characterization of E-HA conjugate Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH 108 Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology Materials and Equipment 124 Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs Statistical Analysis 129	Methodology	83
Characterization of E-HA conjugate Preparation and optimization of vitamin E coated E-HA LPH (EE-HA LPH) In vitro characterization of the prepared EE-HA LPH Statistical Analysis Results and Discussion Synthesis of E hyaluronate (E-HA) conjugate Characterization of E-HA conjugate Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology Materials and Equipment Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs Statistical Analysis 129	Materials and Equipment	83
Preparation and optimization of vitamin E coated E-HA LPH (EE-HA LPH) In vitro characterization of the prepared EE-HA LPH Statistical Analysis Results and Discussion Synthesis of E hyaluronate (E-HA) conjugate Preparation of E-HA conjugate Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology Materials and Equipment Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs 126 Statistical Analysis 129	Synthesis of E hyaluronate conjugate (E-HA)	84
LPH) In vitro characterization of the prepared EE-HA LPH 88 Statistical Analysis Results and Discussion Synthesis of E hyaluronate (E-HA) conjugate 90 Characterization of E-HA conjugate Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology Materials and Equipment Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs Statistical Analysis 129	Characterization of E-HA conjugate	84
Statistical Analysis 89 Results and Discussion 90 Synthesis of E hyaluronate (E-HA) conjugate 90 Characterization of E-HA conjugate 92 Preparation of EE-HALPH 99 Physicochemical characterization of the optimized EE-HA LPH 108 Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology 124 Materials and Equipment 126 Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs 126 Statistical Analysis 129	Preparation and optimization of vitamin E coated E-HA LPH (EE-HA LPH)	86
Results and Discussion Synthesis of E hyaluronate (E-HA) conjugate Characterization of E-HA conjugate Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology Materials and Equipment 124 Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs 126 Statistical Analysis 129	In vitro characterization of the prepared EE-HA LPH	88
Synthesis of E hyaluronate (E-HA) conjugate Characterization of E-HA conjugate Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology 124 Materials and Equipment 124 Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs 126 Statistical Analysis 129	Statistical Analysis	89
Characterization of E-HA conjugate Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology Materials and Equipment Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs 126 Statistical Analysis 129	Results and Discussion	90
Characterization of E-HA conjugate Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology 124 Materials and Equipment 124 Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs Statistical Analysis 129	Synthesis of E hyaluronate (E-HA) conjugate	90
Preparation of EE-HALPH Physicochemical characterization of the optimized EE-HA LPH Conclusions 117 Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology 124 Materials and Equipment 124 Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs 126 Statistical Analysis 129	Characterization of E-HA conjugate	92
Conclusions Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction Methodology Materials and Equipment Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs Statistical Analysis 117 118 119 124 126 126 Statistical Analysis 129	Preparation of EE-HALPH	99
Chapter III. Biological Studies on Different Entecavir Lipid polymer Hybrid Nanoparticles Introduction 119 Methodology 124 Materials and Equipment 124 Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs 126 Statistical Analysis 129	Physicochemical characterization of the optimized EE-HA LPH	108
Nanoparticles Introduction 119 Methodology 124 Materials and Equipment 124 Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs 126 Statistical Analysis 129	Conclusions	117
Methodology 124 Materials and Equipment 124 Preparation of florescence labelled E LPH 126 Biological and toxicological studies of the different E LPHs 126 Statistical Analysis 129	Chapter III. Biological Studies on Different Entecavir Lipid polymer I	_∥ Hybrid
Materials and Equipment Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs Statistical Analysis 124 126 126 127 128 129	Introduction	119
Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs Statistical Analysis 126 126 127	Methodology	124
Preparation of florescence labelled E LPH Biological and toxicological studies of the different E LPHs Statistical Analysis 129	Materials and Equipment	124
Statistical Analysis 129	Preparation of florescence labelled E LPH	126
	Biological and toxicological studies of the different E LPHs	126
Results and Discussion 130	Statistical Analysis	129
	Results and Discussion	130



In vitro hemolytic assay	130
In vitro cytotoxicity studies	131
Cellular uptake studies	132
In vivo biodistribution study of fluorescence labelled EE-HA LPH	137
Organ index	141
Histological examination of major organs	142
Conclusions	144
Summary	145
References	151
Arabic Summary	1

List of Tables

Table No.	Title	Page
1	Investigated variables in the preliminary study for the fabrication of OELPH	27
2	Composition of different formulae used in the preliminary study	27
3	Levels of CPPs, CQAs and QTPP for the preparation of OELPH using the BBD	28
4	The Box Behnken design matrix for the preparation of OELPH	29
5	Recovery of E from PBS (pH 7.4) and DMF.	37
6	Intra-day accuracy and precision for E in PBS pH 7.4 and DMF	38
7	Inter-day accuracy and precision for E in PBS pH 7.4 and DMF	38
8	Characterization of OELPH prepared in the preliminary study	39
9	Experimental design matrix of the critical process parameters and the related critical quality attributes	42
10	Model summary statistics for PS (Y1)	43
11	Model summary statistics for EE % (Y2)	43
12	Quantitative factor effects on the PS expressed as the coefficients of the regression equations	44
13	ANOVA of the obtained data from BBD for the PS of OELPH and associated p-values.	45
14	Quantitative factor effects on the EE% expressed as the coefficients of the regression equations	49
15	ANOVA of the obtained data from BBD for the EE% of OELPH and associated P-values.	50
16	Experimental and predicted PSs of the OELPH	52
17	The experimental and predicted EE % of the OELPH	52
18	Physicochemical characterization of the OELPH	54
19	In vitro release data of E from the OELPH	55
20	Effect of lipid modification on the PS of OELPH	57

21	Effect of lipid modification on EE% of OELPH	59
22	Composition of selected optimum lipid-modified OELPH	59
23	The influence of lipid modification on LE % of OELPH	61
24	Solubility of E in different Lipids	62
25	In vitro release of E from different optimum CH modified LPH	63
26	In vitro release of E from different optimum GMS modified LPH	64
27	Effect of vitamin E coating on physicochemical characteristics of GMS modified LPH.	66
28	In vitro release of E from different vitamin E coated GMS modified LPH	69
29	The effect of serum incubation on optimized EELPH PS and size distribution expressed as PDI	72
30	Characteristics of EELPH at various time intervals following storage at 5°C over a period of 6 months	74
I	Overview of some examples of the successfully prepared PDC	78
II	Examples for different HA-drug conjugates	81
31	Different CPPs with their levels, CQAs and QTPP	87
32	The BBD matrix of EE-HA LPH	87
33	Optimizing the conjugation efficiency of E to HA	94
34	Experimental design matrix related to the critical quality attributes of EE-HA LPH	100
35	Model summary statistics for PS (Y1)	100
36	Model summary statistics for drug association (Y2)	101
37	ANOVA of the obtained data from BBD for the PS of EE-HA LPH and associated p-values	101
38	Quantitative factor effects, expressed as the coefficients of the regression equations for the PS	102
39	Quantitative factor effects, expressed as the coefficients of the regression equations for the association efficiency	105
40	ANOVA of the obtained data from BBD for the drug association of E-HA LPH and associated p-values	105
41	The experimental and predicted PS and drug association of the optimized EE-HA LPH	108

42	Physicochemical characterization of optimized EE-HA LPH	109
43	In vitro release data of E from optimized EE-HA LPH	110
44	The effect of serum incubation on optimized EE-HA LPH PS, size distribution expressed as PDI and ξ.	114
45	Characteristics of optimized EE-HA LPH at various time intervals following storage at 5°C for 6 months	116
46	The obtained MFI after incubating J774 macrophage cells with different DiI-labelled E LPH	134
47	Change in mice body weight post IM of the optimized EE-HA LPH and PBS over 21 days	138

List of Figures

Figure No.	Title	Page
I	The global distribution of HBV	2
II	Scheme of HBV life cycle and druggable targets	3
III	Chemical structure of E	4
IV	Intracellular uptake and metabolism of NAs	5
V	General representation of LPH	15
VI	Schematic representation of LPH preparation by two-step method	16
VII	Schematic representation of LPH preparation by one-step emulsification—solvent evaporation method. (A) Single emulsification—solvent evaporation and (B) double emulsification—solvent evaporation	18
VIII	Schematic representation of LPH preparation by one-step nanoprecipitation method	19
1	Schematic representation of OELPH preparation	26
2	Representative chromatogram of E (0.5µg/ml) in PBS (pH 7.4)	35
3	Representative chromatogram of E (0.5 μ g/ml) in DMF	35
4	Calibration curve of E in PBS pH7.4	35
5	Calibration curve of E in DMF	36
6	Effect of tween 80 concentration on PS and EE % of OELPH	40
7	Effect of stirring time on OELPH PS and EE %	41
8	Plots of the main effect of different significant critical process parameters on PS (Y1)	45
9	Response 3D plot for the interaction of (AC) between PLGA amount and E amount on PS (Y1)	47
10	Response 3D plot for the interaction of (BC) between LEC amount and E amount on PS (Y1)	47
11	Plots of the main effect of different significant critical process parameters on EE % (Y2)	50
12	Response 3D plot for the interaction of (AB) between PLGA amount and LEC amount on EE % (Y2)	51
13	Overlay plots depicting the design space region for the OELPH	52
14	PS Distribution of F1-LEC (A). F2-LEC (B) and F3-LEC (C)	53

15	In vitro release profile of E from the OELPH in PBS (pH 7.4) in the presence of FRS (50% v/v) at 37°C	56
16	The effect of different lipid composition modifications on PS of OELPH	58
17	The influence of lipid modification on ξ of OELPH	60
18	In vitro release of E from different optimum CH modified LPH	64
19	In vitro release of E from different GMS modified LPH	65
20	Effect of vitamin E coating on the PS of OELPH compared to unmodified and GMS modified ones	67
21	Effect of vitamin E coating on LPH ξ compared to unmodified and GMS modified ones	67
22	Effect of vitamin E coating on LPH LE% compared to unmodified and GMS modified ones	68
23	In vitro release of E from vitamin E coated GMS modified LPH	69
24	Transmission electron micrograph of the optimized EELPH	71
25	Atomic force micrograph of EELPH. (A) Planner view and (B) 3-D view	71
26	The effect of serum incubation on EELPH PS, PDI and ξ size, PDI and ξ potential	73
IX	Chemical structure of hyaluronic acid	79
X	Highlights of key features of HA derivatives as drug carrier	80
XI	Schematic representation of coupling reaction between E and HA	82
27	Schematic representation of the conjugation of E with HA	91
28	UV Spectrum of E, HA and E-HA conjugate (15 μg/mL) in PBS pH 7.4	92
29	Calibration Curve of E in PBS pH 7.4 at 254 nm	93
30	¹ H NMR spectra of E (A), HA (B) and E-HA conjugate (C)	95
31	¹³ C NMR spectra of E (A), HA (B) and E-HA conjugate (C)	97
32	Mass spectra of (A) E, (B) HA and (C) E-HA conjugate	98
33	Line plots of the main effect of different significant CPPS on PS (Y1)	102
34	Response 3D plot for the interaction of (AB) between E-HA amount and LEC-GMS on PS (Y1)	103
		_

		1
35	Response 3D plot for the interaction of (AC) between E-HA amount and stirring speed on PS (Y1)	103
36	Response 3D plot for the interaction of (BC) between LEC-GMS amount (B) and stirring speed (C) on PS (Y1)	103
37	Line plots of the main effect of different significant critical process parameters on drug association (Y2)	106
38	Response 3D plot for the interaction of (AB) between E-HA amount and LEC-GMS amount (B) on drug association (Y2)	106
39	Response 3D plot for the interaction of (AC) between E-HA amount and stirring speed on drug association (Y2)	106
40	Overlay plots depicting the design space region for the optimized EE-HALPH	107
41	PS Distribution of optimized EE-HA LPH	108
42	In vitro release profile of E from optimized EE-HA LPH and EELPH in PBS (pH 7.4) in the presence of FBS (50% v/v) at 37°C	111
43	Transmission electron micrograph of optimized EE-HA LPH	112
44	Atomic force micrograph of optimized EE-HA LPH. (A) Planner view and (B) 3-D view	112
45	The effect of serum incubation on optimized EE- HA LPH PS, PDI and ξ	115
XII	Pictorial representation of a liver lobule	119
XIII	The role of Kupffer cells in anti-viral immunity and tissue damage during HBV infection	122
XIV	Role of kupffer cells in immune regulation and viral persistence during HBV infection	123
46	The in vitro hemolysis assay of the optimized EELPH and EE-HA LPH	131
47	Cell viability assay of the optimized ELPH and EELPH (A) and E-HA LPH and EE-HA LPH (B) after incubation for 48h	132
48	In vitro intracellular uptake of the optimized EELPH and EE-HA LPH in J774 macrophage cells by confocal laser scanning microscopy	133
49	Intracellular uptake of the optimized ELPH and EELPH in J774 macrophage cells by flow cytometry	135
50	Intracellular uptake of the optimized E-HA LPH and EE-HA LPH in J774 macrophage cells by flow	136

51	Change in mice body weight post IM injection of the optimized EE-HA LPH and PBS	138
52	In vivo whole body IVIS imaging and biodistribution of the optimized DiR-labelled EE-HA LPH in BALB/c mice after IM injection	140
53	Organ index (%) post IM injection of the optimized EE-HA LPH and PBS	141
54	Histological examination of major organs after biodistribution study	143