Antiviral Peptide Nanocomplexes As Potential Therapeutics For The Treatment Of Infectious Diseases

Jinjin Zhang
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ANTIVIRAL PEPTIDE NANOCOMPLEXES AS POTENTIAL THERAPEUTICS FOR
THE TREATMENT OF INFECTIOUS DISEASES

by

Jinjin Zhang

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Pharmaceutical Sciences Graduate Program

Under the Supervision of Professor Tatiana K. Bronich

University of Nebraska Medical Center
Omaha, Nebraska

September, 2015
ACKNOWLEDGEMENTS

The past 5 years is truly a rough but delightful journey for me to explore the field of the pharmaceutical sciences. There undoubtedly are many ups and downs during this long period, which are shared by many people. At this special moment, I would like to express my sincere appreciation for all of their consistent and strong support.

First and foremost, my deepest gratitude goes to my mentor, Dr. Tatiana K. Bronich, who led me into this fascinating nanoworld. Without her constant support and guidance, it would have been impossible for me to finish my research project. As a dedicated scientist, an inspiring mentor, and an effective team leader, she is definitely my role model, the kind of scientist that I hope to become in the coming years. During the past 5 years, I have been constantly inspired by her hard work, broad knowledge, critical thinking, and creativity. I enjoyed every single discussion between us, which has provided me both professional and personal growth at the same time. From her, I have learned all the fundamental principles in polymer therapeutics as well as their application to address the practical challenges in human diseases. There have been several moments that I felt so lost in my project, but she is always there to guide me with sharp comments and innovative ideas. I have been benefitted so much from her rigorous and instructive training and this benefit will definitely last throughout my whole career. Apart from the scientific training, she has also educated me in communication and presentation skills, working in a highly organized style, and goals setting in professional life. It has been my great pleasure to work under her.
I would like to extend my appreciation to my supervisory committee: Dr. Larisa Y. Poluektova, Dr. Natalia A. Osna, and Dr. Dong Wang for all their continuous guidance and unselfish support to my research work. Dr. Poluektova and Dr. Osna are more like my co-mentors, and both of them have played a major role in my research work as presented in this thesis. With their guidance, we have co-authored several important papers and they have also contributed significantly to my overall development. Dr. Poluektova has shared many invaluable experiences with me regarding the experiment design as well as data analysis and interpretation. With her expertise in the development of humanized mice models, we are able to finish the animal studies smoothly and successfully. I also would like to sincerely thank Dr. Osna for her generous assistance and guidance in all HCV-related cell-based studies. Her patience, enthusiasm, and persistent encouragement always motivated me to move through the struggles with my research. Sincere thanks to Dr. Wang for admitting me to this graduate program and also all his constructive suggestions and questions during my committee meetings.

I am also grateful to other faculty members for their kind support and help. Dr. Jered C. Garrison and his team have helped me to finish radiolabelling of peptide and the animal biodistribution studies. It is truly a pleasure to work with Dr. Garrison and to get inspired by his ideas and dedication to science. Many thanks to Dr. Samuel Sanderson for the help with peptide purification and Dr. Irine Khutsishvili for the assistance in CD spectroscopy.

In graduate school, one of the luckiest things that have happened to me was to join the nanomedicine group together with Swapnil S. Desale, who has always been a continuous source of support and inspiration for me. Starting from learning polymer synthesis together in this lab,
we have witnessed the growth of each other during the past 5 years. I feel extremely grateful to him both for cheering me up through the difficult times and for celebrating with me for every single achievement. Even we are leaving the graduate school soon, our friendship will only become stronger.

I also would like to thank my previous and present Nanomedicine group members Dr. Hardeep Singh Oberoi, Dr. Jong Oh Kim, Kruti Soni, Dr. Fan Lei, Tong Liu, Xinyuan Xi, Anya Brynskikh Boyum, Dr. Jing Tong, Dr. Zhijian He, Dr. Svetlana Romanova, Dr. Shaheen Ahmed, Hangting Hu, Dr. Xiang Yi, Dr. Chantey Morris, and many others for the kind help, friendship, and the opportunity to work with them. Many thanks to the administrative staff (Christine Allmon, Jamie Arbaugh, Keith Sutton, and Katina Winters) for their administration support. I would like to acknowledge the technical assistance from UNMC core facilities in my research and financial support from NIH, UNMC Program of Excellence Graduate Assistantship, and the Department of Veteran Affairs.

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In the end, I am extremely grateful to my parents for their unconditional love, encouragement, and trust in the past 26 years. They always encourage me to chase my own dream and realize my own value, which support me to get through all the difficult times and move forward in my life. Without them, I would not have finished the Ph.D. study. I love them so much
and I feel so blessed to be their child. Thank you so much.

Jinjin Zhang

August, 2015
Hepatitis C Virus (HCV) is recognized as a major burden in global public health, which can be further exacerbated by several cofactors such as human immunodeficiency virus (HIV). Currently, there is no vaccine for HCV. The emergence of potent and highly specific direct-acting antivirals (DAA) has marked a new era in HCV therapy, however, the remaining issues like affordability, genotype dependency, and potential resistance still necessitate the development of additional therapeutic approaches to be used instead or in combination with DAA.

Recently, the antiviral peptide C5A (in our studies designated as p1) and its cationic derivative p41 have been identified as potent antiviral agents. Predominantly due to the α-helicity and amphipathicity, p1 and p41 exhibit submicromolar virocidal effect against HCV, other members of the Flaviviridae, and HIV. However, the clinical translation of peptide drugs is impeded by the susceptibility to degradation, and, particularly for the cationic peptide p41, unfavorable cytotoxicity. To address these limitations, we propose to use the polymeric materials as delivery vehicles for peptide stabilization and reduced toxicity associated with the positive charge.

Antiviral Peptide Nanocomplexes (APN) were developed based on the electrostatic coupling of cationic p41 and biodegradable anionic poly(amino acid)-based block copolymers, and they
have been extensively characterized with a variety of analytical and biophysical techniques. The immobilization of the peptide into APN led to improved stability, reduced cytotoxicity and unaltered anti-HIV/HCV potency of the peptides *in vitro*. Moreover, *in vivo* APN were able to decrease the HIV-1 viral load in mice model. By further modifying the APN surface with liver-targeting ligand galactose (Gal-APN), liver-specific delivery system of p41 was developed as a more selective therapy against HCV. *In vitro*, Gal-APN displayed specific internalization in hepatoma cell lines. Even though liver-targeted and non-targeted APN displayed comparable antiviral activity, Gal-APN offered prominent advantages to prevent HCV association with lipid droplets and suppress intracellular expression of HCV proteins. Moreover, *in vivo* preferential liver accumulation of Gal-APN was revealed in the biodistribution study. The feasibility of DAA and p41 as synergistic combination against HCV was also tested by preparing a series of poly(amino acid)-based micelles loaded with DAA and p41 simultaneously.

The parental peptide p1 was incorporated into the PEG-phospholipid micelles. The formulation was prepared and characterized with multiple techniques. After p1 is encapsulated into micelles, the stability of p1 against the proteolytic degradation was delayed, and the anti-HCV/HIV activity was preserved.

Collectively, we demonstrated that APN approach represents a promising platform for the delivery of antiviral peptide p41 with enhanced stability and reduced toxicity, and its liver-targeted delivery can be achieved by modifying the APN surface with hepatocyte-specific ligand galactose. The encapsulation of DAA and p41 in poly(amino acid)-based micelles may provide potential opportunities as synergistic anti-HCV therapy. We have also developed the
p1-loaded PEG-phospholipid micelles with enhanced stability and preserved antiviral activity. These results demonstrate the power of polymer-based nanoparticulate systems for antiviral peptide delivery and their potential for bench-to-bedside translation.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>%ID/g</td>
<td>Percentage of injected dose per gram</td>
</tr>
<tr>
<td>$^{177}$Lu</td>
<td>Lutetium-177</td>
</tr>
<tr>
<td>$^1$H-NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>APN</td>
<td>Antiviral Peptide Nanocomplexes</td>
</tr>
<tr>
<td>apoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>apoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ASGP-R</td>
<td>Asialoglycoprotein receptor</td>
</tr>
<tr>
<td>AUC</td>
<td>Area-under-the-curve</td>
</tr>
<tr>
<td>BICs</td>
<td>Block ionomer complexes</td>
</tr>
<tr>
<td>BLE-NCA</td>
<td>$\gamma$-benzyl L-glutamate-N-carboxyhydrate</td>
</tr>
<tr>
<td>cART</td>
<td>Combined antiretroviral therapy</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHP</td>
<td>Cholesterol-bearing pullulans</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>Cy5</td>
<td>Cyanine 5</td>
</tr>
<tr>
<td>DAA</td>
<td>Direct-acting antivirals</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCC</td>
<td>$N,N′$-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>D_{eff}</td>
<td>Effective hydrodynamic diameter</td>
</tr>
</tbody>
</table>
DIPEA $N,N$-diisopropylethylamine
DLS Dynamic light scattering
DMAP 4-dimethylaminopyridine
DMEM Dulbecco’s modified Eagle's medium
DMF Dimethylformamide
DMSO Dimethyl sulfoxide
EDC $N$-(3-Dimethylaminopropyl)-$N'$-ethylcarbodiimide hydrochloride
EEA1 Early endosome antigen 1
ELISA Enzyme-linked immunosorbent assay
ER Endoplasmic reticulum
FACS Fluorescence-activated cell sorting
FDA Food and Drug Administration
Fmoc 9-Fluorenylmethyloxycarbonyl
Fmoc-Lys(Fmoc)-OH $N_α,N_ε$-di-Fmoc-L-lysine
FPLC Fast protein liquid chromatography
Gal D-galactose
Gal-APN Galactose-modified Antiviral Peptide Nanocomplexes
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GI Gastrointestinal
GPC Gel permeation chromatography
H&E Hematoxylin and eosin
HBTU $N,N,N',N'$-Tetramethyl-$O$-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
HCl Hydrochloric acid
HCV Hepatitis C Virus
HIV Human immunodeficiency virus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>hu-PBL</td>
<td>Human peripheral blood lymphocytes</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>im</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JFH-1</td>
<td>Japanese fulminant hepatitis-1</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>LC</td>
<td>Loading capacity</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte-derived macrophages</td>
</tr>
<tr>
<td>M&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Number-average molecular weight</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mPEG</td>
<td>Methoxy polyethylene glycol</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>M&lt;sub&gt;w&lt;/sub&gt;</td>
<td>Weight-average molecular weight</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>NCA</td>
<td>N-carboxyanhydride</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
</tbody>
</table>
NMP  N-methyl-2-pyrrolidone
NS5A  Non-structural protein 5A
NTR  Non-translated region
OAS-1  2'-5'-oligoadenylate synthetase 1
ORF  Open reading frame
PACA  Poly(alkyleyanoacrylate)
PBS  Phosphate buffered saline
PDI  Polydisperisty index
PEC  Polyelektrolyte complexes
PEG  Poly(ethylene glycol)
PEG_{2k}-DSPE  1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2 kDa]
PEG-b-PCL  PEG_{5k}-b-poly(ε-caprolactone)
PEG-IFN  PEGylated interferon-α
PEG-PBLE  poly(ethylene glycol)-block-poly(L-glutamic acid γ-benzyl ester)
PEG-PLD  Poly(ethylene glycol)-block-poly(α,β-aspartic acid)
PEG-PLE  poly(ethylene glycol)-block-poly(L-glutamic acid)
PEG-PLE-PLF  poly(ethylene glycol)_{5k}-block-poly(L-glutamic acid)_{30}-block-poly(L-phenylalanine)_{7}
PFA  Paraformaldehyde
PHA  Phytohemagglutinin
PIC  Polyeion complexes
PLGA  Poly(D,L-lactic-co-glycolic acid)
PVP  Poly(vinylpyrrolidone)
qPCR  Quantitative real-time PCR
RBC  Red blood cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase-high pressure liquid chromatography</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SCN-DTPA</td>
<td>S-2-(4-Isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SMA</td>
<td>Poly(styrene-co-maleic anhydride)</td>
</tr>
<tr>
<td>SVR</td>
<td>Sustained virological response</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline containing 0.1% Tween 20</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% tissue culture infectious doses</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet–visible spectroscopy</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
</tbody>
</table>
LIST OF CONTRIBUTORS

1. Chapter II- Andrea Mulvenon performed the AFM analysis and guided the trypsin digestion analysis. Edward Makarov performed the cell studies and animal studies. Jaclyn Hollinger assisted in animal studies. Dr. Sorin Luca contributed the molecular modeling of peptides. Dr. Irine Khutsishvili provided assistance in CD spectroscopy. Dr. Samuel Sanderson helped with peptide synthesis and purification.

2. Chapter III- HCV cell culture studies were performed at Nebraska-Western Iowa Health Care System with Dr. Natalia Osna. Dr. Jered Garrison guided the radiolabelling of peptide as well as the animal biodistribution studies. Susan Brusnahan, Dr. Wei Fan, Dr. Zhengyuan Zhou, Dr. Wen Shi, Wenting Zhang, and Yinnong Jia from Dr. Garrison’s lab provided help in animal biodistribution studies. Dr. Fan Lei has helped with $^1$H-NMR spectra analysis.

3. Chapter IV- HCV cell culture studies were performed at Nebraska-Western Iowa Health Care System with Dr. Natalia Osna. Dr. Svetlana Romanova synthesized the triblock copolymer. Kruti Soni assisted in the diblock copolymer synthesis.

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preparation of the manuscripts.

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CHAPTER I

INTRODUCTION

1.1 Hepatitis C Virus Infection

1.1.1 Global epidemiology and transmission

More than 185 million people worldwide are infected by Hepatitis C Virus (HCV) [1], which accounts for approximately 3% of the global population. Persistent HCV infection is the major cause of chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, etc. [2]. Globally, the HCV infection patterns display geography dependency: high prevalence (>3.5%) is found in Central and Eastern Asia, North Africa, and the Middle East; the areas of moderate prevalence include South and South East Asia, sub-Saharan Africa, Central and South America, Australasia, and Europe [3, 4]. Based on the nucleotide sequence heterogeneity of HCV, the virus is divided into 6 distinct genotypes with multiple subtypes, which creates a major challenge for the development of both vaccines and pan-genotypic drug therapies. Globally, genotype 1 was the most prevalent (46%), followed by genotype 3 (22%), genotype 2 (13%), genotype 4 (13%), genotype 6 (2%), and genotype 5 (1%) [5]. The genotype distribution is also characterized by the regional variations: genotypes 1-3 have a worldwide distribution, while genotypes 4 and 5 mainly are distributed in Africa, and genotype 6 is found in Asia [6, 7].

HCV is parentally transmitted, which most often involves exposure to contaminated needles.
or syringes. In developing countries, HCV transmission mainly results from exposure to infected blood and blood products; whereas HCV infections in the developed countries are associated with the multiple injections in drug users, receipt of transfusions before donor screening, and high-risk sexual activity [8].

1.1.2 HCV virology and life cycle

HCV is an enveloped positive-stranded RNA virus belonging to the *Flaviviridae* family. HCV particles are spherical and 40 to 100 nm in diameter [9]. The principal protein components of the virion are envelope glycoproteins E1 and E2 and core protein. E1 and E2 are anchored to lipid bilayer envelope that surrounds a nucleocapsid composed of multiple copies of core protein and RNA genome (Figure 1.1). HCV RNA genome, which is around 9.6 kb in length, is composed of a 5’ non-translated region (NTR), containing internal ribosome entry site (IRES), an open reading frame (ORF), and a 3’ NTR.
HCV only infects humans and chimpanzees, and hepatocytes are the primary target for infection. Key steps in the life cycle of HCV involve viral entry, uncoating of the genome, translation of HCV proteins, viral genome replication, and the assembly and release of virus (Figure 1.2). The HCV entry is initiated by the binding of viral particles to a series of receptors on hepatocytes surface, including: 1) attachment receptors: low-density-lipoprotein receptor and glycosaminoglycans, and 2) particle entry receptors: CD81, scavenger receptor class B member 1, claudin 1, occludin, and the cholesterol absorption receptor Niemann-Pick C1-like 1 [10]. Followed by the clathrin-mediated endocytosis [11], fusion of the virion envelope with endosomal membranes delivers the genome to the cytoplasm. With the help of ribosome and mediated by IRES, the ORF region of viral genome is translated into a precursor polyprotein of ~3000 amino acids, which is then cleaved by cellular and viral proteases yielding structural proteins (core, E1, and E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and

Figure 1.1 Model structure of HCV. Adapted with permission from Ref. [10].
The functions of HCV proteins are summarized in Table 1.1.

**Table 1.1** HCV proteins and their functions in the viral life cycle.

<table>
<thead>
<tr>
<th>HCV proteins</th>
<th>Main function</th>
<th>HCV proteins</th>
<th>Main function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>Nucleocapsid</td>
<td>NS4A</td>
<td>NS3/4 proteinase co-factor</td>
</tr>
<tr>
<td>E1</td>
<td>Envelope protein</td>
<td>NS4B</td>
<td>NS5A phosphorylation induction of ER “membranous web”</td>
</tr>
<tr>
<td>E2</td>
<td>Envelope protein</td>
<td>NS5A</td>
<td>inhibition of IFN-α?? inhibition of apoptosis??</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Part of replication complex</td>
</tr>
<tr>
<td>p7</td>
<td>Ion channel</td>
<td>NS5B</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>NS2</td>
<td>Component of NS2/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>proteinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS3</td>
<td>NS2/3 proteinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS3/4 proteinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NTPase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNA helicase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNA binding</td>
<td></td>
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</tr>
</tbody>
</table>
Viral replication takes place on a membranous web composed of viral non-structural proteins (NS3/4A, NS4B, NS5A, and NS5B) and host cell proteins termed as “replication complex”, which is located close to the perinuclear membranes. With the synthesized negative-strand RNA as a template and NS5B as the key enzyme, a large amount of positive-strand RNA is created. Lipid droplets are reported to play an essential role in viral assembly and release [12, 13]. The localization of core proteins to lipid droplets recruits non-structural proteins and replication complex to lipid droplets-associated membranes. Therefore, the assembly of HCV viral particles takes place at the junction between the core-coated lipid droplets and replication-complex-rich endoplasmic reticulum (ER) membranes, which allows the multiplied viral RNA to interact with core protein forming the nucleocapsids [14]. Subsequently, the capsids are enclosed by an endoplasmic membrane containing the envelope proteins E1 and E2 and are then released into ER lumen side. At the end, nascent viral particles transit through the p7-buffered secretory pathway, where they undergo maturation and acquire low density before exocytosed to the cell surface. This step is reported to be dependent on the assembly and secretion of very-low density lipoproteins (VLDLs), which export cholesterol and triglyceride from hepatocytes. It has been demonstrated that excreted HCV viral particles are combined with apolipoproteins such as apoE and apoB [15].
Figure 1.2 HCV viral life cycle (a) virus binding and internalization, (b) cytoplasmic release and uncoating, (c) IRES-mediated translation and polyprotein processing, (d) RNA replication, (e) packaging and assembly, (f) virion maturation and release. HCV RNA replication occurs in the membranous web. Adapted from [16] with permission.
1.1.3 HCV-accelerating cofactors

1.1.3.1 HIV/HCV co-infection

Since HIV and HCV share the common routes of viral transmission, 15%-30% of HIV-infected patients are also HCV-positive [17]. HIV/HCV co-infection is linked to higher rates of liver fibrosis, cirrhosis, hepatocellular carcinoma, and overall mortality [18]. Since the advent of combined antiretroviral therapy (cART) has dramatically reduced the HIV-related deaths, the complications of liver diseases become the leading cause of morbidity and mortality in HIV/HCV co-infected patients. Currently, among HIV-infected patients, up to 15% of deaths are due to liver-related mortality. Of these liver failures, 66% were attributed to HCV [18]. Even though cART can significantly prolong the life expectancy HIV patients, co-infected patients exhibit reduced effectiveness of treatment and slower CD4+ T-lymphocyte recovery as compared with HIV mono-infected patients [19]. Additionally, the co-infected patients who receive cART medication also suffer from increased hepatotoxicity. Vice versa, the reduced rate of spontaneous HCV RNA clearance is observed by HIV/HCV co-infection. Before 2011, the standard-of-care for HIV/HCV co-infection is limited to the combination of interferon (IFN) and ribavirin. However, their therapeutic efficacy suffers from the limited virological response as well as severe adverse effects, including fevers, sweats, edema, thrombocytopenia, and depression [20]. This challenge has been overcome with the approval of direct acting antivirals (DAA), which offer significantly improved sustained virological response, IFN-free options, and shorter treatment durations [21]. However, the careful consideration should be taken such as drug-drug interactions.
of DAA with existing cART therapies, potential drug toxicities, and other unknown efficacy [22].

1.1.3.2 Alcohol consumption

Alcohol exposure worsens the course and outcomes of HCV infection. HCV prevalence is 7-10 fold higher in alcoholics than it is in the general population [23], making the combination of HCV infection and alcohol abuse a very common cause of chronic liver disease. Alcohol consumption in HCV infected patients accelerates the liver injury, leading to rapid progression to fibrosis, cirrhosis, and even hepatocellular carcinoma. However, the mechanisms of this synergistic effect are poorly understood [24]. Alcohol also reduces responsiveness of HCV patients to antiviral treatments since only 7% of heavy drinking HCV patients respond to IFN therapy. Both HCV and alcohol suppress the innate immunity in hepatocytes, the primary sites for both viral replication and ethanol metabolism [25]. Thus, there is a high chance that synergistic effect of HCV and alcohol on innate immunity contributes to HCV spread and progressive liver injury.

1.1.4 Antiviral therapy

The major goal of HCV treatment is to achieve the sustained virological response (SVR), which is defined as the persistent absence of HCV RNA in serum 6 months after completing the antiviral treatment [26]. The treatment approach and duration should be accordingly tailored based on various factors, including HCV genotypes, viral load, degree of liver damage, patients’ age and tolerance, immunosuppression, etc.
1.1.4.1 Conventional IFN-based therapy

Until 2011, the combination of PEG-IFN and ribavirin was regarded as the standard of care for HCV treatment regardless of genotypes. The antiviral activity of IFN-α is mainly driven by the activation of innate antiviral immune response: the upregulation of a series of interferon stimulated genes (ISGs) rapidly establishes the antiviral state inside the cells [27]. Unfortunately, the IFN-α monotherapy shows the marginal SVR rates (6%-12% after 6-month treatment) with the relapse frequently observed [28]. To improve the IFN-α circulation half-life, pharmacokinetic profile, and the SVR rate, poly(ethylene glycol) (PEG) was covalently attached to the IFN-α (PEG-IFN). Currently, two types of PEG-IFN products are available on the market: 1) PEG-IFN-α-2a (PEGASYS®; Roche), with a 40 kDa single-branched PEG attached via lysine, and 2) PEG-IFN-α-2b (PEG-INTRON®; Schering-Plough, Kenilworth, New Jersey, USA) with a 12 kDa mono-methoxy PEG conjugated on the histidine residue (Figure 1.3) [29]. The therapeutic outcome is further improved by adding the orally active synthetic guanosine analogue ribavirin with a broad spectrum of antiviral activity, as a synergistic therapy with PEG-IFN. With this combination, the SVR rate among patients with genotype 1 increased to 42-46%, and 76–80% SVR is achieved for genotype 2 and 3 [30, 31]. In spite of improved efficacy, this also means that 40–50% of patients do not respond to the treatment. In addition, the treatment is quite expensive: a 48-week course of standard therapy costs over $20,000. The treatment is also accompanied with severe side effects including fatigue, influenza-like symptoms, hematologic abnormalities, and neuropsychiatric symptoms [32]. Overall, these hurdles necessitate the development of alternative
therapeutic strategies for HCV.

**Figure 1.3** The structure of PEGylated IFN-α-2a (PEGASYS®) and IFN-α-2b (PEG-INTRON®).

### 1.1.4.2 Direct-acting antivirals (DAA) therapy

Since 2011, a more insightful understanding of the HCV genome and proteins greatly facilitates the development of various DAA that specifically target different steps within HCV viral replication cycle. They exhibit high potency, less dependency on host characteristics, all oral regimen, and improved tolerability. Overall, based on the mechanism of action and therapeutic target, DAA are classified into four groups: NS3/4A protease inhibitors (e.g., telaprevir, boceprevir, simeprevir), NS5B nucleoside polymerase inhibitors (e.g., sofosbuvir, mericibabine), NS5B non-nucleoside polymerase inhibitors (e.g., dasabuvir), and NS5A inhibitors (e.g., daclatasvir, ledipasvir). As a milestone, in 2013 FDA approved the combination of sofosbuvir (Sovaldi®, Gilead Sciences) with ribavirin as the oral combination therapy against HCV genotypes 2 and 3 [33] and the triple combination of sofosbuvir with ribavirin and PEG-IFN for
treating HCV genotypes 1 and 4 [34], which led to shorter treatment duration and superior SVR rates. This breakthrough was followed by the approval of the first IFN- and ribavirin-free, once-daily and a single-tablet regimen composed of sofosbuvir and ledipasvir (Harvoni™, Gilead Sciences), which resulted in SVR of 93%-99% in HCV genotype 1 patients [35]. Undoubtedly, the introduction of DAA-based treatment will resolve the burden of HCV-related cirrhosis, hepatocellular carcinoma, etc. Nevertheless, there are still several practical hurdles which should be considered, including: 1) extremely high cost of the treatment; 2) genotype dependency; 3) possible long-term resistance to DAA therapy due to the drug-induced viral mutations.

1.1.4.3 Peptide-based therapy

As an additional or alternative class of therapeutics to the IFN-based and DAA-based therapy, various antiviral peptides have been identified. They target different stages of HCV viral life cycle due to the distinctive antiviral mechanisms. The HCV viral particle entry can be directly blocked by a peptide derived from the human apolipoprotein E (apoE), which comprised a receptor binding fragment and a lipid binding fragment of apoE [36]. It was demonstrated that the submicromolar anti-HCV potency of the peptide is dependent on the peptide length and sequence and is associated with its lipid binding affinity. In addition, a peptide-based vaccine named as IC41 has been developed. It contains 5 synthetic peptides from HCV core, NS3, and NS4 proteins that are conserved across HCV genotypes 1 and 2, with the adjuvant poly(L-arginine) [37]. As shown from the phase II study, this synthetic peptide vaccine is well tolerated, however, limited immunogenicity was observed either as a monotherapy or as a combination therapy with
PEG-IFN and ribavirin [38]. A series of amphiphilic and cyclic peptides made of eight alternating D- and L-α-amino acids, which specifically blocked HCV entry at a post-binding step, were also identified [39]. Based on the structure-activity analysis, it was suggested that antiviral activity is dependent on the intermolecular hydrogen-bond-directed self-assembly into nanotubular assemblies. Cheng et al. discovered an 18-amino-acid virocidal peptide designates as C5A, which is derived from the membrane anchor domain of the HCV nonstructural protein NS5A [40]. Due in large part to its α-helicity and amphipathicity, C5A peptide was found to be effective to inhibit both HIV [41] and HCV infections as well as infections of other human Flaviviridae members. Its antiviral mechanism includes the suppression of the ongoing infections by penetrating through the lipid compositions of the viral membranes to destroy them, and the blockade of the cell-to-cell spread of the virus. All aforementioned reports suggest that the peptide-based drugs represent a promising alternative class of therapeutics to treat the HCV infections.

However, therapeutic peptides pose multiple challenges for their delivery to the site of action [42]. First, the chemical and physical instability of therapeutic peptides remain the major obstacles [43]. Chemical instability is defined as the modification of the peptide by formation or cleavage of covalent bonds, generating new chemical entities, via hydrolysis, oxidation, racemization, isomerization, deamidation, disulfide exchange and β-elimination. Physical instability normally refers to conformation change (dimerization and further aggregation), which could potentially result in denaturation, adsorption to surfaces, aggregation, precipitation, etc. [44]. Second, the successful delivery of peptide-based drugs to the therapeutic target also suffers...
from the rapid renal clearance, reticuloendothelial system recognition, and off-target binding. Third, restricted membrane permeability and degradation in the gastrointestinal tract lead to poor oral bioavailability of most peptide drugs, which explains why the most peptide therapeutics (~75%) are injectables [45, 46].

1.2 Polymer-based vehicles for therapeutic peptide delivery

In an attempt to overcome the aforementioned restraints, polymeric materials have been extensively exploited during the past decade for peptide delivery. As was first demonstrated by Langer and Folkman, the controlled release of proteins was proved to be feasible for more than 100 days via their incorporation into synthetic polymers [47]. Since then, various polymer-based platforms have been explored as delivery vehicles for peptides and proteins including chemical modification (e.g., PEGylation) [48], nano-/micro-particulate encapsulation [49-51], etc. One classical example is insulin, which perhaps is the most studied peptide drug. To achieve the non-invasive delivery, a variety of polymers (e.g., chitosan, dextran, poly(glutamic acid), hyaluronic acid, poly(lactic acid), poly(lactide-co-glycolic acid), polycaprolactone, acrylic polymers and polyallylamine have been exploited to reduce the enzymatic degradation and increase the intestinal permeability of insulin [52-54].

Polymeric materials with highly tunable structures and physicochemical properties offer multiple remarkable advantages for the delivery of peptides, including: 1) improved peptide stability; 2) controlled and sustained release; and 3) improved pharmacokinetics/biodistribution profiles [55-58]. Overall, polymer-based vehicles are attractive carriers for peptide delivery
Aim of this review is to provide an overview of different types of polymer-based nanocarriers that have been developed for peptide delivery.

**Figure 1.4** Overview of polymer-based vehicles for peptide delivery and their advantages.

### 1.2.1 Particulate systems

Numerous studies have shown that the micro-/nano-particulate systems protect peptides from enzymatic degradation, prolong the plasma half-life, and reduce the dosing frequency. As opposed to the polymer-peptide conjugate, they do not require the chemical modification of peptide structure for encapsulation. To select the appropriate particulate system, the properties of peptide drugs such as their solubility, size and structure need to be considered. The administration route of peptide drugs also dictates the choice of the type of the carriers. This part of the review
covers several of the polymeric particulate systems and their application for peptide delivery via different administration pathways (Figure 1.5).

Figure 1.5 Particulate carrier systems for peptide delivery. (A) PLGA-based particles. (B) Polymeric capsules. (C) Polymeric micelles. (D) Polyelectrolyte complexes (left) and block ionomer complexes (right) of cationic peptide with anionic polymer. (E) Hydrogels.
1.2.1.1 PLGA-based particles

Poly(D,L-lactic-co-glycolic acid) (PLGA)-based nano-/microparticles perhaps are the most extensively explored systems for peptides delivery as these polymers are FDA approved, biodegradable, and biocompatible (Figure 1.5 A) [59-61]. The degradation of PLGA copolymers occurs mainly through the hydrolysis of ester bonds, and the rate of degradation is known to be dependent on the molar ratio of the lactic acid to glycolic acid, polymer molecular weight and end groups, as well as particle porosity [62-65]. Therefore, the release kinetics of peptides from PLGA particles can be tuned by adjusting these parameters [66]. On the other side, initial rapid release, incomplete release of native proteins/peptides due to partial degradation, and irreversible adsorption of proteins/peptides to the polymer matrix have been recognized as major issues with this system [64, 67, 68]. The hydrolysis-induced acidic microenvironment during the storage impairs the stability of the encapsulated peptides [55], and this could be addressed by blending with other stabilizer agents including polymers (e.g., cellulose, alginate, polyethyleneimine, PEG) or antacid salts (e.g., Mg(OH)$_2$, MgCO$_3$, ZnCO$_3$) during encapsulation [69, 70]. Another limitation is the recognition of injected hydrophobic PLGA particles by the reticuloendothelial system and their elimination from the blood stream. Hydrophilic polymers (e.g., poly(ethylene glycol), PEG) can be introduced to the particle surface to confer stealthiness and extended circulation half-life. Furthermore, the modification of PLGA particles with ligands capable of recognition of cell-specific surface receptors can be utilized for targeted delivery of bioactive molecules to specific organs [71, 72].
In general, three approaches are used to produce peptides-loaded PLGA particles: double emulsion (w/o/w) technique, phase separation methods and spray drying methods [73]. Notably, peptide drugs are less affected by harsh manufacturing conditions compared to large proteins. Various peptide hormones have been loaded in PLGA microparticles, which act as a depot displaying sustained release over an extended period of time, thereby reducing the dose and the frequency of administration. This is evidenced by numerous PLGA microparticulate products on the market [74] (Table 1.2), which are usually administered subcutaneously or intramuscularly. Lupron Depot® is the first approved injectable PLGA microparticles for sustained delivery of leuprolide (luteinizing-hormone releasing-hormone analogue). To treat advanced prostate cancer, this product is available in 7.5 mg (1 month release), 22.5 mg (3 month release), 30 mg (4 months release), and 45 mg (6 months release). The 14 kDa PLGA (lactic acid to glycolic acid ratio 75:25) is used for the 1 month release product, and the release period is further extended to 3, 4, and 6 months by using the poly(lactic acid) with the molecular weight of 12 kDa-18 kDa [75]. The product consists of lyophilized microparticles, which are reconstituted and administered as a single intramuscular injection. The diluent for reconstitution is composed of suspending agent (carboxymethylcellulose sodium), along with D-mannitol, polysorbate 80, water for injection USP, and acetic acid to control the pH [76].
<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Peptide Drug</th>
<th>Indication</th>
<th>Release Period</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lupron Depot®</td>
<td>Leuprolide acetate</td>
<td>Prostate cancer</td>
<td>1, 3, 4 or 6 months</td>
<td>Abbott</td>
</tr>
<tr>
<td>Trelstar™ Depot</td>
<td>Triptorelin pamoate</td>
<td>Prostate cancer</td>
<td>1 or 6 months</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Decapeptyl</td>
<td>Triptorelin pamoate</td>
<td>Prostate cancer</td>
<td>1 month</td>
<td>Ipsen</td>
</tr>
<tr>
<td>Sandostatin LAR® Depot</td>
<td>Octreotide</td>
<td>Acromegaly</td>
<td>1 month</td>
<td>Novartis</td>
</tr>
<tr>
<td>Somatuline® LA</td>
<td>Lanreotide</td>
<td>Acromegaly</td>
<td>2 weeks</td>
<td>Ipsen</td>
</tr>
<tr>
<td>Suprecur® MP</td>
<td>Buserelin</td>
<td>Endometriosis</td>
<td>1 month</td>
<td>Aventis</td>
</tr>
<tr>
<td>Bydureon®</td>
<td>Exenatide</td>
<td>Diabetes</td>
<td>1 week</td>
<td>AstraZeneca</td>
</tr>
</tbody>
</table>

Compared with microparticles, nanosized PLGA particles display relatively higher cellular uptake and preferential penetration into tissues (e.g., liver, colon, tumor vasculature) [77, 78], and are typically administered through the intravenous route. It has been demonstrated that PLGA nanoparticles can act as a reservoir for antigenic peptides or their combination with adjuvants and, therefore, are very attractive platforms as vaccines or cancer immunotherapeutics with long-lasting effect [79, 80]. Multiple advantages have been reported, including: enhanced immune response due to efficient uptake by antigen presenting cells, sustained release of entrapped antigenic peptide, simultaneous delivery of antigen and adjuvant, etc. [78]. Zhang et al. prepared the PLGA nanoparticles carrying murine melanoma antigenic peptide and toll-like receptor 4 agonist as adjuvant for cancer immunotherapy [81]. As compared to the antigen
peptide mixed with Freund’s adjuvant, the intradermal injection of PLGA nanoparticle-based formulation into mice led to stronger activation of functional cytotoxic T lymphocytes specific to tumor-associated self-antigens. More importantly, *in vivo*, vaccination showed significantly delayed melanoma tumor growth in a prophylactic setting. The dendritic cells-targeted delivery of antigenic peptide (830-844 region of tetanus toxoid) can be achieved by coating the PLGA nanoparticles (200 nm in diameter) with the humanized targeting antibody hD1. As a result, the similar immune responses were achieved from targeted nanoparticles at 10-100 fold lower concentrations than non-targeted nanoparticles [71]. Insulin-loaded PLGA nanoparticles have also been extensively explored for non-invasive routes of insulin administration for treating diabetes [82-84]. For example, after administration of inhalable PLGA-formulated insulin (weight mean diameter of 400 nm) via a sieve type ultrasonic nebulizer to the lung of guinea pigs, the blood glucose level was reduced significantly and the hypoglycemia was prolonged over 48 h, compared to a nebulized aqueous solution of insulin as a reference (6 h) [85]. This result was attributed to the sustained release as well as the wide spread of insulin in the whole lung. The oral delivery of insulin has been also explored using PLGA nanoparticles as carrier systems that are able to protect the drug from enzymatic degradation and increase its permeability through intestinal epithelium. Various strategies have been investigated for this purpose: the encapsulation efficiency of hydrophilic insulin into hydrophobic PLGA nanoparticles was markedly improved by its complexation with various compounds like lipids and hypromellose phthalate [86-88]; favorable encapsulation of insulin was observed in star-branched β-cyclodextrin-PLGA
nanoparticles over linear PEG-PLGA, which led to retention of insulin stability and more sustained release [89].

1.2.1.2 Polymeric capsules

In contrast to the polymeric nano-/microparticles described above with a matrix-type structure, polymeric capsules have inner liquid cores (oil or aqueous solution, depending on the synthetic strategy) surrounded by single-/multi-layered polymeric shells (Figure 1.5 B). Generally, polymeric capsules can be fabricated by template-free or template-assisted techniques [90]. The most frequently applied strategies are the self-assembly of block copolymers (template-free), and the layer-by-layer technique employing a sacrificial template. The major issue with a template-free method is the size polydispersity of the resulting capsules, and this can be addressed by extrusion, sonication, freeze-thaw cycles, etc. Comparatively, the layer-by-layer technique provides a more precise control over the shell properties. The therapeutic agents can be either entrapped in the inner core of the capsule or absorbed on the surface of polymeric shell using various strategies such as: a) drug loading in preformed capsules by temporarily changing shell permeability; b) drug pre-encapsulation into sacrificial core template upon capsule formation, which will leave the drug molecules in the inner cavity when template is removed; and c) incorporating drugs in specific domains of the capsules. In addition, the entrapment can be managed by modulating the core material (oil or aqueous phase) according to peptide properties. The composition and the thickness of polymeric shells determine the capsules permeability and the release rate of entrapped drugs [91, 92]. The triggered drug release can also be achieved by
incorporating stimuli-responsive (e.g., pH-, redox-sensitive, enzyme-cleavable) “building blocks”.

A wide range of different polymers can be selected to prepare polymeric capsules with desired particle size, composition, morphology, shell thickness, and functionality.

Nanocapsules composed of bioadhesive polymers have been widely investigated as carriers for transmucosal delivery. Poly(alkylcyanoacrylate)-based (PACA) nanocapsules have been investigated for oral delivery of various peptides including calcitonin, insulin, octreotide, and cyclosporine A (CsA) [93, 94]. They were prepared by interfacial emulsion polymerization. The peptide encapsulation efficiency as well as release rates were modulated by adjusting the alkyl chain length, molecular weight, monomer concentration, and polymerization kinetics [95]. In addition to the protective effect against enzymatic degradation and controlled release of peptide, the bioadhesive properties of PACA nanocapsules facilitate peptide translocation through the intestinal mucosa resulting in improved oral bioavailability [96]. Different chitosan-based nanocapsules were reported to be effective for the oral administration of salmon calcitonin, a potent hypocalcemic agent, and allow achieving a significant response (~30% peak reduction in serum calcium levels), which was maintained for more than 24 h [97]. These type of nanocapsules were also effective for nasal delivery of the calcitonin [98]. Interestingly, it was demonstrated that chitosan-based nanocapsules fabricated using solvent displacement technique are also useful for loading and delivery a combination of therapeutic peptides, for example they can be loaded with a combination of both hydrophobic peptide such as CsA and the hydrophilic peptide (e.g. insulin, calcitonin) [99].
1.2.1.3 Polymeric micelles

Polymeric micelles are self-assembled nanoconstructs formed from amphiphilic polymers. They have unique core-shell architectures with hydrophobic polymer chains segregating into the core surrounded by a shell of hydrophilic chains such as PEG (Figure 1.5 C) [100]. Hydrophilic shell inhibits protein binding and opsonization during systemic administration, which allows them to circulate in the blood for extended periods of time by evading the mononuclear phagocytic system. Also, modification of the shell with various ligands using different surface chemistries enables the micelles to be targeted to a specific site. Compared with other drug delivery systems, small size (10 to 100 nm) and low polydispersity of micelles enable better penetration into various tissues, for example, by extravasation [101]. The inner hydrophobic core as well as the palisade layer allow the encapsulation of hydrophobic or amphipathic peptides [102]. A wide range of hydrophobic blocks have been explored as drug-loading cores. Examples include polyesters, polyanhydrides, poly(amino acids), phospholipids/long-chain fatty acids, polypropylene oxide (in Pluronics/poloxamers). An important consideration for drug delivery is the relative stability of polymeric micelles: micelles must be stable enough to retain drug cargo upon administration and remain intact long enough to accumulate in sufficient concentrations at the target site. The thermodynamic tendency for micelles to dissociate is primarily controlled by the length of the hydrophobic block, while the kinetic (rate of dissociation) stability depends on many factors, including the size of a hydrophobic block, the mass ratio of hydrophilic to hydrophobic blocks, and physical state of the micelle core and can be further reinforced by
formation of cross-links between the polymer chains [103, 104]. Polymeric micelles have been shown to overcome many challenges related to therapeutic use of peptides including, but not limited to, susceptibility to proteolytic degradation, aggregation in aqueous media, unfavorable release profile, poor biodistribution and pharmacokinetics, limited therapeutic efficacy in vivo, etc. Micelles formed by PEG-phospholipid conjugate (PEG-DSPE) have been utilized to incorporate various therapeutic peptides with the size of 11-42 residues [105-107]. For example, the PEG_{2k}-DSPE micelles loaded with vasoactive intestinal peptide (VIP) with effective diameter of 15 nm were developed as a potential therapeutic modality for rheumatoid arthritis [108]. Interestingly, the association of VIP with PEG-DSPE micelles led to peptide conformation change from random coil to α-helix, which was shown to be beneficial to its stability in aqueous medium [109, 110]. Following intravenous injection, VIP incorporated into the micelles displayed increased half-life in circulation (10.9 h) compared to free VIP (22.6 min), leading to a 13-fold higher drug accumulation at diseased site. However, it should be pointed out that there are certain limitations of this particular system: the peptide loading was strongly dependent on size, secondary structure, and hydrophobicity of the peptide [109]. Zeng et al. designed the cationic micelles prepared from branched polyethylenimine(2 kDa)-stearic acid conjugate (effective diameter of 20-45 nm), which were loaded with melanoma antigen peptide (Trp2) [111]. With cationic polymer micelles alone acting as a robust adjuvant as well as a vector for Trp2 antigen, Trp2-loaded micelles promoted the recruitment of immature dendritic cells as well as their proliferation, maturation, and homing to the draining lymph nodes. Immunizing mice with
these micelles enhanced Trp2-specific cytotoxic T lymphocyte activity and significantly inhibited tumor growth in mouse model of advanced melanoma. CsA is a cyclic lipophilic endecapeptide exhibiting strong immunosuppressive properties. However, its application suffers from very low water solubility (23 µg/ml). Solubilization of CsA into polymeric micelles based on amphiphilic block copolymers [112, 113] and graft copolymers [114] have been explored to achieve clinically relevant CsA concentrations in aqueous media and to improve unfavorable absorption characteristics of CsA. For example, CsA was effectively encapsulated into PEG5k-b-poly(ε-caprolactone) (PEG-b-PCL) reaching aqueous levels of more than 1 mg/mL for CsA [115]. Notably, CsA encapsulation in PEG-b-PCL micelles resulted in elevated CsA blood levels after intravenous administration while limiting the CsA distribution to kidney, liver, and spleen. Xiong et al. investigated the feasibility of using poly(lactic acid)-b-Pluronic F127-b-poly(lactic acid) (Mₙ 29 kDa) micelles for the oral delivery of insulin [116]. According to their study, this micellar formulation was able to maintain a prolonged hypoglycemic effect after oral administration, which was attributed to the delayed GI transit of the insulin-loaded micelles due to their small size and strong interactions of PEG shell with the intestinal wall.

1.2.1.4 Polyelectrolyte complexes

As compared to the delivery platforms based on PLGA or polymeric micelles, which are more favorable to hydrophobic molecules, hydrophilic polymers offer some advantages for peptide delivery as most of the peptides are hydrophilic or amphipathic in nature. Therefore, another promising class of delivery vehicles for peptides is the polyelectrolyte complexes (PECs),
which are composed of polyelectrolyte polymer and peptide/protein of opposite charge (Figure 1.5 D, left). The PECs assembly is predominantly driven by the electrostatic interactions. However, other intermolecular interactions including hydrogen bonding, van der Waal forces, and hydrophobic interactions may also play a role in the complexation process [117]. PECs are an attractive alternative to conventional peptide/protein carrier systems as they can be formulated with high loading efficiency (up to 100% of peptide) under benign conditions that minimize the chances of peptide damage and aggregation. The formation and stability of PECs is dictated by system pH, ionic strength, molecular parameters of polymers (length, structure, charge distribution and density), and peptide/polymer stoichiometric ratios and concentrations [118]. PECs formation leads to particles in the size range of 200-500 nm. The stability of PECs can be further improved by introducing the additional low molecular weight ionic cross-linking agents (e.g., tripolyphosphate, zinc sulfate, calcium chloride) or the oppositely charged polymers [119-121]. Kissel and co-workers utilized cationic polysaccharide chitosan and its derivatives with the molecular weight higher than 25 kDa for the synthesis of PEC-based carriers for insulin which is negatively charged above its isoelectric point (pI 5.3) [122]. It was demonstrated that chitosan methylation and PEGylation significantly improved the stability of insulin in the PECs. Moreover, the PECs could protect insulin from degradation even at 50 °C and in the presence of trypsin. All complexes could be lyophilized without influencing the particle size, complex concentration, and stability of insulin. The interchain electrostatic bonds can be further strengthened by adding the third component like anionic polymers (e.g., alginate, dextran sulfate,
hyaluronate) [123] or cross-linkers (e.g., tripolyphosphate, carboximethyl-β-cyclodextrin) [124-126].

A very special class of polyion complexes based on doubly hydrophilic copolymers containing ionic and non-ionic blocks (“block ionomers”) was proposed in the mid 90’s as promising carrier systems for peptide and proteins [127, 128]. Such complexes are spontaneously formed in aqueous solutions upon electrostatic binding of block ionomers with oppositely charged macromolecules and termed “polyion complex micelles”, or “block ionomer complexes” (BICs) [128-130]. BICs form core-shell nanoparticles with complex cores of neutralized polyions and proteins and hydrophilic polymer shells (Figure 1.5 D, right). Ionic block lengths, charge density, and ionic strength of solution affect the formation of stable BICs and, therefore, control the amount of the protein that can be incorporated within the micelles [129, 131]. It was demonstrated that proteins/enzymes incorporated in BIC displayed extended circulation time, are stable against proteolytic degradation in blood and inside cells and preserved their activity [132, 133]. Our laboratory has developed and characterized several BIC formulations of an amphipathic α-helical peptide, a positively charged analog of C5A peptide derived from the HCV NS5A protein, with a reported virocidal activity. Anionic PEG(5 kDa)-b-poly(L-aspartic acid) or PEG(5 kDa)-b-poly(L-glutamic acid) block copolymers with the anionic segment around 10-20 monomer units were used for peptide immobilization into BIC. The self-assembled antiviral peptide nanocomplexes were ca. 35 nm in size, stable at physiological pH and ionic strength, and retained in vitro antiviral activity against HCV and HIV. Moreover, incorporation of the peptide
into BIC attenuated its cytotoxicity associated with the positive charge. We demonstrated that these BIC were able to decrease the viral load in mice transplanted with human lymphocytes and HIV-1-infected without signs of toxicity to the animals [134].

1.2.1.5 Hydrogels

Hydrogels are generally defined as the three-dimensional and cross-linked polymeric networks that, due to their hydrophilic nature, can absorb large amount of water [135-137]. Based on the size, hydrogels can be in the form of macroscopic networks or confined to smaller dimensions like microgels or nanogels (Figure 1.5 E) [138]. They represent a distinct class of drug carriers exhibiting high loading capacity, biocompatibility, responsiveness to external factors (e.g., ionic strength, pH, and temperature), and potential for controlled release. Besides, the functionalization of the hydrogel particles with targeting ligands can further facilitate their selective accumulation in the target tissue or cells [139, 140]. An incorporation of peptides into such hydrophilic systems makes them less susceptible to conformation change and potential aggregation, which tend to happen at the hydrophobic interfaces [141]. A number of hydrogel particulate systems have been synthesized and characterized with respect to proteins/peptides delivery. Among polymers of natural origin, polysaccharides (e.g. chitosan and its derivatives, hyaluronic acid, poly(γ-glutamic acid), pullulan) are most commonly used for the preparation of hydrogels. Synthetic biocompatible polymers such as poly(vinyl alcohol), poly(vinyl pyrrolidone), and poly(N-isopropylacrylamide) have frequently been used in the development of potential delivery systems [142]. Diverse synthetic strategies for hydrogels preparation involving physical
or chemical methods of cross-linking have also been elaborated [138, 143, 144]. Physical self-assembly of hydrogels involves electrostatic interactions [145], hydrophobic interactions [146], and/or hydrogen bonding of the interactive polymers [147]. The more stable networks can be fabricated using covalent cross-linking. The coupling of reactive functional groups can take place during polymerization of monomers or can be used for cross-linking of macromolecular precursors. The most commonly used covalent cross-linking strategies include, but are not limited to radical polymerization, amide-based cross-linking [148], photo-induced cross-linking [149], quaternization of amino groups [150], thiol-disulfide exchange reaction [151], and click chemistry [152]. The porosity, charge density and other characteristics of the gel particles can be systemically varied using covalent cross-linking. Cleavable linkages and degradable polymer backbones or pendant groups are often used to make hydrogels degradable and trigger the drug release.

The majority of micro-/nanogels that have been investigated for peptide delivery are based on physically cross-linked polymers [153-155]. The entrapment of peptides into such hydrogels is predominantly driven by electrostatic interactions, and the loading capacity of these carriers depends on hydrogel and peptides net charges and charge densities [156]. Besides, the binding and release of peptides from these carriers are also determined by network properties (cross-linking density, network homogeneity, dimensions), peptide size/length and conformation, hydrophobicity, etc. [136].

The delivery of insulin has been extensively studied using hydrogel particles based on
polysaccharides (e.g., pullulan, chitosan, dextrin) [155, 157], poly(methacrylic acid) [158], poly-γ-glutamic acid [159], etc. Insulin loading of up to 80% was achieved using positively charged chitosan nanogels prepared based on the ionotropic gelation of chitosan with tripolyphosphate containing poloxamer 188 (1.0% w/v) as the surfactant. [160]. Chitosan gel particles with the size of 250-400 nm enhanced the intestinal absorption of insulin compared to free insulin after oral administration in alloxan-induced diabetic rats that led to a prolonged hypoglycemia effect. Akiyoshi and co-workers have developed self-assembled nanogels composed of cholesterol-bearing pullulans (CHP) (pullulan (M₆ 55 kDa) substituted with 2.1 cholesterol molecules per 100 glucose units) with a particular focus on encapsulation of various proteins and peptides [147, 161-163]. They demonstrated that up to 10 insulin molecules can be complexed with CHP nanogels (20-30 nm in diameter). The thermal denaturation and subsequent aggregation of insulin were effectively suppressed upon complexation. The complexed insulin was also significantly protected from enzymatic degradation. However, the decrease in blood glucose levels after intravenous injection of insulin-loaded CHP nanogels in rats was not significantly different from that observed for free insulin that was attributed to the relatively fast release of insulin from the complex [147, 164]. These challenges were further addressed by incorporating CHP nanogels into hybrid macrogels [165, 166], wherein the sustained release of insulin was achieved. With insulin immobilized into the nanoaggregates of α,β-poly(N-hydroxyethyl)-DL-aspartamide modified with hydrophobic segments (C₄), anionic groups (COOH), and cysteine residues, its oral administration was proved to be feasible due to
the elevated stability against the gastric degradation [167]. Moreover, Sonaje et al. reported the insulin-loaded pH-sensitive and self-assembled nanogels, which are composed of the mixture of positively-charged chitosan (80 kDa) and negatively-charged poly-γ-glutamic acid (60 kDa), showing high encapsulation efficiency of 71.8 ± 1.1% [159, 168]. In diabetic rat models, effective penetration of the insulin-loaded nanogels through the mucus layer of intestinal epithelium was observed due to the mucoadhesive property of chitosan. Subsequently, insulin was released from the disintegrated nanogel into the systemic circulation due to pH-dependent dissociation of chitosan/poly-γ-glutamic acid complex matrix. Overall, the unique characteristics of hydrogels such as hydrophilicity, tunable size, interior network for the incorporation of various biomolecules, and large surface area available for functionalization make them very attractive platform for the drug delivery applications.

1.2.2 Injectable implants

In terms of parenteral controlled-release formulations, injectable implants are an alternative to microparticles and preformed implants. They are the liquid formulations comprised of biodegradable polymers and therapeutic agents. After intramuscular or subcutaneous injection via a syringe, the low-viscosity solution is delivered to the malignant site. Triggered by environmental factors, it subsequently transformed in situ to solid or semi-solid drug depot that releases entrapped drugs over an extended period of time [169]. Simultaneously, the polymer matrix begins to degrade and gradually becomes metabolized and excreted, eliminating the need
for surgical removal of the implants. Generally, injectable implants are applicable when the diseased site is accessible via injection. As compared with micro-/nanoparticulate drug delivery systems, the manufacturing process is less complex and mainly requires dissolution and dispersion. Additionally, lower administration frequency, smaller gauge needle, and tissue biocompatibility potentially can improve patient compliance. With many advantages listed above, injectable implants have emerged as a tangible delivery platform for therapeutic peptides [170].

Generally, the mechanisms of implants formation can be divided into 3 categories: 1) *in situ* polymer precipitation (phase separation), which can be initiated by solvent removal, temperature change, or pH change; 2) *in situ* cross-linking, wherein polymers form networks by a variety of ways including photo-irradiation, chemical cross-linking, or physical cross-linking of specific monomers; and 3) *in situ* solidification, which are either hot melts that solidify upon cooling to physiological temperature or liquid crystalline polymers, which self-assemble in aqueous solutions ([Figure 1.6]) [171].

![Figure 1.6](image-url) The injectable implants formation mechanisms. Adapted with permission from Ref. [170].
So far, the majority of the marketed products are developed based on *in situ* polymer precipitation systems. Atrigel® technology is one of the earliest *in situ* forming depots developed by Dunn et al. [172]. It contains the biodegradable carrier PLGA, which is hydrophobic and dissolved in water-miscible and biocompatible organic solvent (e.g., N-methyl-2-pyrrolidone). Both PLGA concentration and molecular weight affect the viscosity of the liquid formulation. Upon subcutaneous injection, the water-miscible organic solvent migrates into surrounding tissues and water penetrates into the organic phase, which results in a fast phase inversion and precipitation of the polymer at the injection site. The properties of organic solvent mainly influence the dynamics of this process. When drugs are blended into the polymer solutions, they also remained entrapped in the polymer matrix upon precipitation and their release from the implant is highly dependent on the PLGA molecular weight, composition, and concentration. Based on this platform, injectable implant systems that release leuprolide acetate over 1, 3, 4 or 6 months (Eligard®, Sanofi Aventis, USA) are currently available for the management of advanced prostate cancer. For 1 month release, the formulation is composed of 7.5 mg of leuprolide acetate and PLGA copolymer (lactic acid to glycolic acid ratio 50:50) dissolved in N-methyl-2-pyrrolidone (NMP) (NMP to polymer ratio 1.94). The drug release rate can be further delayed (3, 4, and 6 months) by increasing the lactic acid to glycolic acid ratio and by decreasing the ratio of NMP to polymer [173]. Another marketed product (ReGel®, BTG, UK) is based on low molecular weight PLGA(1.5 kDa)-PEG(1 kDa)-PLGA(1.5 kDa) triblock copolymer. Once injected, it undergoes sol-gel transition at physiological temperature (above lower critical
transition temperature) [174]. Notably, this technology does not use organic solvents and may be more suitable for incorporation of peptides/proteins. Indeed, injectable implants based on thermosensitive gelation were investigated for delivering various peptide drugs including insulin [175], glucagon-like peptide-1 [176], CsA [177], etc. For example, the constant release rate of insulin from ReGel® was achieved for over 2 weeks after single subcutaneous injection. However, the complete release was hampered by random peptide aggregation, which can be suppressed by using zinc-insulin complexes [175].

Apart from the commercial products, a lot of efforts have been made to develop in situ-forming injectable implants based on a diverse range of polymers as well as gelling mechanisms. However, some challenges remain to be addressed for their successful clinical translation such as initial burst release and potential peptide instability caused by protein-polymer interactions. Furthermore, control over the drug release and polymer matrix degradation rates is limited due to the highly variable process of in situ implant formation.

1.2.3 Polymer-peptide conjugates

Polymer-peptide conjugates are hybrid materials that exhibit properties of both the biomolecules and synthetic polymers. Covalent attachment of polymers to peptides/proteins is known to improve their efficacy by increasing lifetimes in vivo and decreasing immunogenicity [178-180]. The peptide modification points, linkers, modification degree and the conjugation chemistry are all important design considerations having a dramatic impact on the properties of
resulting conjugates and their *in vivo* performance [178, 181]. Recent advances in synthetic polymer chemistry have facilitated the possibility of site-specific conjugation of polymers to the proteins/peptides without compromising their activity. Lysine and cysteine residues of a peptide as well as N- and C-terminus modifications and disulfide bridge insertion are commonly exploited for direct-covalent attachment of the polymers. Among various polymers exploited for attachment to peptides/proteins, PEG is the most successfully used polymer for development of protein therapeutics. Readers are referred to several excellent and more detailed reviews on various peptide/protein conjugation chemistries including PEGylation [156, 180, 182-185]. For the purposes of this review, we will highlight the major PEGylation strategies and focus on some examples of PEG-peptide conjugates and their applications, followed by some other polymer-peptide conjugates in pre-clinical developments.

Types of PEGylation chemistries are broadly classified into first-generation and second-generation [181]. Examples of first-generation PEG derivatives include amine reactive PEG that can be easily attached to the amino groups of lysine residues of a peptide (Figure 1.7) [184]. Most first-generation PEG chemistries used acylation (Fig. 4), accompanied by loss of charge. Alkylation, on the other hand, using PEG-aldehyde maintains a positively charged amine.
Unfortunately, these PEGylation methods have lacked the ability to produce pure mono-functional PEG derivatives [185]. In addition, it was shown that unstable bonds formed between the biomolecule and PEG can lead to degradation of the conjugate during manufacturing and injection [186]. Among second-generation PEGylation strategies, thiol reactive PEGs have been explored most extensively due to more selective polymer attachment without interfering with the biological function of the peptide, while maximally decreasing its immunogenicity [187-189]. PEG-maleimide, PEG-vinylsulfone, PEG-iodoacetamide, and PEG orthopyridyl disulfide are thiol reactive PEGs that have been used for cysteine modification (Figure 1.8). The enzymatic (e.g., glucose oxidase) or chemical (e.g., sodium periodate) oxidation of N-terminal serine or threonine residues generates reactive aldehyde groups, which can be further conjugated with PEG hydrazide or amine derivatives [179].
Figure 1.8 Second-generation of PEGylation with thiol reactive PEG derivatives.

Another popular second-generation PEG is the heterobifunctional PEG having two different terminal groups, which can be used as a spacer to link two functional groups, or to introduce targeting ligands [181]. In addition to the linear PEG, branched polymers (Figure 1.9) have also been reported and proven useful for protein modification (e.g. PEG–interferon α-2a, Pegasys®) [190, 191].

Figure 1.9 Two examples of branched PEGs (PEG2).
Collectively, PEGylation technology has proven its pharmacological advantages and acceptability, but the technology still lags in providing a commercially attractive, generic process to produce highly specific PEGylated therapeutic products with a high yield. With growing interest from emerging biotechnology, there is great scientific and commercial interest in improving present methodologies and in introducing innovative PEGylation strategies for peptide modification.

PEGylated peptides have already entered the clinic for the treatment of a range of diseases. Somavert (pegvisomant) is a PEGylated human growth hormone antagonist marketed by Pfizer to treat acromegaly, a rare hormonal disease, in which excessive insulin-like growth factor-1 causes soft-tissue enlargement [192]. Pegvisomant consists of 5 linear chains of 5 kDa PEG covalently linked to peptide through lysine and phenylalanine residues. Omontys (peginesatide), developed by Affymax and Takeda, is an erythropoietic agent, a functional analog of erythropoietin. It was approved by the US FDA for treatment of anemia associated with chronic kidney disease in adult patients on dialysis (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm297464.htm). Two 20 kDa PEG are conjugated to the serine terminals of peptide. Clinical trials have demonstrated that peginesatide, administered monthly, was as effective as epoetin, administered one to three times per week, in maintaining hemoglobin levels in patients undergoing hemodialysis. A number of PEGylated peptides are in clinical trials (Table 1.3). Peglispro is insulin PEGylated with the 20 kDa polymer at lysine B28 via a urethane linkage developed by Lilly Research Laboratories. In a
recently-completed Phase III clinical trial, Peglispro demonstrated superiority in maintaining the blood glucose level compared to Lantus (a commercial insulin analogue) in patients with Type 1 diabetes [193]. Peglispro is currently under Phase III development for the treatment of both Type 1 and Type 2 diabetes. CBX129801, developed by Cebix Inc., is a PEG-conjugate of human C-peptide that can be administered subcutaneously once weekly. Currently it is in Phase II clinical trial for the treatment of Type 1 diabetes patients with mild-to-moderate diabetic peripheral neuropathy. Thymalfasin pegol developed by Jiangsu Hansoh Pharmaceutical is a PEGylated Thymalfasin for the treatment of Hepatitis C, which is currently in Phase III clinical trial in China. These examples clearly demonstrate the usefulness of PEGylation in the improvement of therapeutic value of peptide drugs.

**Table 1.3** Representative PEGylated peptides on market or in clinical trials.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Status</th>
<th>Company</th>
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<tbody>
<tr>
<td>Somavert (pegvisomant)</td>
<td>PEGylated human growth hormone antagonist</td>
<td>Approved</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Omontys (peginesatide)</td>
<td>PEGylated erythropoietin</td>
<td>Approved</td>
<td>Affymax and Takeda</td>
</tr>
<tr>
<td>Peglispro</td>
<td>PEGylated insulin</td>
<td>Phase III</td>
<td>Eli Lilly</td>
</tr>
<tr>
<td>CBX129801</td>
<td>PEGylated human C-peptide</td>
<td>Phase II</td>
<td>Cebix Inc.</td>
</tr>
<tr>
<td>Thymalfasin pegol</td>
<td>PEGylated Thymalfasin</td>
<td>Phase III</td>
<td>Jiangsu Hansoh Pharmaceutical</td>
</tr>
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</table>
Apart from PEGylation, there have been other attempts to conjugate polymers to the therapeutic peptides in the pre-clinical development stage [194-197]. The extended circulation time and the sustained release of the cytotoxic EGFR-lytic peptide was achieved after its conjugation to the carboxymethyl dextran (15-20 kDa) through a formation of disulfide bond in pancreatic cancer model [195]. Yamamoto et al. demonstrated the 15-fold increased plasma half-life of YIGSR peptide (inhibitor of adhesion and invasion of tumor cells to extracellular matrix) when it was conjugated to poly(vinylpyrrolidone) (PVP) through an amide bond [196]. In another study, YIGSR was conjugated to poly(styrene-co-maleic anhydride) (SMA) which led to the decrease of degradation and increase of antimetastatic effect on lung metastasis of B16-BL6 melanoma cells [197]. Conjugation was achieved through anhydride group of SMA and free amine group of peptide.

**1.3 PEG-poly(L-amino acid) block copolymers as polymer therapeutics**

Polymers based on natural L-amino acids are an attractive class of materials given their biocompatibility, controlled biodegradability and metabolizable degradation products. Incorporation of amino acids as a building block into synthetic polymers not only allows for adjusting hydrophilic/hydrophobic properties of the resulting polymers and tuning their degradability, but also imparts chemical functionalities to facilitate further modification with bioactive molecules (e.g., drugs, imaging probes or targeting ligands).

**1.3.1 Chemistry of PEG-poly(L-amino acid) block copolymers**
The most common and efficient method to produce this class of block copolymers with sufficient molecular weight is the ring opening polymerization of N-carboxyanhydrides (NCAs) [198]. NCAs can be easily synthesized through a reaction between amino acids and phosgene or a phosgene derivative (e.g., triphosgene), and the polymerization is commonly initiated from a terminal amine on PEG (Scheme 1.1).

Scheme 1.1 Synthesis of polypeptides via α-amino acid N-carboxyanhydride ring-opening polymerization.

In recent years, the advances in NCA polymerizations, either using metal initiators or improved conventional initiators, allowed for the synthesis of broad range of block copolymers and side-chain functionalized polypeptides with controlled characteristics (molecular weight, sequence, composition, and molecular weight distribution) [199]. In addition, the diversity of synthetic polymer chemistries and “click” reactions offer the ability to covalently link a variety of different polymers together and to prepare hybrid materials with functional macromolecular architectures and tunable physicochemical properties to meet various requirements of biomedical applications.

1.3.2 Application of PEG-poly(L-amino acid)-based micelles in drug delivery
So far, the polymeric micelles self-assembled from PEG-poly(L-amino acid) block copolymers have been extensively applied as carrier systems to deliver various bioactive molecules (e.g., small molecules, proteins, peptides, nucleic acids, reporter molecules) [200]. The doxorubicin-loaded polymeric micelles based on PEG-\textit{b}-poly(aspartic acid) named as NK911 was pioneered by Kataoka and co-workers. In this system, the aspartic acid units are partially substituted with doxorubicin (ca. 45%). With the hydrophobic block formed, the hydrophobized micelles were further loaded with free doxorubicin physically. The improved stability and sustained release of micelles were achieved through a strong interaction between the conjugated and physically encapsulated doxorubicin. This formulation has entered phase I clinical trials in 2001. The results showed that NK911 exhibits longer circulation half-life, a larger area under the curve, and lower toxicities as compared to conventional formulation [201]. To date, NK911 has proceeded to the phase II clinical trials in Japan to treat the metastatic pancreatic cancer [202]. In addition, as previously described (section 1.2.1.4), the BICs can be formed through self-assembly of charged poly(L-amino acid) segment of block copolymer and oppositely charged macromolecules, which will lead to the enhanced stability against proteolytic degradation and prolonged circulation half-life.

1.4 Conclusion

Despite the fact that great success has been achieved to treat HCV with modern DAA approaches, the efficacy and safety of DAA to treat HCV in the presence of accelerating cofactors
(e.g., HIV, alcohol) remained to be evaluated with numerous clinical trials going on. Other practical hurdles as mentioned above also necessitate the development of alternative or additional therapeutic approaches to DAA. The antiviral peptide is one of such candidates, however, its successful clinical translation is hampered by a number of factors, which are mainly due to the low stability issue. Polymer-based carriers have already demonstrated their excellent potential for the delivery of therapeutic peptides both in preclinical and clinical settings. The recent advances in polymer chemistry have allowed the development of a diverse range of nanomaterials of various sizes, shapes, surface chemistries, and targeting properties, which offer exciting prospects for novel formulations, providing non-invasive delivery, stabilization, and immunogenicity reduction for therapeutic peptides. Notably, each of these polymer-based vehicles has their own pros and cons, and their applicability for peptide delivery is predominantly determined by the nature of peptides. Among all the drug delivery systems, those based on the self-assembled PEG-poly(amino acid)-based block copolymer micelles have great potential due to the small size (10-100 nm) and well-defined core-shell structure leading to the protection of active agent in the core by a hydrophilic polymer shell. The ionic poly(amino acid) block enabled the efficient encapsulation of the oppositely charged entities such as polypeptides into the micellar core. We propose that the self-assembled antiviral peptide nanocomplexes (APN) would combine several key characteristics including easy and efficient peptide loading, nanoscale size, and protection from disintegration, which will ascertain a long-lasting biological function of peptides.
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CHAPTER II

ANTIVIRAL PEPTIDE NANOCOMPLEXES AS A POTENTIAL THERAPEUTIC MODALITY FOR HIV/HCV CO-INFECTION

2.1 Introduction

In the United States and Europe approximately one-third of HIV-1 infected individuals are co-infected with HCV [1-4]. The prevalence of HCV co-infection is about 70-95% in current or former drug users and transfused hemophiliac patients, and became a leading cause of morbidity and mortality amongst this patient population [2, 5-7]. Both viruses potentiate each other’s progression, complicate therapeutic application of antiretroviral drugs due to increased liver toxicity and interferon-based treatment for HCV, due to myelotoxicity, severe cognitive impairment and psychotic complications [1, 3, 8-16]. New therapeutic modalities are urgently required for treatment of HIV/HCV co-infection. Not many compounds are known to be effective antiviral (HCV) or antiretroviral (HIV) agents. The specific blockade of two viruses by one compound is precluded by several factors. There are diverse virus-susceptible host cells – macrophages, lymphocytes, and hepatocytes. The simultaneous drug targeting of multiple pathways of virus-host cell interactions and dissemination is also challenging. However, non-specific virocidal compounds could find their niche as antiviral/antiretrovirals and their inclusion in combination of therapeutics with different mechanisms of action could be beneficial.

For HCV treatment antiviral peptide therapeutics potentially may reduce the time needed for achievement of stable virologic responses and be effective in IFN-a-non-responders [17-19].

A virocidal peptide derived from the membrane anchor domain of the HCV nonstructural protein NS5A (C5A) was found to be effective to inhibit both HIV [20] and HCV infections as well as infections with other human Flaviviridae members in cell culture systems [21]. The mechanism includes prevention of initiation of infection by destroying the virus and suppressing ongoing infections by blocking the cell-to-cell spread of the virus. It has been suggested that C5A recognizes cellular components of virus membranes most likely associated with the membrane lipid composition. Even demonstrating a low toxicity and immunogenicity [20, 21], the therapeutic in vivo application remains questionable due to sensitivity to proteolysis and immune activation properties [22]. A technology based on incorporation of proteins and polypeptides into polyion complexes with oppositely charged block copolymers, block ionomer complexes (BIC), can be used to deliver potent polypeptides while mediating the clinical delivery challenges. Proteins, peptides, and enzymes may be encapsulated in BIC to preserve activity and promote stability in the body [23-25]. When pH of the solution exceeds the isoelectric point, a protein becomes charged and can form protein-polyelectrolyte complexes with an oppositely charged block ionomer (Figure 2.1A). Stability of the protein incorporated into BIC can be further improved by introducing hydrophobic groups to block copolymer or by cross-linking [25, 26]. The pH and salt sensitivity of BIC also provide a unique opportunity to control the triggered release of the protein [27]. Structure-activity relationship analysis of modified C5A (SWLRDIWDWICEVLSDFK), further abbreviated as p1, performed by Cheng and co-authors
revealed that a cationic derivative of this peptide (SWLRRIWRWICKVLSRFK), abbreviated as p41, with a net charge of +6 at pH 7, also displayed virocidal activity against HCV. The objective of this work was to characterize the broadness of antiviral properties of cationic peptide p41, to immobilize it into a peptide/polyion BIC using negatively charged block copolymers of poly(ethylene glycol) (PEG) and poly(amino acids), and to demonstrate in vitro and in vivo activity of an antiviral peptide nanocomplex (APN).

2.2 Materials and methods

2.2.1 Materials

Peptides SWLRDIWDWICEVLSDFK (p1), SWLRRIWRWICKVLSRFK (p41), p41-Cy5, SWLRRIWRWISKVLSRFK (sp1), and SWRLRIWRWICKSVLRFK (sh) were custom synthesized by AnaSpec (Fremont, CA). Block copolymers of methoxy-poly(ethylene glycol)-block-poly(α,β-aspartic acid) (PEG-PLDₙ) and methoxy-poly(ethylene glycol)-block-poly(L-glutamic acid) (PEG-PLEₘ) with PEG molecular mass of 5,000 Da and different length of anionic segments (n and m represent the degree of polymerization of PLD and PLE blocks) were obtained from Alamanda Polymers, Inc. (Madison, AL). The list of block copolymers used in this work and their molecular characteristics are presented in Table 2.1. For in vitro application peptides were dissolved in DMSO at 10mM concentrations and adjusted to the final concentrations of 2.5 mM-10 mM by phosphate buffered saline (PBS) or culture medium.
Table 2.1 Physicochemical characteristics of block ionomers.

<table>
<thead>
<tr>
<th>Copolymer $^a$</th>
<th>Molecular weight</th>
<th>Polydispersity index</th>
<th>Average number of units in ionic block $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-PLD$_{10}$</td>
<td>6400</td>
<td>1.03</td>
<td>10</td>
</tr>
<tr>
<td>PEG-PLD$_{20}$</td>
<td>7200</td>
<td>1.05</td>
<td>20</td>
</tr>
<tr>
<td>PEG-PLE$_{10}$</td>
<td>6500</td>
<td>1.15</td>
<td>10</td>
</tr>
<tr>
<td>PEG-PLE$_{25}$</td>
<td>8800</td>
<td>1.20</td>
<td>25</td>
</tr>
<tr>
<td>PEG-PLE$_{40}$</td>
<td>11100</td>
<td>1.01</td>
<td>40</td>
</tr>
</tbody>
</table>

$^a$ The average molecular weights, polydispersity indices, and the average number of monomer units in polyacid blocks were provided by manufacturer. The molecular weight of PEG block was 5000.

2.2.2 Synthesis of APN

APN were prepared by mixing buffered solutions (phosphate buffer, 10 mM, pH 7, or PBS) of p41 peptide and anionic block copolymer at various compositions. The composition of the mixtures ($Z_-/+$) was calculated as a molar ratio of carboxylic groups in the copolymer to the amino groups in lysine and arginine residues of the peptide. As an example, the p41/PLD$_{20}$ APN at composition of $Z_-=1$ was prepared by mixing 5 µl p41 (5 mg/ml) and 2.38 µl PEG-PLD$_{20}$ (10 mg/ml) in the presence of buffer.

2.2.3 Dynamic Light Scattering (DLS)

Electrophoretic mobility and hydrodynamic diameters of APN were determined by DLS using Nano ZS Zetasizer (Malvern Instruments, UK) at a fixed 173° scattering angle. All measurements were performed in automatic mode at 25°C. Software provided by the manufacturer which employs cumulants analysis and non-negatively constrained least-squares
particle size distribution analysis was used to determine the intensity-mean z-averaged particle diameter ($D_{\text{eff}}$), polydispersity index (PDI), and ζ-potential. All measurements were performed at least in triplicate to calculate mean values ± SD. Complexes were prepared in various buffer systems of 10mM concentration: citrate buffer - pH 5 and 6; phosphate buffer - pH 7, HEPES – pH 8, and Tris buffer - pH 9. Concentration of peptide in the complexes was 50 µM.

2.2.4 Atomic Force Microscopy (AFM) Analysis

Samples for AFM imaging were prepared by depositing 5 µl of an aqueous dispersion of complexes (0.125 mg/ml) onto positively charged 1-(3-aminopropyl) silatrane mica surface (APS-mica) or freshly cleaved negatively charged mica for 2 minutes followed by surface washing with deionized water and drying under argon atmosphere. The AFM imaging in air was performed with regular etched silicon probes with a spring constant of 42 N/m using a Multimode NanoScope IV system (Veeco, Santa Barbara, CA) operated in a tapping mode. The images were processed, and the widths and heights of the particles were measured using Femtoscan software (Advanced Technologies Center, Moscow, Russia).

2.2.5 Liquid Chromatography Analysis

Formation of APN (p41/PEG-PLD_{20}) was directly monitored by size exclusion chromatography (SEC) using an ÄKTA FPLC (Amersham Biosciences). APN or its components (100 µl, 50 µM on peptide basis) were applied on a Superose 12 10/300 GL column (GE Healthcare Bio-Sciences AB, Uppsala Sweden). PBS (pH 7.4, 0.14 M NaCl) was used as an eluent at the flow rate of 0.5 ml/min. Copolymer was detected by refractive index (Knauer RI 2300) and peptide was detected by UV ($\lambda = 280$ nm) at room temperature.
2.2.6 Analytical Ultracentrifugation

Sedimentation equilibrium experiments were performed using a Beckman Optima XL-I analytical ultracentrifuge equipped with AN-60Ti rotor as previously reported [28]. Six-sectored centerpieces were used with loading volumes of 110 µl for the APN samples and 125 µl for the reference solutions. The peptide concentrations were 41 µM. The sedimentation equilibrium data were collected using UV absorption optics at 280 nm at the rotor speed of 5000 rpm and 20°C. Data analysis was performed using the Beckman XL-A/XL-I software package within Microcal, ORIGIN Version 4.

2.2.7 Circular dichroism (CD) spectroscopy

The CD spectra were recorded by using an Aviv circular discroism spectrometer (model 202SF, Aviv Associates Inc., Lakewood, NJ). The spectra were measured at 25°C using a 1 mm pathlength cell over a wavelength range from 190 to 300 nm in 10 mM phosphate buffer (pH 7.0) with and without 0.14 M NaCl. Data were collected at 1 nm intervals with a scan rate of 15 nm/min. All spectra were acquired in triplicate and averaged. The spectrum of an appropriate buffer control sample was then subtracted from each of the sample spectra. The final spectral data were converted to mean molar ellipticities. The peptide concentrations were 100 µM.

2.2.8 Trypsin digestion analysis

APN stability against proteolytic digestion was studied using Trypsin Spin Columns (Sigma). Columns were first prepared by washing with enzyme reaction buffer and centrifugation according to the product protocol. For sample digestion, 100 µl of buffered solution of p41, corresponding APN (Z+/=2), or PEG-PLD20 (20 mg based on peptide equivalents) were applied
on the trypsin spin columns. The pH was kept at approximately 8 during the whole procedure. The samples in the column were incubated at room temperature for two time points, 5 min and 13 min, respectively, followed by elution with enzyme reaction buffer or water and centrifugation at 3000 rpm for 2 min. Eluates were analyzed by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and gel electrophoresis. The MALDI–TOF MS measurement was performed by UNMC mass spectrometry and proteomics Core Facility.

2.2.9 Hemolytic activity

Human blood was collected in BD heparinized vacutainers, centrifuged at 13500x g to separate the red blood cells (RBC). The pellet was washed three times using 150mM saline solution. After the third wash, the RBC solution was diluted with 100 mM PBS solutions to make the final RBC concentration into $10^8$ per 200 ml while maintaining pH at 7.4. APN, peptides, or copolymer were added to 200 ml of RBC suspensions at different concentrations (2.5 mM, 5 mM and 10 mM on peptide basis), gently mixed and incubated for 60 min in a 37°C water bath. The release of hemoglobin was determined after centrifugation (13,500x g for 5 min) by spectrophotometric analysis of the supernatant at 541 nm. The hemolysis of RBC in PBS solutions and in 1% v/v Triton X-100 solution were used as negative and positive controls, respectively. The observed hemolytic activity of each tested compound was normalized to that of the positive control, 1% v/v Triton X-100 solution, as a 100% hemolysis.

2.2.10 Immunofluorescent staining and confocal microscopy

Monocytes were grown in suspension cultures using Teflon flasks or cultured as adherent cells in poly-D-lysine/fibronectin-coated LabTek chamber slides were purchased from BD
Biosciences (San Diego, CA). Rabbit Abs to EEA1 were purchased from Cell Signaling Technologies (Danvers, MA). Rabbit monoclonal Abs against PEG were purchased from Epitomics, Inc. (Burlingame, CA). The secondary Abs conjugated to Alexa Fluor 488, 594 and ProLong Gold anti-fading solution with 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Life Technologies Corporation (Grand Island, NY). For immunofluorescent staining, cells were washed three times with PBS and adherent cells were fixed with 4% paraformaldehyde solution (PFA) in PBS at room temperature for 30 min. Cells were treated with blocking/permeabilizing solution (0.1% Triton, 5% BSA in PBS) and quenched with 50 mM NH₄Cl for 15 min. Cells were washed once with 0.1% Triton in PBS and sequentially incubated with primary and secondary Abs at room temperature. Non-specific cross binding of secondary Abs was tested prior to immunostaining. Slides were covered in ProLong Gold anti-fading reagent with DAPI and imaged using a 63X oil lens in a LSM 510 confocal microscope (Zeiss). Cell suspension was stained for FACS utilizing BD Bioscience protocol for intracellular staining (cat. # 560098) and samples were analyzed by BD FACSArray™ Bioanalyzer.

2.2.11 In vitro anti-HCV activity

The infectious full-length genotype 2a HCV clone JFH1 that replicates and produces infectious virus particles in cell culture was used in these studies as previously described [29]. Huh-7.5 cell line provided by Dr. Charles Rice (The Rockefeller University, New York, NY) was propagated in DMEM supplemented with 10% fetal bovine serum and 1% nonessential amino acids. Cells were infected with JFH1 virus (MOI 0.1) overnight, virus was removed and cells were incubated for 7 days in fresh media. The peptides (10 mM stock solution in DMSO),
copolymers and APN were diluted to peptide equivalent concentrations of 10, 5 and 2.5 mM, incubated in complete culture medium with cells for 1 h and then were removed before infection (single pretreatment) or were added to the culture medium post infection every 48 h with media exchange (multiple treatments). To confirm the intracellular levels of HCV infection, real-time PCR was performed as described [30]. In brief, HCV RNA quantification was performed using a StepOne Realtime PCR system amplifying a highly conserved sequence in the 5’ UTR of the viral genome. Total viral RNA was extracted using the MagMax Viral RNA Isolation Kit (Applied Biosystems), and first-strand cDNA synthesis performed using a high capacity RNA-to-cDNA kit (Applied Biosystems). The following primers and probe for this consensus sequence were designed using PrimerExpress Software v2.0 (Applied Biosystems): 5’UTRF GACCGGGTCTTTTCTTGGAT; 5’UTRR CCAACACTACTCGGCTAGCAGTCT; probe FAM-ATTTGGGCGTGCCCCCGC-NFQ. Positive and negative controls were included in all runs. To measure the intracellular expression of HCV core protein by flow cytometry infected Huh7.5 cells were detached by EDTA-containing Cell stripper and then permeabilized using BD Pharmigen buffer set (cat # 560098). After permeabilization, cells were stained with monoclonal antibody to HCV core protein 1 µl/well (clone C7-50, Thermo Fisher Scientific Inc., IL, USA). After 1 hr incubation, cells were additionally stained with anti-mouse IgG-PE and analyzed by FACSDiva (BD Biosciences Immunocytometry Systems).

2.2.12 In vitro anti-HIV-1 activity

Human monocytes and peripheral blood lymphocytes (PBL) were obtained from leukopheresis of HIV-1, HIV-2 and hepatitis B seronegative donors and purified by
countercurrent centrifugal elutriation as previously described [31]. Monocyte-derived macrophages (MDM) were cultured in DMEM supplemented with 10% heat-inactivated pooled human serum and 1% glutamine (Sigma-Aldrich, St. Louis, MO), 10 mg/ml ciprofloxacin (Sigma-Aldrich), and 1000 U/ml of purified recombinant human macrophage colony stimulating factor (M-CSF) [31]. The CCR5 coreceptor utilizing HIV-1\textsubscript{ADA} strain was propagated using MDM. CXC4-utilizing lymphocyte-tropic HIV-1\textsubscript{LAI} strain was propagated on phytohemagglutinin (PHA)-stimulated PBL in the presence of interleukin-2 (BD Bioscience, San Jose, CA) (PHA/IL-2 lymphoblasts). Cells were split 1:2 and infected 3 days after stimulation. Viral preparations were screened and found to be negative for endotoxin (<10 pg/ml) (Associates of Cape Cod, Woods Hole, MA) and mycoplasma (Gen-Probe II; Gen-Probe, San Diego, CA). PBL and MDM cell cultures were infected with HIV-1 at MOI 0.01.

To test drug efficacy TZM-bl cells (JC53BL-13, NIH AIDS Research and Reference Reagent Program) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units of penicillin and 100 µg/ml of streptomycin. Cells were allowed to reach 70% confluence, then detached by 25 mM trypsin/EDTA for 5 min at 37°C. Detached cells were seeded in 96-well plates at 1×10^4 cell/well and used in experiments when they reached 40% confluence. Cells were pretreated with drugs (copolymer, p1, p41 and APN) at peptide equivalent concentrations of 2.5, 5 and 10 µM for 2 h, washed and infected. After infection cells were cultured for additional 48 h prior to β-galactosidase-positive cell number detection (per manufacturer’s instructions, Invitrogen). Bright field images were acquired using a Nikon Eclipse TE300 microscope (Nikon) and virus-infected blue cells were counted. PHA/IL-2 lymphoblasts were pre-treated with APN
and peptides at a concentration of 2.5 and 5 µM for 2 h and infected with HIV-1_{LAI} for 4 h, then drugs and inoculum were removed and media with drugs were added for 4 consecutive days. All treatments were done in quadruplicates. MDM were plated in 96-well flat-bottom plates (10^6 cells/ml), maturated in the presence of M-CSF for 7 days and used in 6 parallels for all treatments. Cells were incubated with peptide, copolymer and APN at 10 mM for 2 h and drugs were removed before infection, or were added to the culture medium every 48 h with media exchange. Supernatants were collected at days 5, 7 and 10 post infection. HIV-1 replication in MDM and PBL cell cultures was detected by reverse transcriptase (RT) activity [32] and adjusted to the cell viability determined by the standard tetrazolium dye method (MTT assay).

### 2.2.13 In vivo anti-HIV-1 activity

*In vivo* anti-HIV-1 activity was tested on 4-week-old NOD/scid-γc null (NSG, The Jackson Laboratories, stock # 005557) reconstituted with human peripheral blood lymphocytes (hu-PBL-NSG) as described before [33-35]. Briefly, hu-PBL 20x10^6 cells/mouse were injected intraperitoneally (i.p.), 7 days later animals were injected in the caudal thigh muscle intramuscularly (i.m.) with p41 or APN (at a dose of 50 mg of peptide in 50 ml volume). Control animals received saline. One group of mice was left uninfected, three other groups (treated with saline, p41 or APN, n = 6 per group) were inoculated i.p. with HIV-1_{ADA} at 10^4 of 50% tissue culture infectious doses (TCID_{50}) 30 minutes later. APN, p41 or saline were administered for the next 6 days i.m. and animals were sacrificed 24 h after the last i.m. injection. Spleen tissue samples were collected for flow cytometry analysis and RNA extraction. HIV-1_{gag} RNA expression was determined using real time PCR assays with primers and probes previously
described [36]. All PCR reagents were obtained from Applied Biosystems. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as an endogenous control.

2.2.14 Flow Cytometry

APN and p41 uptake by macrophages was determined by FACS. In a 96-round-bottom plate MDM and compounds were incubated at 37°C for 5-120 minutes. Plate was transferred on ice, cells were washed with ice-cold buffer, and intracellular staining for PEG was performed for cells treated with APN and PEG-PLD<sub>20</sub>. Cells exposed to p41-Cy5 were washed and fixed in 2% PFA solution.

FACS analysis of animal splenocytes was conducted as previously outlined by Gorantla and colleagues [37]. In short, spleens were extracted from the mice at sacrifice and crushed through a 40-µm cell strainer to obtain single cell suspensions. Splenocytes thus isolated were stained for human cells using antibodies to CD45, CD3, CD4, CD8. Appropriate isotype controls were used, and all antibodies were obtained from BD Pharmingen (San Diego, CA, USA). Cells were analyzed using BD LSR II with BD FACS Diva software (BD Immunocytometry Systems, Mountain View, CA, USA). All animals had comparable levels of PBL engraftment.

2.2.15 In vivo evaluation of toxicity and immunogenicity of peptides and APN

Toxicity and immunogenicity of peptide and APN were tested on C57Bl/6 mice by 7 daily i.m. administrations (50 mg peptide or equivalent dose of APN, n = 3 per group) with two week follow up observation. Serum was collected for the detection of antibodies to peptide/polymer by ELISA. ELISA plates were coated with 100 mg/ml of p41, APN and polymer in phosphate buffer
solution overnight, washed and blocked with 3% bovine serum albumin for 1 h. Serial dilutions (1:20-1:2400) of heat-inactivated serum were added for 2 h. Anti-mouse IgM and anti-mouse IgG were detected with reagents and protocol obtained from Bethyl Laboratories, Inc. (Montgomery, TX). Reaction was calculated as differences in end point titers between experimental and saline-treated animals. Tissues (liver, kidney, lung, spleens and brains) were collected in 4% PFA for fixation, embedded in paraffin and analyzed after H&E staining for pathomorphological changes.

2.2.16 Statistical analysis

Data were analyzed using ANOVA and Student’s t test for comparisons. A value of $p < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Characteristics of APN

P41 peptide has a net charge of +6 at pH 7 and is therefore cationic at physiological conditions. Peptide - block ionomer complexes (APN) were prepared by simple mixing of buffered solutions (10 mM phosphate buffer, pH 7 or PBS pH 7.4) of p41 and anionic block copolymer (PEG-PLD or PEG-PLE), which electrostatically bind to each other (Figure 2.1 A). Formation of complexes was confirmed by gel filtration chromatography (Figure 2.2), sedimentation equilibrium analysis (Figure 2.3), and DLS (Table 2.2). P41 was almost completely incorporated into the complexes at the stoichiometric composition of the mixtures. The APN particles were found to be very small (average diameter of approximately 35 nm),
uniform (monomodal, relatively narrow particle size distribution with polydispersity indices (PDI) in the range of 0.1-0.2), and had slightly negative $\zeta$-potential. In a sharp contrast, p41 had a tendency to form positively charged large aggregates (around 900 nm in diameter) in diluted aqueous solutions. The complexes formed by the block ionomer with longer ionic chains (PEG-PLE$_{40}$) appear to be larger. Furthermore, the systems formed by this block ionomer were more polydisperse. The formation of nanosized APN was further confirmed by AFM. A typical image of p41/PEG-PLD$_{20}$ APN is shown in Figure 2.1 B. Analysis of the images revealed that APN appeared to be round-shaped particles with a narrow distribution in size and an average of height of approximately 2-3 nm and width in the range of 20-40 nm depending on copolymer structure. It should be noted that imaging in air usually provides lower numbers for the height because of the drying process, but provides higher numbers for the width, due to the tip-convolution effect. Furthermore, interaction between particles and positively charged mica surface might also result in additional flattening and affect dimensions measured by AFM. In contrast to APN, free p41 peptide deposited on negatively charged mica form large non-structured aggregates (Figure 2.1 B). These observations were in good agreement with the DLS data.

APN formation was further examined using gel filtration chromatography. The retention times for the free p41 and PEG-PLD$_{20}$ were 36.0 min (UV) and 25.5 min (RI), respectively, while APN were eluted at 15.2 min (UV). A similar peak at elution time of 15.8 min was detected in RI profile of APN. The relative area-under-the-curve (AUC) corresponding to p41, PEG-PLD$_{20}$ and APN mixtures of different compositions were calculated and are presented in Figure 2.2. The
AUC analysis as a function of the composition of p41/PEG-PLD$_{20}$ mixture (expressed as $Z_{+/−}$ ratio) revealed a gradual increase in the peak area corresponding to APN (Figure 2.2 C) and concomitant disappearance of the peak corresponding to p41 (Figure 2.2 A) as the relative amount of the copolymer in the p41/PEG-PLD$_{20}$ mixture was increased. The peak area for both APN and p41 remained practically constant at $Z_{+/−} > 1.3$. This indicates that p41 was almost completely incorporated into the APN in the vicinity of stoichiometric charge ratio. At $Z_{+/−}$ ratios greater than 1 an increase in the PEG-PLD$_{20}$ AUC (Figure 2.2 B), corresponding to unbound copolymer, was also observed. These data suggested that an excess of the copolymer beyond the stoichiometric charge ratio ($Z_{+/−} = 1$) was not incorporated into APN. The incorporation of excess of the anionic block copolymers into the APN and tethering of a greater number of PEG chains at the core-shell interface may be unfavorable due to an increase in lateral tension between PEG chains.

The molecular weight average and stoichiometry for p41/PEG-PLD$_{20}$ APN ($Z_{+/−} = 1$) was determined by sedimentation equilibrium. Representative plot of optical density at equilibrium as a function of radius for p41/PEG-PLD$_{20}$ APN ($Z_{+/−} = 1$) is shown in Figure 2.3. Partial specific volume ($\bar{v}$) for APN was determined using the solvent-density-variation method of Edelstein and Schachman by parallel determination of the concentration distributions at sedimentation equilibrium in H$_2$O and D$_2$O solutions of APN and was calculated to be 0.558 ml/g [38]. Molecular weight average for p41/PEG-PLD$_{20}$ APN ($Z_{+/−} = 1$) was obtained from single-component fit to sedimentation equilibrium using the computed value of $\bar{v}$. As it is seen a single-component model showed very good convergence (Figure 2.3, red line), as demonstrated
by the minimum deviation seen in the residuals (upper panel, Figure 2.3). The measured molecular weight of the stoichiometric p41/PEG-PLD20 APN was ca. 687.2 kDa, which suggests that they contained about 148 p41 molecules and 44 copolymer chains.

Figure 2.1 (A) Schematic representation of spontaneous formation of APN as a result of electrostatic coupling of the cationic peptide and anionic block copolymer. (B) Tapping-mode AFM images of (left) p41/PEG-PLD20 APN and (right) p41. Scan size is 2 mm. The insert shows an image of an individual APN particle. Bar equals 30 nm. Samples were deposited onto (left) positively charged APS-modified mica or (right) freshly cleaved negatively charged mica.
Figure 2.2 Relative area-under-the-curve (AUC) for (A) p41, (B) PEG-PLD$_{20}$, and (C) APN calculated from SEC data as a function of composition of mixture ($Z_{-/+}$). Samples were loaded onto a column and eluted using PBS (pH 7.4, 0.14 M NaCl). The peptide concentrations were 46 µM.
Figure 2.3 Sedimentation equilibrium analysis of p41/PEG-PLD$_{20}$ APN ($Z_{+/+} = 1$). (Lower panel) The sample concentration distribution in H$_2$O at equilibrium is shown as a function of radius: sedimentation equilibrium data (circles) and single-component model fit (solid red line). Figure inset shows molecular weight and the variance of all data points. (Upper panel) Residuals of the fit. The peptide concentration is 41 µM. Rotor speed is 5000 rpm.
Table 2.2 Physicochemical characteristics of APN

<table>
<thead>
<tr>
<th>Sample</th>
<th>$D_{\text{eff}}$ (nm)$^b$</th>
<th>PDI$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>p41</td>
<td>902</td>
<td>0.785</td>
</tr>
<tr>
<td>p41/PEG-PLD$_{10}$</td>
<td>32.9 ± 3.5</td>
<td>0.14</td>
</tr>
<tr>
<td>p41/PEG-PLD$_{20}$</td>
<td>36.5 ± 5.5</td>
<td>0.12</td>
</tr>
<tr>
<td>p41/PEG-PLE$_{10}$</td>
<td>31.1 ± 1.7</td>
<td>0.09</td>
</tr>
<tr>
<td>p41/PEG-PLE$_{25}$</td>
<td>43.4 ± 6.1</td>
<td>0.17</td>
</tr>
<tr>
<td>p41/PEG-PLE$_{40}$</td>
<td>71.1 ± 6.9</td>
<td>0.24</td>
</tr>
</tbody>
</table>

$^a$ Complexes were prepared in PBS (pH 7.4, 0.14 M NaCl) at $Z_e/-=1$.

$^b$ Effective diameter ($D_{\text{eff}}$) and polydispersity indices (PDI) were determined by DLS at 25°C (n=3).

The dimensions of APN particles in aqueous dispersions practically did not change upon pH variation between pH 5-9 (Table 2.3). APN maintained their colloidal stability and exhibited no aggregation for a prolonged period of time (at least two weeks) in the absence of salt (10 mM phosphate buffer) and within 24 h at physiological concentrations of salt (0.14 M NaCl). Beyond this time formation of heterogeneous particle population was detected by DLS followed by slow aggregation (Table 2.4).
Table 2.3 Effect of pH on physicochemical characteristics of APN (P41/PEG-PLD20). Complexes were prepared at Z-/+=1 in various 10 mM buffer systems: citrate buffer (pH 5 and 6), phosphate buffer (pH 7), HEPES buffer (pH 8), and Tris buffer (pH 9). Data are means ± SD (n = 3).

<table>
<thead>
<tr>
<th>pH</th>
<th>D_{eff} (nm)</th>
<th>PDI</th>
<th>Particle size distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>37.6 ± 3.7</td>
<td>0.23 ± 0.07</td>
<td>Bimodal</td>
</tr>
<tr>
<td>6</td>
<td>33.9 ± 0.3</td>
<td>0.16 ± 0.04</td>
<td>Unimodal</td>
</tr>
<tr>
<td>7</td>
<td>33.2 ± 0.9</td>
<td>0.09 ± 0.03</td>
<td>Unimodal</td>
</tr>
<tr>
<td>8</td>
<td>40.5 ± 4.7</td>
<td>0.47 ± 0.36</td>
<td>Bimodal</td>
</tr>
<tr>
<td>9</td>
<td>34.6 ± 0.9</td>
<td>0.23 ± 0.05</td>
<td>Bimodal</td>
</tr>
</tbody>
</table>

* All measurements were performed at 25°C using a Nano ZS Zetasizer (Malvern Instruments, UK). Average effective diameter (D_{eff}) and polydispersity indices (PDI) were determined at a scattering angle of 173°.

Table 2.4 Physicochemical characteristics of APN as a function of time. Complexes were prepared in PBS (pH 7.4, 0.14 M NaCl) at Z-/+=1. Data are means ± SD (n = 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>D_{eff}(nm)/PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0h</td>
</tr>
<tr>
<td>p41/PEG-PLD_{10}</td>
<td>32.9±3.5/0.14</td>
</tr>
<tr>
<td>p41/PEG-PLD_{20}</td>
<td>35.5±5.0/0.10</td>
</tr>
<tr>
<td>p41/PEG-PLE_{10}</td>
<td>31.1±1.7/0.09</td>
</tr>
<tr>
<td>p41/PEG-PLE_{25}</td>
<td>43.4±6.1/0.17</td>
</tr>
<tr>
<td>p41/PEG-PLE_{40}</td>
<td>71.1±6.9/0.24</td>
</tr>
</tbody>
</table>
As expected, CD spectra of the p41 in aqueous solution indicated a notable degree of helical structure (Figure 2.4). It exhibited a double minima at ~208 and 222 nm along with a positive ellipticity at ~195 nm, features that are typical of α-helices. The absolute magnitude of these peaks was markedly increased upon binding of p41 to PEG-PLD_{20}. Importantly, PEG-PLD_{20} copolymer at these conditions is characterized by a single broad negative ellipticity centered at ~202 nm, indicative of an unordered structure. These data suggest that incorporation of p41 into APN did not only alter the inherent propensity of the peptides to form α-helical structures but also enhanced the helical content of p41, which is necessary for their antiviral activity.
Figure 2.4 Circular dichroism (CD) spectra of p41 (dashed line), PEG-PLD$_{20}$ copolymer (dotted line), and APN (p41/PEG-PLD$_{20}$) (solid line) in (A) 10 mM phosphate buffer (pH 7.4) and (B) PBS (pH 7.4, 0.14 M NaCl). The peptide concentrations were 100 µM. Complexes were prepared at $Z_{v0}=1$. 
2.3.2 Stability against trypsin digestion

APN stability against proteolytic digestion was studied using Trypsin Spin Columns followed by MALDI-TOF MS (Figure 2.5). The incorporation of p41 into APN resulted in partial protection of peptide against proteolytic digestion by trypsin. Indeed, the MS spectra of p41/PEG-PLD20 APN digested for 5 and 13 min showed the existence of intact p41 with the peak at 2433 (m/z) along with the peaks belong to the p41 fragments (identified as SWLR and IWR, respectively). In contrast, at similar conditions p41 alone was completely disintegrated. Higher stability of APN against protease degradation was additionally confirmed by gel electrophoresis (Figure 2.6).
Figure 2.5 APN displayed enhanced stability against proteolytic degradation. MALDI-TOF spectra represent intact p41 and APN and inserts represent spectra of digested p41 and APN after incubation on trypsin column for 13 min. Spectra show the existence of intact p41 with the peak at 2433 (m/z) along with the p41 fragments (561 and 474) in APN sample, whereas p41 was completely degraded. Red ovals indicate non-digested p41 and arrows show the protected p41 after APN digestion.
Figure 2.6 Gel analysis of APN (p41/PEG-PLD\textsubscript{20}) stability against proteolytic degradation by trypsin. Lanes: (1) protein ladder (2-250 kDa); (2) p41; (3) APN. Lanes 4-7 correspond to samples after incubation on trypsin column for different time period: (4) p41, 5 min; (5) APN, 5 min; (6) p41, 13 min; (7) APN, 13 min. The gel was stained with Commassie Blue.
2.3.3 Hemolytic activity of APN

Considering that cationic p41 is toxic to negatively charged cell membranes, the red blood cell hemolysis assay of p41 and APN was conducted to evaluate their membrane destabilizing activity (Figure 2.7). In this study 1% v/v Triton X-100 and PBS were adopted as the control groups for 100% and 0% hemolysis, respectively. Anionic block copolymers and parental p1 peptide (a net charge of -2 at pH 7) did not cause significant levels of hemolysis at all concentrations in the experimental range. As expected, p41 induced hemolysis in a dose-dependent manner. At 10 µM concentrations p41 revealed 56.70 ± 0.03% of hemolysis. Regardless of concentration, the hemolytic activity of p41 immobilized in APN was significantly reduced compared to free p41.
**Figure 2.7** Hemolytic activity of APN and its constituents as a function of concentration. APN, peptides, or copolymer at different concentrations (peptide equivalents) were mixed with human RBC for 1 h in isotonic solution and hemolytic activity was normalized to positive control, 1% \( \text{v/v} \) Triton X-100 solution, as a 100% hemolysis. Data presented as mean ± SD (n = 3).
2.3.4 Cellular uptake of APN

Cellular uptake of APN was evaluated in Huh7.5 hepatoma cells and monocyte-derived macrophages (MDM). Exposure of Huh7.5 cells to Cy5-labeled p41 led to significant intracellular accumulation of free peptide. However, after continuous incubation for 4 h the damage of cell monolayer was observed and cells started to undergo apoptotic death (Figure 2.8) suggesting the cytotoxic activity of the free cationic peptide. In contrast, APN were less efficiently taken by Huh7.5 cells, but did not induce cytotoxicity or affect the monolayer integrity (Figure 2.8). The accumulation kinetics in MDM, highly active phagocytic cells, suggested a rapid and time-dependent uptake of APN while uptake of PEG-PLD_{20} copolymer alone was negligible (Figure 2.9 A). Notably the free peptide was taken up in MDM almost completely in 5 min (95% of MDM were Cy5-positive). The reduced uptake efficiency of APN at the same time point (45.8% of MDM were loaded with APN) can be attributed to the steric hindrance of PEG chains on the surface of the particles that prevent their interactions with the cells. In order to understand the mechanisms of APN uptake we utilized the attached culture of MDM (Figure 2.9 B, C). Cells were co-incubated with APN for 5 - 120 minutes, washed, fixed and stained with antibodies to PEG and early endosome antigen 1 (EEA1). PEG-positive granules were colocalized with the surface cellular membranes by 5 minutes of incubation (data not shown). After 2 h PEG-positive staining of surface membranes was increased and intracellular colocalization with EEA1 was found. This allows suggesting that APN could be endocytosed by early endosomes and possibly sorted to late endosomes and lysosomes, as well as for recycling to the plasma membrane.
Figure 2.8 Uptake of APN in Huh7.5 cells. Cell cultures were exposed to 10 μM concentrations of p41-Cy5 and APN (p41-Cy5/PEG-PLD_{20}) for 1, 2 and 4 h, washed, fixed and confocal images were captured at 40x objective. Red staining corresponds to p41-Cy5, and cell nuclei were stained with DAPI (blue). Apoptotic cells are indicated by white arrows.
Figure 2.9  APN uptake by macrophages. (A) The time-dependent uptake of APN and PEG-PLD$_{20}$ by MDM in suspension was determined by intracellular staining with rabbit monoclonal antibodies against PEG followed by FACS analysis. The p41-Cy5 uptake was measured by direct fluorescence. (B, C) Detection of APN uptake by attached MDM culture by staining for PEG (red) and co-localization with earlier endosomal antigen 1 (EEA1, green) at 2 h of incubation. Cell nuclei were stained with DAPI (blue). The set of images consists of a single color and overlay (B) and a side view of the XZ optical line scan through the red line, and a side view of the YZ optical line scan through the green line (C). ON side view optical image, yellow color indicates PEG colocalized with EEA1-stained vesicles. Magnification is at 63x/1.4 oil DIC M27. Scale bar 10 µm.
2.3.5 Anti-HCV activity of peptides and APN

Anti-HCV activity of p41 and APN was evaluated against JFH1 virus (MOI 0.1) in Huh7.5 cell line. The cell infectivity and antiviral potency of formulations were determined using RT-PCR (HCV-RNA expression) and FACS analysis (staining of intracellular HCV core protein). As shown in Figure 2.10 A, a significant reduction in intracellular viral RNA was detected in Huh7.5 cells pretreated for 1 h with APN (p41/PEG-PLD, at a dose equivalent to 10 μM p41) prior to HCV infection compared to infected control cells. Notably, the potency of p41 to inhibit the establishment of HCV infection was significantly enhanced after its incorporation into APN compared to free peptide. To assess whether APN can suppress an ongoing HCV infection, the cells that were incubated for 1 h with different doses of APN and then infected with virus and cultured in the presence of APN for 7 days (APN at the corresponding concentrations were replenished at culture medium exchange every 48 h). As shown in Figure 2.10 B, the APN decreased the number of HCV infected cells in a concentration-dependent manner with no sign of toxicity, and at a dose equivalent to 10 μM p41 the infected cells were completely eradicated.

Moreover, the anti-HCV activity of p41-based APN was further confirmed by including more controls. First, anti-HCV activity of APNs prepared from p41 and another two variants of p41 was tested. Two derivatives include single mutant sp1 peptide (SWLRRIWRWISKVLSRFK) in which cysteine residue was substituted to serine, and scrambled form sh peptide (SWRLRIWRWICKSVLRFK), whose amphipathicity was reduced by swapping the positions of its amino acids. The molecular models of p1, p41, sp, and sh peptides are shown in Figure 2.11 for comparison.
When intracellular expression of HCV core protein was quantified by FACS, only the p41-based APN decreased the number of HCV infected cells (~50% reduction), while sp1- or sh-based APN were not active (Figure 2.1A). Moreover, the copolymer alone did not exert any antiviral effect (Figure 2.1B). Meanwhile, it is worth mentioning that prolonged exposure of the infected cells to free peptides in similar treatment regimen resulted in extensive cell loss, thereby confirming the propensity of APN to suppress the cytotoxicity of cationic peptide. Overall, these data suggest that APN are able to inhibit HCV infection both extracellularly and within infected cells.
Figure 2.10 APN anti-HCV activity *in vitro*. (A) Intracellular HCV RNA after 1 h pre-treatment of Huh7.5 cells with p41 and APN at a dose equivalent to 10 µM peptide prior HCV infection. APN demonstrated enhanced ability to suppress HCV infection. Data presented as mean ± SD (n = 3); * - p < 0.05. (B) FACS analysis of Huh7.5 cells pretreated for 1 h with APN at various peptide concentrations before infection with following 3 additional treatments during culture medium exchange. APN significantly suppressed HCV core antigen expression.
Figure 2.11 Molecular model of α-helical peptides from front (left) and back (right) side using the VMD software. (A) p1 peptide (SWLRIWDWICEVLSDFK). (B) p41 peptide (SWLRRRIWRWICKVLSRFK). (C) single mutant sp1 peptide (SWLRRRIWRWICKVLSRFK). (D) scrambled sh peptide (SWRLRIWRWICKSVLRFK) with reduced amphipathicity. Nonpolar residues: gray; cationic residues (R and K): blue; anionic residues: red; cysteine: gold. All the models were built based on α-helix templates. Figure is courtesy of Dr. Sorin Luca.
Figure 2.12 FACS analysis (staining of the intracellular HCV core protein) of Huh7.5 cells. (A) Anti-HCV activity of several APNs based on p41 and its two variants: single mutant sp1 peptide (SWLRRRIWRWISKVLSRFK) and scrambled non-amphipathic sh peptide (SWRLRIWRWICKSVLRFK). Cells were pretreated with different APNs (2.5 μM on peptide basis) for 2 h, infected with JFH-1 virus (MOI 0.1) overnight, and then cultured in the presence of APNs for another 48 h post-infection. Only the APN based on p41 decreased the number of HCV infected cells while sp1- or sh-based APN were not active. APN displayed lower toxicity compared to free peptides, as indicated by low cell number after the treatment. (B) The block copolymer alone (PEG-PLD20) does not exert any anti-HCV effect.
2.3.7 Anti-HIV activity of peptides and APN in vitro

As was shown HCV C5A protein-derived peptide (in our studies designated as p1) added with HIV-1 to TZM-bl cells for 4 h prevents HIV infection via neutralization (destabilization) of both free and cell-bound viral particles [20, 21]. We tested the ability of the peptides (both p1 and p41), copolymer alone, and APN (p41/PEG-PLD_{20}) to prevent infection after 2 h pretreatment of cells, assuming prolonged peptide activity in the complex. We found that pretreatment of TZM-bl cells with copolymer alone or p1 did not prevent the infection. Interestingly, in this cell model p41 exhibited stronger antiviral activity compared to parental p1 peptide that was further significantly enhanced by its incorporation in APN (Figure 2.13 A). Also, pretreatment of activated PBL for 2 h before infection following by incubation of cells with 2.5 and 5 mM of APN (on p41 basis) had superior activity compared to the free peptides (Figure 2.13 B). When MDM were pretreated for 2 h with 10 mM of peptides or APN before infection and drugs were added every other day with culture medium exchange, only APN suppressed viral replication by more than 90% (Figure 2.13 C). It is also important to note that similarly to Huh7.5 cells the multiple treatments of infected MDM with free peptide were toxic to the cells while no significant changes in MDM viability were detected upon treatments with APN (Figure 2.14). These data suggest that incorporation of p41 into APN substantially potentiate and prolongate an antiviral activity of peptide against HIV.
Figure 2.13 APN suppress HIV-1 replication in cell cultures. (A) Pretreatment of TZM-bl cells for 2 h before HIV-1ADA infection with p41 showed reduction of the number of infected cells. Protective effects of APN were observed even at the lower concentrations of peptide. (B) APN pre-treatment exhibited superior protection of PHA-activated human lymphocytes from HIV-1ADA infection compared to p41 and p1. (C) APN exhibited the most potent antiviral activity and suppressed the ongoing HIV infection. MDM were pre-treated for 2 h, HIV-1ADA infected overnight, washed and additional treatment with APN and peptides at a dose equivalent to 10 µM peptide were performed every other day. RT activity was adjusted to MTT values/well. Data presented as mean ± SD (n = 4); * - p < 0.05 statistically significant differences between p41 and APN.
Figure 2.14 MDM were pretreated for 2h with 10 µM of peptides or APN before HIV-1 infection and drugs were added every other day with culture medium exchange (black bars). MTT assay was done at the end of experiment (day 10). The viability of APN-treated HIV-1-infected MDM was comparable with uninfected cells (open bar) and exceeded viability of cells treated with p41. Data presented as mean ± SD. * - p < 0.01.
2.3.8 In vivo anti-HIV activity of APN

Initially, the toxicity of APN and their constituents was assessed by i.m. administration in 6-week-old C57Bl/6 mice. Animals were inoculated with the corresponding formulation everyday over the 7 days followed by two-week observation of their well-being. No alterations in animal behavior, body weight or hypersensitivity reactions were observed during the experiment. Histopathological analysis by light microscopic examination of H&E stained tissue sections did not reveal any pathological changes as a result of treatments.

As a proof of concept that APN can suppress HIV-1 replication after systemic administration in vivo, we performed an experiment where NSG mice were transplanted with human hu-PBL. In 30 min prior i.p. virus inoculation mice were injected i.m. with 50 µg of free p41 or its APN format. Animals were then treated with p41 or APN on daily basis for the next six days, and were euthanized on day 7 post infection. An additional group of animals was treated with saline as a control. As shown in Figure 2.15, within 7 days the total number of human lymphocytes in the spleen of animals infected with HIV-1 was not significantly reduced compared to uninfected animals (Figure 2.15 A). However, the number of CD3+CD4+ cells significantly declined compared to uninfected control group (24.8 ± 0.6% versus 32.6 ± 2.5%, \(p = 0.033\)) (Figure 2.15 B, C). The drop in CD4:CD8 cell ratio was also observed in infected animals (0.38 ± 0.01 versus 0.56 ± 0.09 for uninfected group, \(p = 0.013\)) suggesting the loss of CD4+ cells due to HIV infection (Figure 2.15 D). Consistent with these results, the presence of high levels of HIVgag RNA expression was detected within spleen tissue of the infected animals (Figure 2.15 E). These readouts, known to be associated with HIV-1 infection in hu-PBL-NSG mice, were all
affected by treatments with APN. Significant reduction in viral RNA expression was detected in spleen tissue of animals treated with either APN or p41 alone (Figure 2.15 E). However, APN treatment was more effective in inhibition of virus replication. Indeed, the median normalized to GAPDH RT-PCR values in APN-treated group were 2.08 ($p = 0.004$) compared to 690 in HIV-infected control group, while the p41 treatment reduced this value only to 120 ($p = 0.026$). In particular, in four of six animals in APN-treated group the $HIV_{gag}$ expression was significantly suppressed ~ by $2 \log_{10}$ (in the range of 0.63 to 2.48). In agreement with APN anti-viral effects the percentage of CD3+CD4+ cells in this group was not different from uninfected and significantly higher than in infected or p41-treated animals (Figure 2.15 B, C). Although treatment with p41 alone resulted in reduced viral RNA expression, it was not protective for CD3+CD4+ cell number. A similar pattern was observed for CD4:CD8 cell ratio (Figure 2.15 D). Thus, these observations confirm that APN possess anti-HIV activity in vivo.
Figure 2.15 Anti-HIV activity of APN in vivo. NSG mice were transplanted with hu-PBL and 7 days later i.p. inoculated with HIV-1_{ADA} 10^4 TCID_{50}. In 30 min prior virus inoculation mice were injected i.m. with 50 mg of free p41 or its APN format. Animals were then treated with p41 or APN on daily basis for the next 6 days, and were euthanized on day 7 post infection. The uninfected (open bars) and HIV-infected (black bars) animals with similar engraftment of human cell (CD45+ cell % in spleen, A) were compared for CD4+ and CD8+ T cell numbers (B, C), CD4:CD8 ratios (D) and HIVgag RNA tissue expression (E). APN treatments significantly protected CD4+ cells and suppress HIVgag expression in spleen tissue. Gray lines in panel E represent median of viral RNA normalized to GAPDH values for treated groups of animals (n = 6). * - p < 0.01, # - p < 0.03, ## - p < 0.004 compared to HIV-infected control animals.
2.4 Discussion

A virocidal peptide (C5A) was found to be effective in inhibition of HCV and HIV infections as well as other *Flaviviridae* members *in vitro* [21]. This peptide derived from the membrane anchor domain of the HCV nonstructural protein NS5A prevents an initiation of HCV infection by destroying the virus and suppresses ongoing infections by blocking the cell-to-cell spread of the virus. It was suggested that C5A recognizes cellular components of virus membranes, most likely their lipid composition. A cationic derivative of this peptide (p41) was also found to display virocidal activity against HCV. The limitations to the use of these peptides as therapeutics are their rapid elimination from circulation, inactivation by proteases present in the body, as well as unfavorable toxicity profile typical for cationic peptides. To overcome these restraints and ensure prolonged stability of p41 molecule in an active form, the cationic antiviral peptide p41 was incorporated into polyion complexes, APN, with anionic biodegradable PEG-poly(amino acid) block copolymers (*Figure 2.1 A*). Electrostatic coupling of the negatively charged carboxylic groups of the block copolymer and positively charged amino groups of p41 leads to the formation of hydrophobic domains, which segregate in aqueous media into a peptide/polyion core of polyion complex micelles. Water-soluble nonionic segments (here PEG) prevent aggregation and macroscopic phase separation. As a result, these APN self-assemble into particles of nanoscale size and form stable aqueous dispersions at the physiological conditions (pH, ionic strength). We found that the length of the anionic segment of the block copolymer need to be around 10-20 monomer units to ensure the formation of well-defined particles with unimodal distribution and low polydispersity. At these conditions the sizes, charge and
morphology of the resulting APN did not depend on the chemical structure of the block copolymers. This study demonstrated that binding of p41 to the block ionomer resulted in stabilization of α-helical structure of the peptide that is functionally linked to its virocidal activity. Notably, incorporation of p41 into the cores of the complexes was able to significantly reduce cytotoxicity and hemolytic activity associated with cationic peptides. Moreover it delayed proteolytic degradation of the peptide. The enhanced stability of APN against inactivation by proteases in combination with decreased cytotoxicity may result in extended circulation time and allow their administration at higher doses.

APN demonstrated time-dependent cellular accumulation in both macrophage and Huh7.5 cell models. PEGylation is known to reduce interaction of particles with cells due to the formation of a hydrophilic stealth coating around the particles leading to reduced uptake [39]. Thus, it was not surprising that APN were taken up by the cells at a slower rate than free peptide. Our data suggest that following the entry in macrophages APN trafficked to early endosomes. Both viruses are known to exploit the cell-encoded pathways of intercellular vesicle trafficking, exosome exchange, for both the biogenesis of viral particles and transmission [40-45]. Thus, it is likely that APN might pursue intracellular pathways similar to viruses and destroy them in earlier endosomes or other endocytic compartments before they enter the cytoplasm or are degraded in lysosomes. Similarly to non-formulated peptide, APN were able to inactivate both HIV and HCV upon direct interaction in media. The infectivity of the viruses treated in such a way was significantly reduced. We also observed that APN retained intracellular anti-HCV and anti-HIV activity. Notably, APN significantly exceeded anti-viral activity of the non-modified peptides.
when cells were infected after pretreatment with APN. The prolonged antiviral effect of APN in cells (up to 48 h post-withdrawal of the treatment) is most likely due to its improved stability. We believe that the block copolymer chains in the APN can sterically protect p41 molecules against degradation by intracellular proteases. This is further supported by the fact that in spite of the lower uptake of APN compared to free peptide, the internalized fraction remained more active over time. When administered in vivo APN appeared to be well tolerated by the animals, as judged by their general behavior and absence of toxicity signs. APN was able to suppress viral replication and prevent loss of CD4+ cells in spleen of the treated mice, while naked peptide did not have such CD4+ cell protective activity. Overall, we demonstrated that the antiviral peptides incorporated into the protective polymer scaffold exhibited increased stability, bioavailability, retained virocidal activity, and have therapeutic potential. Despite the absence of histological evidences of toxicity, the observed high levels of p41-induced in vitro hemolytic activity and possible death of activated human lymphocytes cannot be excluded and may preclude the therapeutic application of cationic p41 peptide even in the form of APN. Nevertheless, presented data supports the hypothesis that incorporation of antiviral peptides into block ionmer complexes can address the challenges of protein therapeutic delivery by improving stability, reducing toxicity, and increasing bioavailability.

2.5 Conclusion

Well-defined nanocomplexes of positively charged antiviral amphipathic α–helical peptides were prepared by electrostatic coupling with anionic biodegradable block copolymers based on
poly(amino acids). Our in vitro studies suggest that incorporation of cationic peptides into APN substantially attenuate their intrinsic cytotoxicity while preserving an antiviral activity against both HIV and HCV viruses. As a proof-of-concept, we demonstrated that APN were able to decrease the viral load in mice transplanted with human lymphocytes and HIV-1-infected without signs of toxicity to the animals. The unique self-assembly behavior and the simplicity of the preparation make the APN approach an extremely promising platform for the delivery of therapeutic peptides. The distinctive virocidal mechanisms of action of amphipathic α-helical peptide and proposed strategy of therapeutic delivery by APN may provide a potentially broad applicability in combination with standard therapeutics for HIV/HCV drug resistance, advanced end-stage liver disease, and for the reduction of HCV viral rebound after liver transplantation for monoinfected patients. APN inclusion in the therapeutic strategies may also shorten the treatment of HCV monoinfected patients with either IFNα or DAA and reduce the risk of non-responsiveness due to viral mutations.

2.6 References


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CHAPTER III

LIVER-TARGETED ANTIVIRAL PEPTIDE NANOCOMPLEXES AS POTENTIAL ANTI-HCV THERAPEUTICS

3.1 Introduction

More than 185 million people worldwide are infected with the Hepatitis C Virus (HCV) [1]. This blood-borne pathogen leads to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, and it is also the primary cause of liver transplantation in the United States [2]. In spite of being such a fatal disorder, the standard of care until 2011 was restricted to the combination of pegylated interferon-α (PEG-IFN-α) and ribavirin, which exhibited limited sustained viral response and poor tolerability [3]. The emergence of direct-acting antivirals (DAA), which specifically block HCV replication, has marked a new era in HCV therapy [4]. However, the challenges such as affordability, genotype selectivity and possible long-term resistance to DAA therapy due to viral mutations [5] still necessitate the development of alternative anti-HCV drug candidates. As an option, therapeutic peptides are revolutionizing the paradigm of drug development with their prominent bioactivity [6], and, in fact, several anti-HCV peptides, targeting different stages of HCV life cycle, have been discovered [7-9]. One such, the 18-mer C5A, which is derived from the membrane anchor domain of HCV nonstructural protein

NS5A and exhibits broad spectrum antiviral activity with submicromolar potency in vitro [10, 11]. The characteristics of α-helicity and amphipathicity allow this peptide to neutralize the viral particles by destabilizing the lipid composition of viral membranes without genotype dependency [12]. Its cationic derivative, with 4 residues in C5A replaced with positively charged amino acids (lysine and arginine), which we refer to as p41, also displays equivalent antiviral potency. However, the susceptibility to proteolysis and the unfavorable toxicity profile, typical for cationic peptides, remain the obstacles in their translation to clinics.

In an attempt to overcome these restraints, our group has successfully developed the well-defined antiviral peptide nanocomplexes (APN) through the immobilization of cationic p41 into nanoscale block ionomer complexes with oppositely charged poly(amino acid)-based block copolymers [13]. The p41 encapsulation into APN led to higher proteolytic stability, reduced cytotoxicity and unaltered antiviral potency of the peptides. The self-assembly behavior and preparation simplicity make the APN an extremely promising approach for peptide delivery. Using the APN platform, a more selective delivery of p41 to hepatocytes as primary sites for HCV replication can be achieved through targeting the asialoglycoprotein receptor (ASGP-R), which is a well-defined endocytic receptor primarily expressed on parenchymal liver cells [14-16]. Therefore, we hypothesize that the decoration of the APN surface with the ligand β-D-galactose (Gal), which possesses high binding affinity to ASGP-R (Kd ~ 10^{-3}–10^{-4} M) [17, 18], will target APN to the liver, thereby providing a basis for organ-specific anti-HCV therapy development.
Here, we have successfully prepared a series of APN with varying densities of the Gal ligand on the surfaces (Gal-APN) and evaluated their antiviral activities in cell culture systems. Biodistribution studies demonstrated preferential liver accumulation of Gal-decorated APN proving the effectiveness of this targeting approach for the delivery of antiviral peptides.

3.2 Materials and Methods

3.2.1 Materials

Peptide p41 (SWLRRIWRWICKVLSRFK) and Cy5-labeled p41 were custom synthesized by AnaSpec (USA). α-(9-Fluorenylmethoxycarbonyl)amino-ω-carboxy succinimidyl ester poly(ethylene glycol) (Fmoc-PEG-NHS, $M_W$ (PEG) = 5000 g mol$^{-1}$, $M_w/M_n$ = 1.02) was purchased from Jenkem Technology (China). Amberlyst® 15 hydrogen form, molecular sieve UOP type 3 Å, L-glutamic acid γ-benzyl ester, D (+)-galactose, propargylamine, L(+)-ascorbic acid sodium salt and copper (II) sulfate pentahydrate, 98+%, A.C.S. reagent were obtained from Sigma-Aldrich (U.S.). Silica gel (for chromatography, 0.03-0.200 mm, 60 Å), 2-bromoethanol, ethyl acetate, methanol, dichloromethane (DCM), dimethylformamide (DMF), tetrahydrofuran (THF) were purchased from Acros Organics (USA). S-2-(4-Isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid (SCN-DTPA) was ordered from Macrocyclics (Dallas, TX, USA). Lutetium-177 trichloride was obtained from PerkinElmer (USA).

3.2.2 Synthesis of Gal-terminated poly(ethylene glycol)-block-poly(L-glutamic acid)
copolymers (Gal-PEG-b-PLE)

Gal-PEG-b-PLE copolymer was synthesized using the following steps (Scheme 3.1):

Scheme 3.1 Synthesis of Gal-PEG-b-PLE via NCA-based ring-opening polymerization and subsequent click chemistry.

3.2.2.1 Synthesis of propargyl-PEG-NH₂

Fmoc-PEG-NHS (0.5 g, 0.1 mmol) was dissolved under stirring in 5 ml of DCM at 0°C. The propargylamine (80 µl, 1.2 mmol) dissolved in 5 ml of cold DCM was added dropwise and the solution was stirred for 24 h. The solvent was removed at reduced pressure and the product was dialyzed against distilled water (MWCO 2 kDa) followed by lyophilization to give
Fmoc-PEG-propargyl. Standard 20% v/v piperidine/DCM solution (3 ml) was used to remove the Fmoc-protecting group. After stirring for 2 h at room temperature, DCM was removed by rotaevaporation, reaction mixture was redissolved in water and after filtration dialyzed against distilled water (MWCO 2 kDa) for 2 days, and then lyophilized to obtain propargyl-PEG-NH₂.

3.2.2.2 Synthesis of propargyl-PEG-b-PLE

The BLE-NCA (γ-benzyl L-glutamate-N-carboxyanhydride) monomer was prepared as described previously [19]. Benzyl-protected propargyl-PEG-b-PBLE was synthesized via ring-opening polymerization of BLE-NCA monomer using propargyl-PEG-NH₂ as a macroinitiator. Propargyl-PEG-NH₂ (20 mg, 0.004 mmol) was dissolved in 300 µl of anhydrous DMF with 3 µl of triethylamine (TEA) (5 equivalent of PEG) and then added into water-free and oxygen-free ampules. BLE-NCA (26.3 mg, 0.1 mmol, the feed molar ratio of propargyl-PEG-NH₂ to BLE-NCA was 1:25) dissolved in 250 µl of DMF was added dropwise under nitrogen. The ampule was sealed and the reaction was allowed to proceed at 40°C for 48 h. The solvent was evaporated under vacuum and the residue was dissolved in THF followed by addition of 2 N NaOH to deprotect glutamate residues. After stirring for 4 h at 40°C, THF was removed at reduced pressure, the residual solution was neutralized by 1 M HCl, dialyzed using a dialysis membrane (MWCO 3.5 kDa) against distilled water for 48 h, and lyophilized to obtain propargyl-PEG-b-PLE.

3.2.2.3 Synthesis of 2’-azidoethyl-O-D-galactopyranoside

The 2’-azidoethyl-O-D-galactopyranoside was synthesized according the procedure reported
by Geng et al. [20]. Briefly, the Amberlyst® 15 hydrogen form (8.0 g) and molecular sieve (4.0 g) suspended in 2-bromoethanol (50 ml) were refluxed for 30 min followed by addition of D(+)galactose (8.0 g). After another 3 h reflux, the product formation was confirmed by TLC (ethyl acetate/methanol, 7/1, v/v, Rf: 0.3), reaction mixture was filtered, and rotaevaporated. The crude 2’-bromoethyl-D-galactopyranoside was further purified by flash column chromatography on silica gel. The obtained 2’-bromoethyl-D-galactopyranoside (3.0 g, 10.5 mmol) and sodium azide (2.1 g, 21 mmol) were further dissolved in water/aceton (7.5 ml/45 ml) and refluxed for 20 h. After removal of solvents by rotaevaporation, the residue was suspended in ethanol and filtered to remove an excess of salts (sodium azide and sodium bromide). The filtrate was concentrated under vacuum to obtain the final product as a white solid. The structure was characterized by 1H-NMR [Bruker AVANCE III instrument (400 MHz)] and mass spectrometry (Qtof Micro electrospray ionization mass spectrometer, Waters, USA).

3.2.2.4 Galactosylation via click reaction

The 2’-azidoethyl-O-D-galactopyranoside (3.12 mg, 20 µmol) was reacted with propargyl-PEG-b-PLE (20 mg, 4 µmol) dissolved in 100 µl of a water and methanol (1:1) mixture in the presence of CuSO₄ ⁵H₂O (62.42 µg, 0.25 µmol), tris-(hydroxypropyltriazolylmethyl)amine (218.5 µg, 0.5 µmol) as the stabilizing agent and ascorbic acid (495.28 µg, 2.5 µmol) for 2 days at room temperature. The product was dialyzed against distilled water (MWCO 3.5 kDa) for another 2 days to remove all the unreacted small molecules and then lyophilized to produce Gal-PEG-b-PLE. The content of residual copper
present in the product was determined using the inductively coupled plasma mass spectrometry (ICP-MS, NexION 300Q, PerkinElmer) by diluting standards and samples in double distilled 2% (v/v) nitric acid.

3.2.2.5 Polymer characterization

The composition of propargyl-PEG-b-PBLE was confirmed by \(^1\)H-NMR in DMSO-\(d_6\) at 80°C using a Bruker 400 MHz spectrometer. Gel permeation chromatography (GPC) measurements to determine the molecular weights and polydispersity (PDI = \(M_w/M_n\)) of the polymers were carried out at 40°C using a Shimadzu liquid chromatography system equipped with TSK-GEL® column (G4000HHR) connected to Shimadzu RI and UV/vis detectors. DMF was used as mobile phase at a flow rate of 0.6 ml/min. Poly(ethylene glycol) standards (Agilent Technologies, USA) with a molecular weight range of 106 - 34,890 were used to generate the standard curve. The Gal conjugation efficiency was determined by phenol-sulfuric acid method in microplate format [21]. Briefly, 150 µl of concentrated sulfuric acid was added to 50 µl of each sample in a series of standard aqueous solutions of D(+)Galactose with concentrations ranging from 0.02 mM to 4 mM or Gal-PEG-b-PLE, followed by adding 30 µl of 5% phenol in water. Samples were incubated for 5 min at 90°C in a water bath. After cooling to room temperature for 5 min, the absorbance at 490 nm was measured using SpectraMaxM5 spectrophotometer (Molecular Devices Co., USA).

3.2.3 Gal-APN preparation and characterization

Gal-APN (Z\(_{/+}\) = 1) were prepared by mixing buffered solutions (PBS, pH 7.4) of p41 and
anionic block copolymers (Gal-PEG-b-PLE and propargyl-PEG-b-PLE) as described earlier [13]. The composition of the mixtures ($Z_{\pm}$) was calculated as a molar ratio of carboxylic groups in the copolymers to the amino groups of lysine and arginine residues of the peptide. To obtain the APN with varying Gal densities on the surface, Gal-PEG-b-PLE and propargyl-PEG-b-PLE were combined in different molar ratios (Scheme 3.2). Cy5-labeled formulations were prepared in the same way by introducing 10 mol% of Cy5-labeled p41 into the APN. For all in vitro cellular studies, APN concentration was 5 µM on the basis of p41. Intensity-mean $z$-averaged particle diameter ($D_{\text{eff}}$) and polydispersity index (PDI) of the APN were determined by dynamic light scattering (DLS) using Nano ZS Zetasizer (Malvern Instruments, UK). All measurements were performed in automatic mode at 25°C at a fixed 173° scattering angle. Software provided by manufacturer was used to calculate the $D_{\text{eff}}$ and PDI of APN. Data are presented as mean values ± SEM (n = 3).

3.2.4 Western Blot analysis

The cell lysates of HepG2, Huh 7, primary human hepatocytes, Huh 7.5, and JFH-1-infected Huh 7.5 were prepared by using 1x SDS sample buffer and were quantified with a Micro BCA™ protein assay kit (Pierce, USA). After cell lysates were mixed with β-mercaptoethanol with the ratio of 9:1, an equal amount of protein from each lysate was subjected to SDS-PAGE and then transferred to PVDF membrane at 80 mA and 4°C for overnight. Next day, membranes were blocked with 10% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h at room temperature. The membranes were then incubated with rabbit polyclonal ASGP-R1
antibody (Thermo Scientific, USA) diluted 1:1000 for overnight at 4°C and then with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Thermo scientific, USA) with 1:5000 dilution for 1 h. Both primary and secondary antibodies were diluted with TBST containing 10% nonfat milk. The membranes were washed in TBST for 30 min before the immunoblots were developed with ECL (Pierce, USA). An equal protein load was confirmed by immunoblot with β-actin.

Scheme 3.2 Schematic illustration of the preparation of Gal-APN.

3.2.5 Cell culture

Huh 7, Huh 7.5, HepG2 (hepatocarcinoma cell lines) and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Primary human hepatocytes isolated from a 61-year-old female
with 70% viability were obtained through the Liver Tissue Cell Distribution System (Pittsburg, PA) funded by NIH (contract # HSN276201200017C). They were seeded on the coverslips coated with collagen IV and cultured in William's E medium supplemented with nicotinamide, HEPES, sodium bicarbonate, sodium pyruvate, glucose, glutamine (Acros), L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, penicillin/streptomycin (Sigma), dexamethasone (Alexis Biochemical), ITS+Premix (BD) and 10% FBS. All cells were cultured at 37°C, 5% CO₂.

3.2.6 Cellular uptake studies

Huh 7 or HepG2 cells (10,000 cells/well) or primary human hepatocytes (300,000 cells/well) were grown on sterile glass coverslips placed in 24-well plates and exposed to Cy5-labeled Gal-APN, APN or p41. After determined time periods, the cells were rinsed, fixed with 4% paraformaldehyde (PFA) at room temperature for 30 min, and stained with 4',6-diamidino-2-phenylindole (DAPI). The slides were mounted with ProLong® Gold Antifade (Invitrogen, USA) and images were acquired using a LSM 710 confocal microscope (Carl Zeiss, USA). In select experiments, cells were exposed to Cy5-labeled Gal-APN with surface Gal densities ranging from 5% to 70% for 2 h, followed by washing and visualization under the confocal microscope. All the treatments were performed in duplicate. About 20 images containing 1,000-1,500 cells were collected for each time point. ImageJ (NIH) software was used for the quantification of the fluorescence from Cy5-labeled APN or p41. Mean pixel intensities in each image were normalized to the total cell number by counting the number of DAPI-labeled nuclei. For the competition assay, HepG2 (100,000 cells/well in 24-well plates) were pretreated
with free D(+)-galactose (100 mM) for 0.5 h then co-incubated with APN formulations for another 1 h and imaged as described above.

For assessment of cellular uptake by flow cytometry, Huh 7 cells (400,000 cells/well) were exposed to Cy5-labeled Gal(35%)-APN or APN for 2 h, washed with PBS, trypsinized at 37°C, and harvested by centrifugation (1500 rpm, 5 min). Thereafter, the cells were resuspended in PBS, stained using LIVE/DEAD (Invitrogen, USA) to assess cell viability, fixed with 4% PFA and the median fluorescence intensity (Cy5) of each sample was measured by flow cytometry (Becton Dickinson LSR II) and analyzed using FlowJo software (Tree Star Inc.). At least 10,000 live cells from each sample were acquired in linear mode, gated to exclude debris and dead cells, and visualized in logarithmic mode. For the competition assay, D(+)-galactose (100 mM) was added to the cells for 0.5 h followed by co-incubation with Gal(35%)-APN or APN and the experiment was carried out as described above.

3.2.7 In vitro anti-HCV activity

To study the antiviral efficacy of Gal-APN in cell culture systems, full-length genotype 2a HCV clone JFH-1 (provided by Dr. Takaji Wakita) that replicates and produces infectious viral particles was used as a source of virus [22]. Huh 7.5 hepatoma cell line, which support HCV replication in vitro, was obtained from Dr. Charles Rice (The Rockefeller University, New York, NY) and was used in all experiments requiring viral infection. After attachment of 200,000 Huh 7.5 cells/well to 6-well plates, cells were infected with JFH-1 virus (MOI 0.1) overnight, then virus was removed and cells were replenished with fresh medium containing APN or p41 for
another 48 h (post-treatment regimen). To study virocidal effects, JFH-1 virus stock solution was pre-incubated with APN or p41 for 1 h at 37°C and then these mixtures were used to infect Huh 7.5 cells at MOI 0.1 overnight. Supernatant was removed and cells were replenished with fresh media for another 48 h. HCV RNA quantification was performed using a StepOne Realtime PCR system amplifying a highly conserved sequence in the 5’ UTR of the viral genome. Total cellular RNA was extracted using the TRIzol reagent (Invitrogen, USA) and was subsequently converted to cDNA using Verso cDNA Synthesis Kit (Thermo Scientific, USA). The following primers and probe for this consensus sequence were designed using PrimerExpress Software v2.0 (Applied Biosystems): 5’UTRF GACCGGGTCCTTTCTTGGAT; 5’UTRR CCAACACTACTCGGCTAGCAGTCT; probe FAM-ATTTGGGCGTGCCCCCGC-NFQ. Positive and negative controls were included in all runs. The relative HCV RNA expression level in infected cells was quantified after normalization to cellular GAPDH mRNA levels.

3.2.8 Assessment of intracellular HCV localization

Huh 7.5 cells (50,000 cells/well in 24-well plates) were treated with Gal-APN, APN or p41 for 5 h, infected with JFH-1 virus (MOI 0.1) overnight, virus was removed and cells were incubated for another 48 h in fresh media. After treatment, cells were fixed with 4% PFA for 15 min at 37°C and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. Goat serum in PBS (5%) was used for blocking for 30 min. For co-localization analysis of HCV core protein with lipid droplets, cells were incubated with primary HCV core antigen antibody (clone: C7-50) (Thermoscientific, Cat# MA1-080) for 1 h with dilution of 1:300. For co-localization
analysis of NS5A with lipid droplets, cells were incubated with HCV NS5A antibody (1:2000) for 1 h. After washing, cells were probed with the mixture of corresponding Alexa Fluor 594-labeled secondary antibody for NS5A or HCV core and BODIPY for lipid droplets (1:100) for another 1 h. At the end, nuclei were labeled with DAPI and cells were subjected to confocal imaging analysis.

3.2.9 The effect of Gal-APN on ISGs expression

Huh 7.5 cells (200,000 cells/well) were seeded on 6-well plate for overnight. Cells were treated with Gal(35%)-APN (5 µM) for 48 h after overnight viral infection (MOI 0.1), and 4 h before harvesting, ISGs were activated by IFN-α (100 units/ml). Total cellular RNA was isolated using TRizol reagent and subsequently converted to cDNA. Antiviral ISGs [2’-5’-oligoadenylate synthetase 1 (OAS-1) and viperin] abundance and HCV RNA replication were quantified by real-time qPCR.

3.2.10 Biodistribution study

3.2.10.1 Synthesis of $^{177}$Lu-DTPA-p41

Prior to radiolabeling, the radiometal chelator (DTPA) was non-selectively conjugated to the amine groups on the p41 peptides. Briefly, 1 mg of p41 (0.14 µmol) and TEA (0.29 µl, 5 equivalent of p41) were dissolved in DMF. Upon stirring, SCN-DTPA (1.5 equiv of p41) in DMF was added dropwise to solution. The reaction mixture was kept stirring at room temperature for overnight. DTPA-p41 was characterized and peak-purified by RP-HPLC/MS. The UV detector connected to HPLC was set at 280 nm and 220 nm. The mobile phase was composed of water
containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). For all purifications, the initial gradient of 62% A:38% B linearly decreased to 40% A:60% B over a 12 min time period. At the end of the run time for all HPLC experiments, the column was flushed with the gradient of 5% A:95% B and re-equilibrated to the starting gradient. To synthesize \(^{177}\text{Lu-DTPA-p41}\), 30 µg of DTPA-p41 was incubated with 2.5 mCi \(^{177}\text{LuCl}_3\) in ammonium acetate buffer (1 M, 100 µl, pH 5.5) for 1 h at room temperature. The resulting \(^{177}\text{Lu-DTPA-p41}\) was peak-purified by RP-HPLC equipped with Flow Count radiometric detector system (Bioscan, USA) and UV detector (220 nm and 280 nm). The isolated radiochemical yield was 10%-20%. L(+)‐ascorbic acid sodium salt (10 mg) was added to the purified radioconjugate to reduce radiolysis. To remove the bulk of the acetonitrile and concentrate the radiolabeled peptide, dry nitrogen was applied to the mixture of \(^{177}\text{Lu-DTPA-p41}\) and non-labeled p41.

\(^{177}\text{Lu-labeled APN formulations were prepared by following the same procedure as described in 2.3.}

### 3.2.10.2 Animal studies

Biodistribution study of APN (targeted and non-targeted) and p41 were carried out on BALB/c mice (average weight, 25 g). Each animal was intravenously injected with 50 µg of \(^{177}\text{Lu-labeled p41}\) or equivalent dose of \(^{177}\text{Lu-labeled Gal-APN or APN}\) (n = 6 per group) carrying 5 µCi radioactivity in 100 µl saline. After the designated time (15 min and 4 h), the mice were sacrificed, and tissues (liver, spleen, kidney, lung and bone) were excised, rinsed and weighed. The \(^{177}\text{Lu radioactivity of excised organs, tail, urine + bedding, and carcasses was measured using}\)
a gamma counter, and the percentage of injected dose per gram (%ID/g) was calculated for each organ.

Perfused liver was dissociated in the presence of collagenase using a gentle MACS dissociator (Mitenyi Biotec, Germany). The dissociated liver was centrifuged at 700 rpm for 5 min to obtain the pellet of hepatocytes. Subsequently, non-parenchymal cells (NPCs) were harvested by further centrifuging supernatant for 5 min at 1500 rpm. The $^{177}$Lu radioactivity of hepatocytes and NPCs was measured using a gamma counter. The cell number and viability were determined by the trypan blue exclusion method.

### 3.2.11 Statistical analysis

The data were expressed as mean ± standard deviation. For comparison between two groups, the data were analyzed using student’s $t$ test. For multiple groups, one-way analysis of variance (ANOVA) was used. Differences were considered statistically significant at a value of $p < 0.05$.

### 3.3 Results

#### 3.3.1 Preparation and characterization of Gal-APN

The synthesis of Gal-modified PEG-$b$-PLE copolymer is illustrated in Scheme 3.1. To this end, propargyl-terminated PEG-$b$-PLE block copolymer was synthesized via ring-opening polymerization of BLE-NCA monomer using propargyl-PEG-amine as a macrorinitiator. The chemical composition of the resulting block copolymer was confirmed by $^{1}$H-NMR (Figure 3.1). The degree of polymerization of the PLE block was 25 (Table 3.1). From the GPC analysis, the
weight-average molecular weight ($M_w$) and the molecular weight distribution ($M_w/M_n$) of propargyl-PEG-$b$-PLE were determined to be 10,618 and 1.08, respectively (Table 3.1 and Figure 3.1). Pursuing Gal conjugation to the copolymer using click chemistry, the azide-modified Gal was synthesized by the following two-step modification [20]: D(+) galactose reacted with 2-bromoethanol first to give the bromo substituent, which was subsequently converted to Gal azide with sodium azide. With its molecular mass confirmed by mass spectrometry (Figure 3.2), Gal azide and propargyl-PEG-$b$-PLE were reacted in the presence of copper (II) sulfate and sodium ascorbate in water/methanol mixture at room temperature. $^1$H-NMR (Figure 3.1) confirmed the conversion of the alkyne groups to the triazole. The Gal conjugation yield was quantified using phenol-sulfuric acid method [21], and it showed that 67% ± 6% of the copolymer was successfully modified with Gal after click reaction. Due to the concern of copper toxicity, the absence of residual copper after 2 days’ exhaustive dialysis was confirmed by ICP-MS (data not shown).
Figure 3.1 $^1$H-NMR spectra for (A) propargyl-PEG-NH$_2$, (B) propargyl-PEG-b-PBLE, (C) propargyl-PEG-b-PLE, and (D) Gal-PEG-b-PLE. Peak assignments are as follows:

(A) $^1$H-NMR (DMSO-$d_6$, 25°C): $\delta$ ppm = 8.1 (1H, s, -NHCO-), 3.8-4.0 (2H, m, -CH$_2$-), 3.2-3.8 (4H, m, -CH$_2$CH$_2$O-), 3.07 (1H, s, C$_2$H$\equiv$C-).

(B) $^1$H-NMR (DMSO-$d_6$, 80°C): $\delta$ ppm = 8.1-8.3 (1H, br, CH$\equiv$C-CH$_2$-NHCO-), 7.8-8.0 (2H, m, -NHCO-), 7.1-7.4 (5H, m, PhH), 4.9-5.2 (2H, m, PhCH$_2$-), 4.3 (2H, s, CH$\equiv$C-CH$_2$-), 3.8-4.0 (1H, m, -CH$_2$-), 3.3-3.8 (4H, m, -OCH$_2$CH$_2$O-), 2.2-2.4 (2H, br, -COCH$_2$-), 1.9-2.2 (2H, br, -CH$_2$CH-).

(C) $^1$H-NMR (D$_2$O, 25°C): $\delta$ ppm = 4.2-4.6 (1H, s, -CH-), 4.1-4.2 (2H, s, -COCH$_2$O-), 4.0-4.1 (2H, s, CH$\equiv$C-CH$_2$-), 3.5-3.9 (4H, m, -OCH$_2$CH$_2$O-), 2.49 (2H, s, -COCH$_2$-), 1.8-2.3 (2H, m, -CH$_2$CH-).

(D) $^1$H-NMR (D$_2$O, 25°C): $\delta$ ppm = 8.1 (1H, s, -CH$_2$N(N=)CH=C(N=)CH$_2$O-), 4.9 (1H, m, saccharide a-H), 4.2-4.6 (1H, br, -CH-), 4.1-4.2 (2H, s, -COCH$_2$O-), 4.0-4.1 (2H, s, -CH$_2$N(N=)CH=C(N=)CH$_2$O-), 3.5-4.0 (4H, m, -OCH$_2$CH$_2$O-), 2.4-3.0 (2H, br, -COCH$_2$-), 2.0-2.4 (2H, br, -CH$_2$CH-).

(E) Typical GPC elution curves of precursor propargyl-PEG-NH$_2$ (magenta) and propargyl-PEG-b-PBLE (black).
**Figure 3.2** Mass spectra of 2’-Azidoethyl-O-D-galactopyranoside. The theoretical molecular weight is 249 Da. The main peak shown in the spectra is 249 + 23 (Na) = 272 Da.

**Table 3.1** Characteristics of propargyl-PEG-b-PBLE determined by $^1$H-NMR and GPC.

<table>
<thead>
<tr>
<th>propargyl-PEG-b-PBLE</th>
<th>Feeding Ratio</th>
<th>Degree of Polymerization</th>
<th>$M_w$ (g/mol)</th>
<th>$M_n$ (g/mol)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPC</td>
<td>1:25</td>
<td>26</td>
<td>10618</td>
<td>9800</td>
<td>1.08</td>
</tr>
<tr>
<td>$^1$H-NMR</td>
<td></td>
<td>25</td>
<td>10475</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Gal-APN were prepared as described before [13] by simple mixing of buffered solutions of cationic p41 and anionic block copolymers (Gal-PEG-\(\text{b-PLE}\) and propargyl-PEG-\(\text{b-PLE}\)) which electrostatically bind to each other (Scheme 3.2). The Gal density on the surface of the Gal-APN was controlled by adjusting the molar ratio of Gal-PEG-\(\text{b-PLE}\) and propargyl-PEG-\(\text{b-PLE}\) copolymers. In this way, we prepared a series of Gal-APN with various proportions of Gal moieties ranging from 5% to 70%. APN prepared from p41 and propargyl-PEG-\(\text{b-PLE}\) was used as a non-targeted control for comparison. Gal-APN displayed a slight increase in size compared to non-targeted APN as more Gal moieties were introduced onto the APN surface (from 89 nm to 108 nm), and in all cases exhibited uniform size distributions (PDI less than 0.2) as determined by DLS (Table 3.2).

Table 3.2 Physicochemical characteristics of Gal-APN

<table>
<thead>
<tr>
<th>Gal Density</th>
<th>(D_{\text{eff}}) (nm)(^b)</th>
<th>PDI(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>89 ± 1</td>
<td>0.13</td>
</tr>
<tr>
<td>14%</td>
<td>96 ± 5</td>
<td>0.14</td>
</tr>
<tr>
<td>28%</td>
<td>98 ± 4</td>
<td>0.10</td>
</tr>
<tr>
<td>35%</td>
<td>103 ± 5</td>
<td>0.13</td>
</tr>
<tr>
<td>42%</td>
<td>98 ± 2</td>
<td>0.15</td>
</tr>
<tr>
<td>56%</td>
<td>93 ± 2</td>
<td>0.13</td>
</tr>
<tr>
<td>70%</td>
<td>108 ± 1</td>
<td>0.20</td>
</tr>
</tbody>
</table>

\(^a\) Complexes were prepared in PBS (pH 7.4, 0.14 M NaCl) at \(Z_{+/}=1\).
\(^b\) Effective diameter \((D_{\text{eff}})\) and polydispersity indices (PDI) were determined by DLS at pH 7.4, 25°C \((n = 3)\).
3.3.2 Uptake of Gal-APN in hepatoma cell lines

The internalization of Gal-APN was studied in Huh 7 cells with relatively low ASGP-R expression and in HepG2 cells, which overexpress the ASGP-R (Figure 3.3). We prepared a fluorescence-labeled APN by incorporation of 10 mol% of Cy5-labeled p41 into complexes as a tracer (Table 3.3). The confocal microscopy suggested that Gal(35%)-APN were taken up rapidly into the Huh 7 cells, whereas the non-targeted APN were internalized more gradually (Figure 3.4 A). Indeed, nearly all of the cells became Cy5-positive after 2 h incubation with Gal(35%)-APN, while at least 5 h were needed for all cells to take up APN (Figure 3.5). Notably, p41 alone displayed the least efficient internalization. The quantification of cell-associated fluorescence (Figure 3.4 B) indicated that both Gal(35%)-APN and APN displayed time-dependent cellular accumulation, but the cellular content of Gal(35%)-APN was significantly ($p < 0.001$) greater than of non-targeted APN. No difference in cellular uptake between Gal(35%)-APN and APN was observed in the ASGP-R negative HeLa cell line (Figure 3.6). Importantly, the higher cellular uptake occurred for the Gal-APN with 5%-35% Gal moieties on their surface (Figure 3.4 C). Above this percentage, the uptake of Gal-APN was decreased. To further elucidate whether the cellular association of Gal-APN depends on binding to ASGP-R on the hepatocytes surface, Huh 7 cells were treated with Gal(35%)-APN or APN in the presence of excess free galactose (100 mM). Flow cytometric analysis revealed that the uptake of Gal(35%)-APN was suppressed to the level of the non-targeted APN in the presence of free galactose (Figure 3.4 D). This clearly demonstrates the involvement of a specific receptor-mediated uptake mechanism.
Figure 3.3 Western blot analysis of ASGP-R expression level in hepatoma cells (non-infected and infected) and primary human hepatocytes. Lanes: (1) HepG2 cells; (2) Huh 7 cells; (3) Huh 7.5 cells; (4) Huh 7.5 cells after 1 day infection; (5) Huh 7.5 cells after 2 days infection; (6) Huh 7.5 cells after 3 days infection; (7) primary human hepatocytes; (8) HeLa cells (negative control).

Table 3.3 Physicochemical characteristics of Cy5-labeled APN\textsuperscript{a} for confocal imaging analysis.

<table>
<thead>
<tr>
<th>Samples</th>
<th>$D_{\text{eff}}$ (nm)\textsuperscript{b}</th>
<th>PDI\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal(35%)-APN</td>
<td>144 ± 6</td>
<td>0.25</td>
</tr>
<tr>
<td>APN</td>
<td>163 ± 2</td>
<td>0.20</td>
</tr>
</tbody>
</table>

\textsuperscript{a}10 mol% Cy5-p41 were introduced into complexes.

\textsuperscript{b}p41 concentration was 5 µM.

\textsuperscript{c}Effective diameter ($D_{\text{eff}}$) and polydispersity indices (PDI) were determined by DLS at pH 7.4, 25°C (n = 3).
**Figure 3.4** Uptake of Gal-APN by Huh 7 cells. (A) Representative confocal microscopy images of Huh 7 incubated with Cy5-labeled Gal(35%)-APN, APN or p41 for 2 h at 37°C. The merged images of Cy5-labeled p41 (red) and DAPI-stained nuclei (blue) (objective 40x). (B) Quantification of internalization kinetics of Gal(35%)-APN, APN and p41 in Huh 7 cells. Mean fluorescence intensity per cell was calculated from images taken at indicated time points. Asterisks show statistically significant differences between Gal(35%)-APN and APN at the corresponding time points. (C) Gal-APN uptake as a function of Gal density following 2 h incubation. *** indicates significantly higher uptake vs. non-targeted APN. (D) Inhibitory effect of free Gal on the uptake of Gal(35%)-APN as analyzed by flow cytometry. Huh 7 cells were treated with free Gal (100 mM) for 0.5 h, followed by co-incubation with either Gal(35%)-APN (blue) or APN (black) for another 2 h. Cells treated with Gal(35%)-APN (grey) or APN (red) were used as controls. In all experiments, cells were treated with APN and peptide at a dose equivalent to 5 µM of p41. Data are presented as mean ± SEM (n = 15-20); *p < 0.05, ***p < 0.001, NS - not significant.
**Figure 3.5** Representative confocal microscopy images (40x) of Huh 7 cells incubated with Cy5-labeled Gal(35%)-APN, APN or p41 (equivalent to 5 µM of p41) for 5 h at 37°C. The merged images of Cy5-labeled p41 (red) and cell nuclei stained with DAPI (blue).

**Figure 3.6** Representative confocal images (20x) of HeLa cells (ASGP-R negative) after 1 h incubation with Gal(35%)-APN and APN at 37°C. The peptide concentration is 5 µM. The cellular nuclei were stained with DAPI (blue), and p41 was labeled with Cy5 (red).
Contrary to our expectation, the internalization kinetics and cellular uptake of Gal(35%)-APN and APN in HepG2 cells that abundantly express ASGP-R (Figure 3.3) were not significantly different during continuous incubation with the complexes (Figure 3.7 A). Similar behavior of Gal(35%)-APN was also observed in primary human hepatocytes (Figure 3.8). Nevertheless, the competition experiments demonstrated that the uptake of Gal-APN into HepG2 cells was inhibited by the presence of soluble Gal (Figure 3.7 B and Figure 3.9). By contrast, no attenuation in internalization was observed in the cells treated with non-targeted APN under the same experimental conditions. These findings indicate that uptake of targeted Gal-APN is at least partially mediated by interaction with ASGP-R on cultured HepG2 cells.
Figure 3.7 Uptake of Gal-APN into HepG2 cells. (A). Time-dependent uptake of Gal(35%)-APN and APN at 37°C as quantified from confocal images (objective 20x). Data are presented as mean ± SEM (n = 15−20). (B). Representative confocal images of HepG2 cells incubated with Cy5-labeled Gal(35%)-APN and APN in the absence or presence of free galactose. For competition experiments, cells were pre-treated for 0.5 h with 100 mM of galactose followed by co-incubation with corresponding APN for 1 h. The merged images of Cy5-labeled p41 (red) and cell nuclei stained with DAPI (blue) (objective 20x). In all experiments, cells were treated with APN at a dose equivalent to 5 µM peptide at 37°C.
Figure 3.8. Uptake of Gal-APN into primary human hepatocytes. (A). Representative confocal images (40x) of freshly isolated primary human hepatocytes after 2 h and 5 h incubation with Cy5-labeled Gal(35%)-APN and APN (red) at a dose equivalent to 5 µM of peptide. The cellular nuclei were stained with DAPI (blue). (B). Internalization kinetics of Gal(35%)-APN and APN as quantified by ImageJ from confocal images. Data are presented as mean fluorescence per cell ± SEM (n = 15-20); **p < 0.01, ***p < 0.001.
Figure 3.9 In HepG2 cells, the internalization of Gal(35%)-APN was effectively blocked by free Gal (blue) compared to the cells treated only with Gal(35%)-APN (grey). In contrast, APN uptake remained the same in the absence (red) or the presence (black) of free galactose. ASGP-R were blocked by free galactose (100 mM) for 0.5 h before 1 h co-incubation with Gal(35%)-APN or APN (at a dose equivalent to 5 µM p41). The median fluorescence intensity (Cy5) of each sample was acquired by Becton Dickinson LSRII cytometer (BD Biosciences, USA) and analyzed by FlowJo (Tree Star Inc., USA). At least 10,000 cells from each sample were acquired in linear mode, gated to exclude debris and dead cells, and visualized in logarithmic mode.
3.3.3 Gal-APN anti-HCV activity in cell culture systems

Anti-HCV activity of Gal(35%)-APN and APN was evaluated against JFH-1 virus (MOI 0.1) in the Huh 7.5 cell line. MOI 0.1 has been demonstrated as the most efficient for JFH-1 virus in Huh7.5 cells by others [10]. As was previously demonstrated, both p41 and APN displayed dose-dependent antiviral activity [13]. To rule out any confounding effects due to cytotoxicity of free p41, all formulations (Gal-APN, APN and p41 alone) were used at a dose equivalent to 5 µM peptide which was shown to be sufficient to suppress HCV infection without inducing cytotoxicity [13]. The antiviral potency of formulations was determined by quantifying the intracellular HCV RNA replication using RT-qPCR. To assess whether APN can suppress an ongoing HCV infection and block cell-to-cell viral spread, the cells were first infected with virus and then treated with formulations (at a dose equivalent to 5 µM p41). As shown in Figure 3.10 A, both Gal(35%)-APN and APN were able to suppress viral replication after 48 h of cell treatment. It is likely that the lack of the differences in magnitude of the antiviral response between targeted and non-targeted APN might be due to the strong virocidal activity of p41 peptide, which is not affected by targeted delivery of the peptide. To test this hypothesis, virus was pre-incubated with formulations for 1 h and then Huh 7.5 cells were infected with this mixture. As shown in Figure 3.10 B, in contrast to the viral replication observed in control cultures, the pre-incubation of both Gal(35%)-APN and APN with virus stock induced 99% inhibition of intracellular viral RNA accumulation. Overall, it appeared that in terms of reducing viral RNA replication in cell culture systems, Gal-APN is as effective as APN.
**Figure 3.10** *In vitro* anti-HCV activity of Gal-APN. (A) APN suppress the ongoing HCV infection. Cells were infected with JFH-1 virus (MOI 0.1) overnight and then treated with Gal(35%)-APN, APN or p41 for 48 h. (B) Virocidal effect: JFH-1 virus (MOI 0.1) was pre-incubated with respective formulations for 1 h at 37°C, then cells were infected with these mixtures overnight, washed, and cultured for another 48 h. For all experiments, intracellular HCV RNA levels were determined by RT-qPCR and normalized to GAPDH mRNA levels. Data are presented as %control ± SEM (n = 3) in comparison to HCV RNA level in non-treated infected cells (100%); ***p < 0.001, NS - not significant.
We attempted to further elucidate the antiviral mechanism of p41 that is beyond the virocidal effect and interferes with the intracellular viral life cycle. Since amphipathic α-helical p41 is derived from the membrane anchor domain of NS5A [10], we hypothesized that p41 competes with NS5A and HCV core proteins for binding to lipid droplets, thereby preventing the assembly and release of mature viral particles. As expected, infected cells exhibited high co-localization of HCV core and NS5A proteins with lipid droplets (Figure 3.11 A and C). When cells were treated with APN or free peptide for 5 h before exposure to HCV, the expression of HCV core protein and NS5A, as well as their localization to lipid droplets, were substantially reduced. Notably, in both cases the strongest inhibition of co-localization with lipid droplets was observed after treatment with Gal(35%)-APN, which significantly ($p < 0.001$) exceeded the inhibition effects of APN and p41 (Figure 3.11 B and D).
Figure 3.11 Gal-APN suppress association of HCV proteins with lipid droplets in infected Huh 7.5 cells. (A, C) Representative confocal microscopy images of the co-localization of HCV core (A) or NS5A (C) proteins with lipid droplets. Cells were pretreated with Gal(35%)-APN, APN or p41 (at a dose equivalent to 5 µM of p41) for 5 h before overnight infection (MOI 0.1). In 48 h, cells were analyzed using BODIPY to stain lipid droplets (green) and immunofluorescence with Alexa Fluor 594-labeled antibodies either to HCV core or NS5A proteins (red). Cell nuclei were stained with DAPI (blue). Co-localization of lipid droplets with HCV core or NS5A proteins defined as yellow overlap of green and red fluorescence (objective 63x and 20x). (B, D) Quantitative analysis of co-localization of lipid droplets with HCV core (B) or NS5A (D) proteins from the images (objective 20x). Data are presented as mean ± SEM (n = 15-20), **p < 0.01, ***p < 0.001, NS - not significant.
In addition to the direct antiviral effect of Gal-APN, the indirect aspect of antiviral action was also investigated based on the activation of interferon-sensitive genes (ISGs) (Figure 3.12). ISGs were induced by 4 h exposure to low dose of interferon alpha (IFN-α, 100 U/ml), which itself did not affect HCV RNA levels. Meanwhile, cells were treated with Gal(35%)-APN 48 h post-infection with or without IFN-α exposure. Gal-APN itself had no effect on ISGs activation, however, the accumulation of ISGs (viperin and 2’5’ oligoadenylate synthase-1) was significantly enhanced by Gal-APN after triggering of IFN-α signaling.
**Figure 3.12** The effect of Gal-APN on ISGs expression. After incubation with Gal(35%)-APN (at a dose equivalent to 5 µM of peptide), cells were treated with IFN-α (100 U/ml) for 4 h before harvesting to activate ISGs expression. ISGs [2'-5'-oligoadenylate synthase 1 (OAS-1) and viperin] activation and HCV RNA were presented as %control ± SEM (n=3), where 100% were ISGs or HCV RNA expression in infected cells only exposed to IFN-α; ***p < 0.001.
3.3.4 Biodistribution studies

As a proof of concept, the in vivo biodistribution of Gal(14%)-APN, APN, and p41 was tested in BALB/c mice using $^{177}$Lu-DTPA-p41 as a probe. No changes in size were observed for APN incorporating DTPA-labeled p41: all the complexes produced had diameters between 90 and 96 nm with narrow polydispersity, indicating that the macroscopic characteristics of APN were not affected by the radiolabelling step. Therefore, it was appropriate to verify the effects of Gal-ligands on APN biodistribution profile by using these complexes. Since no significant differences were observed in the uptake of Gal-APN within 5%-35% range of ligand surface densities, Gal(14%)-APN with an average level of galactosylation were selected for further in vivo studies. Following intravenous administration of the radiolabeled compounds, biodistribution studies showed that all formulations were rapidly captured by the liver (~13 %ID/g) within 15 min (Figure 3.13 A) with no significant differences observed during this short time frame. However, at 4 h after injection, significantly ($p < 0.001$) higher hepatic disposition of Gal(14%)-APN (6.0 ± 1.6 %ID/g) was observed compared to APN (2.6 ± 1.7 %ID/g) and p41 (1.4 ± 0.3 %ID/g) (Figure 3.14 A). The trend remained the same after the liver was perfused. The slight drop of radioactivity after perfusion implied that the majority of the drugs were associated with the liver tissue. The liver-to-blood ratios in animals injected with the liver-targeted Gal-APN was calculated to be 6.1 ± 2.1 and was significantly ($p < 0.001$) higher compared to non-targeted APN (2.1 ± 0.8) and free p41 (1.3 ± 0.6). Our preliminary data showed that radiolabeled formulations were associated with both liver hepatocytes and non-parenchymal cells (consisting
primarily of Kupffer cells and sinusoidal endothelial cells). However, preferential accumulation in hepatocytes was observed for Gal(14\%)-APN (9.2\% ± 2.3\% of total dose accumulated in the liver), while for APN and free p41, these values were 5.8\% ± 1.4\% and 3.7\% ± 0.1\%, respectively (Figure 3.13 B). When compared to APN and p41, a slightly higher extent of Gal(14\%)-APN accumulation was also observed in the spleen and lung, due to these organs contain high numbers of macrophages expressing lectin receptors (Figure 3.14 B). Overall, the \textit{in vivo} data demonstrates the increased hepatic localization of Gal-APN, most likely as a result of Gal-APN binding to ASGP-R expressed on the surface of hepatocytes.
Figure 3.13 Biodistribution study of radiolabeled Gal(14%)-APN, APN, and p41 in BALB/c mice. (A) Liver accumulation of drugs (before and after perfusion) at 15 min post i.v. injection. Data are presented as mean percentage of injected dose per gram of tissue (%ID/g) ± SEM (n = 3). (B) Intrahepatic cellular localization at 4 h post-injection. *p < 0.05, NS – not significant.
Figure 3.14 Biodistribution of radiolabeled Gal-APN in vivo at 4 h post i.v. injection. (A) Quantification of the liver (before and after perfusion) and blood concentrations of the Gal(14%)-APN, APN and p41 (50 µg p41 or its APN format per animal). The values in parenthesis indicate the mean liver (perfused)-to-blood ratio. (B). Tissue distribution of Gal(14%)-APN, APN and p41. Apart from liver, a higher accumulation of Gal(14%)-APN was found in lung and kidney. All results were interpreted as mean percentage of injected dose per gram of tissue (%ID/g) ± SEM (n = 6); *p < 0.05, **p < 0.01, ***p < 0.001, NS - not significant.
3.4 Discussion

The targeting of bioactive molecules to specific organs is an attractive strategy to increase local concentration of therapeutics at the anticipated site of action, while reducing off-target effects. Viral replication in HCV infection occurs predominantly in the hepatocytes. The ASGP-R, a C-type lectin receptor presented in abundance on the sinusoidal surface of hepatic parenchymal cells, mediates the binding and removal of glycoconjugates containing exposed terminal galactosyl or N-acetylgalactosaminyl sugars. Despite that ASGP-R has been also detected on extra-hepatic cells such as peritoneal and liver residential macrophages, in human kidney, thoroid and activated T cells, the ASGP-R has been intensely validated as a potential target for drug and gene delivery to the liver \[23-25\]. For example, it was demonstrated that N-glycosylated human interferon beta directed to ASGP-R efficiently inhibited hepatitis B virus infection \textit{in vivo} \[26\]. Coulstock et al. \[27\] demonstrated increased targeting to the liver of interferon genetically fused with a ASGP-R-specific antibody following systemic intravenous administration.

In our previous \textit{in vitro} studies, we demonstrated that incorporation of cationic p41, an antiviral peptide derived from the membrane anchor domain of HCV nonstructural protein NS5A, into nanosized APN substantially increases peptide proteolytic stability, reduces its intrinsic cytotoxicity, while preserving antiviral activity against HCV. Here, exploiting a concept of the liver-targeted delivery, we designed ASGP-R-directed APN coated with Gal ligands to deliver an antiviral HCV-specific peptide to hepatocytes, which is anticipated to eventually benefit treatment outcomes. A series of Gal-decorated APNs with various densities of Gal ligands was
prepared by controlling the molar ratio of Gal-PEG-b-PLE/propargyl-PEG-b-PLE copolymers.

The targeting properties of Gal-decorated APN were evaluated in hepatic cells with differential expression of ASGP-R. Interestingly, the uptake efficiency of Gal-APN did not correlate with ASGP-R expression level. Rapid and preferential accumulation of Gal-APN over APN was observed in Huh 7 cells with relatively low receptor expression. Moreover, an efficient uptake of Gal-APN was observed when the density of the Gal ligand did not exceed 35%. The affinity of the ASGP-R is a consequence of oligovalent interactions with its physiological ligands, so-called “cluster glycoside effect” [28]. Studies using natural and synthetic ligands have illustrated the importance of the special arrangements of the terminal Gal residues for the binding affinity to ASGP-R. Based on affinity studies of several asialoglycopeptides and synthetic cluster glycosides with defined sugar arrangements and geometries it was concluded that appropriate sugar spacing (at least 15Å) is required for optimal receptor recognition [28-30]. It is possible that increase in ligand density on the Gal-APN above 35% can lead to formation of structures with shorter intergalactose distances and results in lower binding affinity of the corresponding Gal-APN.

Furthermore, observed ligand density effects can be also related but not limited to improper orientation of the ligand, steric hindrance of neighboring molecules or competitive behaviors for the binding of the receptor. Similar negative cooperative binding effects were previously reported for other ligand-decorated nanoformulations such as liposomes [31, 32], nanogels [33], polymeric micelles and dendrimers [34], and superparamagnetic iron oxide nanoparticles [35].

Surprisingly, there were nearly no differences in the uptake of targeted and non-targeted
APN in cells with higher density of ASGP-R (~76,000 and 3,000 ASGP-R/cell for HepG2 and Huh 7, respectively [36]). Nevertheless, the uptake of Gal-APN but not APN was significantly inhibited by co-incubation with free Gal in both cell lines. The latter indicates that uptake of galactosylated APN into hepatic cells involves a specific ASGP-R-mediated mechanism, while non-targeted APN utilize some alternative endocytotic pathway. This can possibly be attributed to the difference in composition of the outer shell of the APN. However, the functional relationship between APN composition, ligand density and ASGP-R expression on hepatic cells remains to be established.

Huh 7.5 cells, as an in vitro model for HCV infection, were used to evaluate the antiviral activity of Gal-APN. The life cycle of HCV requires its penetration into hepatocytes via receptors for viral entry, replication, and degradation of a viral polypeptide into smaller structural and non-structural viral proteins, which then are assembled on lipid droplets in the vicinity of endoplasmic reticulum (ER) into full infectious particles. These particles then leave the cells in the complexes with very low density lipoproteins, which are “sensed” by new intact hepatocytes leading to spread of infection [37, 38]. Thus, in the liver, HCV may persist extracellularly as well as intracellularly. In our study, we demonstrated that antiviral activity of p41 (as a part of APN and Gal-APN) also consists of two components: extracellular virocidal component related to viral membranolytic activity of the peptide and intracellular component, which presumably affects viral assembly on lipid droplets. No difference was found between the capability of Gal-APN and APN to reduce intracellular HCV RNA replication when the formulations were either added to
the cells after preincubation with virus or were applied post-infection. This may be attributed to the dominant virocidal effect of p41 before its penetration into hepatocytes, which corroborates the results reported by Cheng et al. [10] that amphipathic α-helical peptides derived from the membrane anchor domain of the HCV NS5A protein, including p41, destabilize the viral lipid membranes. This extracellular virocidal component may not depend on targeted APN delivery via ASGP-R in the context of in vitro experiments. However, in vivo, targeted delivery of APN to hepatocytes would provide additional benefit of specific accumulation of Gal-APN and its antiviral payload in the ASGP-R-expressing hepatocytes that are a primary site of HCV replication. In fact, based on our biodistribution experiments, p41 peptide is mainly delivered to liver and hepatocytes via Gal-APN, where, as expected, it will ultimately provide the desired antiviral effect and prevent the spread of extracellularly secreted virus to the neighboring non-infected cells. Besides, the co-staining of HCV core and NS5A proteins with lipid droplets after the treatment with targeted or non-targeted APN sheds light on the additional intracellular antiviral mechanism of p41 that is beyond its virocidal action. Specifically, when the formulations were delivered to the cells before viral entry (avoiding preliminary extracellular contact of APNs with HCV), it prevented the binding of both core and NS5A proteins to lipid droplets via the common amphipathic α-helical domain. This binding of HCV proteins to lipid droplets is necessary for assembly and release of fully assembled infectious viral particles [37, 38]. The decreased association between viral proteins and lipid droplets may be vital for the virus assembly. Here, we show that Gal-APN demonstrated the advantages over APN and free p41 to
inhibit this step as a prerequisite for virion production. Furthermore, we also observed the reduced expression of HCV core and NS5A proteins after Gal-APN treatment. Currently, we have not identified the exact intracellular compartment, where HCV interacts with p41. Since both the viral particles and Gal-APN enter hepatocytes via receptor-mediated endocytosis [39, 40], and the destabilization of APN and p41 release can occur probably due to the protonation of carboxylic groups of PLE chains in the APN after acidification in endosomes, we cannot exclude that, in part, inactivation of virus may also be attributed to virocidal effects of APN in this compartment. In addition, arginine-rich α–helical structure of p41 as well as its membranolytic properties may facilitate the endosomal release of these peptides into the cytosol [41]. We also cannot exclude the possibility that p41 may access the HCV replication complex on the ER as a result of multiple fusion of APN/p41-containing vesicles, using lipid droplets or heat shock proteins, or other endocytosis tools as trafficking vehicles. These mechanisms definitely require more detailed investigations.

The effects of antiviral small molecules (including recently discovered DAA) are potentiated by activation of host innate immunity, namely, activation of ISGs that encode generation of endogenous antiviral proteins. This activation of innate immunity diminishes the chance of relapses of chronic HCV infection. It is likely that in addition to disturbance of HCV assembly on lipid droplets, Gal-APN might provide an indirect effect on IFN-α-induced activation of ISGs that encode the expression of antiviral proteins. In the infected cells, HCV core and NS5A proteins suppress IFN-α signaling and prevent activation of ISGs, thereby abolishing the synthesis of
intracellular antiviral proteins and diminishing anti-HCV protection [42-44]. While the major antiviral mechanism of Gal-APN is distinct from exogenous IFN-α, we demonstrated that Gal-APN indirectly potentiate the endogenous IFN-α signaling, thereby increasing its downstream antiviral ISGs activation in liver cells (Figure 3.12). We strongly believe that apart from the potent extracellular virocidal action, the combination of advanced intracellular effects of Gal-APN and subsequent ISG activation may potentiate in vivo antiviral activity of Gal-APN.

In vivo, using radiolabeled peptides, we subsequently showed that the galactosylation of APN offered certain advantages including preferential liver uptake as well as accumulation in hepatic parenchymal cells (hepatocytes). It appeared that the biodistribution profile of Gal-APN and APN is highly dose-dependent: if lower dose were administered due to inaccurate injection, the distribution of p41 in all excised organs (%ID/g) was correspondingly decreased (data not shown). This is reconciled with previous findings that the lower plasma concentration of protein-based block ionomer complexes makes them more susceptible to dissociation due to the dilution in the bloodstream and interaction with charged serum proteins [45]. For the Gal-APN, the levels of radioactivity in all other tissues assayed were lower than the liver, which was in line with what was obtained for both the APN and p41. The relatively high level of radioactivity detected in the kidney can be attributed to renal excretion of the peptides that was already apparent 15 min following injection of p41 or its formulations.

Interestingly, slightly but significantly higher uptake of Gal-APN compared to non-targeted APN or free p41 was detected in the spleen and lungs (Figure 3.14). Since galactosylation did not
alter the physicochemical properties of APN, such as size and charge, this indicates the increased binding of Gal-APN to galactose-specific C-type lectins expressed on macrophages in these organs rather than enhanced opsonization [46-48]. Collectively, our findings suggest that preferential hepatic uptake of Gal-APN was a result of ASGP-R binding on the surface of hepatocytes and that galactosylated APN can be used to affect the biodistribution of therapeutic antiviral peptides. However, we should acknowledge that ASGP-R function and expression level in the liver of HCV patients are influenced by many factors including oxidative stress, toxic substances (such as alcohol), viral infection (Figure 3.3), etc. [49]. Therefore, hepatic distribution of Gal-APN under pathological conditions should be evaluated, which will be crucial for treatment outcome. To serve this purpose, currently, our group is in the process of establishing a HCV animal model using urokinase-type plasminogen activator (uPA)-NOG mice with reconstituted humanized liver [50].

In the frame of this study, we have not aimed to compare the efficiency of Gal-APN with DAA that already demonstrated their clinical specificity and efficiency. However, apart from the predominant liver accumulation, another advantage of Gal-APN (due to the strong virocidal effects of the peptide) might be the lack of dependence on high rate of HCV mutations, which with time may limit viral sensitivity to DAA. Potentially, Gal-APN can be used for the development of drug combinations that target different aspects of the viral life cycle. APN could complement the DAA potency, interfere with multiple viral genotypes, overcome resistance development and, eventually, reduce treatment duration.
3.5 Conclusion

In the present study, we designed and synthesized the galactosylated nanocomplexes of antiviral peptides as a platform for targeted therapy against HCV. *In vitro*, Gal-APN displayed favorable ASGP-R-mediated uptake by hepatoma cell lines and primary human hepatocytes. Even though both targeted and non-targeted APN efficiently suppress HCV RNA replication in cell culture systems, Gal-APN displayed a significantly stronger ability than APN to prevent intracellular binding of HCV core and NS5A proteins to lipid droplets, which is known to be an essential step for viral assembly and release. The preferential hepatic accumulation of Gal-APN in animals further indicates the possibility of improved therapeutic outcomes *in vivo*. Overall, the results presented here demonstrate the potential of Gal-decorated APN as a specific therapeutic strategy against HCV.

3.6 References


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CHAPTER IV

THE DELIVERY OF ANTIVIRAL PEPTIDE AND DIRECT-ACTING ANTIVIRALS COMBINATION AS SYNERGISTIC THERAPY FOR HCV

4.1 Introduction

Great success in HCV therapy has been achieved with the development of a series of direct-acting antivirals (DAA) [1]. DAA specifically target different steps within the HCV viral life cycle, and the major targets include 4 categories: NS3/4A protease, NS5A, NS4B, and NS5B polymerase [2]. DAA exhibit high potency, simplified dosing regimen, improved tolerability, etc. However, the practical hurdles such as high cost, genotype dependency, and potential resistance due to high rate of viral mutations drive us to pursue additional therapeutic approaches to be used instead or in combination with DAA [3]. The cationic peptide p41 is one of such candidates displaying submicromolar anti-HCV potency. Its distinct antiviral mechanism of action is driven by the α-helicity and amphipathicity that can neutralize the viral particles by destabilizing the lipid composition of viral membranes without genotype dependency [4]. Therefore, the virocidal amphipathic peptide, such as p41, is expected to complement the therapeutic outcome of DAA, by targeting different intracellular aspects of the viral life cycle. As demonstrated in previous chapters, the antiviral peptide nanocomplexes (APN) platform has been successfully developed by our group for the delivery of p41 [5, 6]. Therefore, we anticipated that, by further introducing DAA into the current APN-based system, APN could complement the DAA potency, interfere with multiple viral genotypes, overcome resistance development and eventually reduce treatment
duration.

To serve this purpose, two DAA as model drugs were selected (Figure 4.1). The first one is daclatasvir (BMS-790052), which is a first-in-class and highly selective NS5A inhibitor [7]. Its antiviral mechanism is to suppress the viral genome replication by altering the subcellular localization of NS5A into functional replication complex [8]. It displays picomolar antiviral potency against the replicons expressing a broad range of HCV genotypes and the JFH-1 genotype 2a infectious virus in cell culture [7, 9]. The other candidate is the HCV polymerase inhibitor lomibuvir (VX-222), which binds to the thumb II allosteric pocket of the HCV RNA-dependent RNA polymerase [10]. Lomibuvir exhibits non-competitive and selective inhibition in HCV NS5B of genotype 1a and 1b at nanomolar level. It is currently undergoing the clinical evaluation in an all-oral triple combination with protease inhibitors, telaprevir and ribavirin, as a therapy for genotype 1 HCV patients [11].

In this chapter, a series of poly(amino acid)-based block ionomer complexes (BIC) were prepared and characterized to facilitate simultaneous encapsulation and delivery of both the antiviral peptide p41 and the potent DAA. The structural versatility of the poly(amino acid)-based block copolymers allows us to incorporate DAA either physically or chemically. Furthermore, the feasibility of p41+DAA combination as a synergistic therapy against HCV was evaluated. By using this strategy, the sustained release as well as the superior anti-HCV effect of p41+DAA were expected, which might improve the clinical benefit of the HCV therapy.
4.2 Materials and methods

4.2.1 Materials

Both daclatasvir and lomibuvir were purchased from the Selleck Chemicals (Houston, TX) with the structures shown in Figure 4.1. Peptide SWLRRIWRWICKVLSRFK (p41) was custom synthesized by AnaSpec (Fremont, CA). Stearic acid (98%) was ordered from the Alfa Aesar (MA, USA). The propargyl-poly(ethylene glycol)$_{5k}$-block-poly(L-glutamic acid)$_{25}$ (PEG-PLE) was synthesized as described in Chapter III. The triblock copolymer methoxy-poly(ethylene glycol)$_{5k}$-block-poly(L-glutamic acid)$_{30}$-block-poly(L-phenylalanine)$_{7}$ (PEG-PLE-PLF) was synthesized as described before [12]. The methoxy-poly(ethylene glycol)$_{5k}$-block-poly(L-glutamic acid)$_{25}$ (mPEG-PLE) was purchased from the Alamanda Polymers, Inc. (Madison, AL). N,N'-dicyclohexylcarbodiimide (99%) (DCC), 4-(dimethylamino)pyridine (≥99%) (DMAP), N$_{\text{a}}$N$_{\text{c}}$-di-Fmoc-L-lysine (Fmoc-Lys(Fmoc)-OH), N,N-diisopropylethylamine (DIPEA), and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were obtained Sigma-Aldrich (USA). Ethanol, N-Hydroxysuccinimide (NHS), acetonitrile (HPLC), water (HPLC), anhydrous dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and tetrahydrofuran (THF) were obtained from the Acros Organics (USA).
4.2.2 Preparation of BIC micelles loaded with p41 and daclatasvir (PEG-PLE/(p41+daclatasvir))

The complexes composed of p41 and PEG-PLE (APN) were prepared as described in Chapter II. Briefly, the buffered solutions (PBS, pH 7.4) of cationic p41 and anionic block copolymer were mixed ($Z_{+/-} = 1$), and they will self-assemble into nanosized APN. Subsequently, daclatasvir was solubilized into the cores of the APN using an extraction method [13]: a thin film of daclatasvir (prepared by evaporation of an ethanol solution of daclatasvir) was incubated with aqueous dispersion of APN (48 h at room temperature). Unincorporated daclatasvir was removed by centrifugation at 8,000 rpm for 5 min (Centrifuge 5242, Eppendorf). The size distribution was determined by DLS as described in Chapter II. The amount of daclatasvir loaded in APN was quantified via reverse-phase HPLC using an Agilent Eclipse XDB C18 5 µm column (250 mm × 4.6 mm) and Agilent 1200 HPLC system. Mobile phase composed of 45% water and 55% acetonitrile was applied at a flow rate of 1 ml/min. Detection wavelength was set at 254 nm. Drug loading capacity was calculated as percent ratio of mass of incorporated drug to total mass of drug-loaded APN without water.
4.2.3 Preparation of (daclatasvir+p41)-loaded triblock copolymer micelles (PEG-PLE-PLF/p41/daclatasvir)

The synthesis of triblock copolymer PEG-PLE-PLF has been reported [12], and the concentration of carboxylate groups in the copolymer samples was estimated by potentiometric titration. First, p41-encapsulated micelles were prepared by adding p41 to an aqueous dispersion of micelles with the molar ratio of carboxyl groups of polymer to the amino groups of p41 ($Z_+/\epsilon$) equaling 1. The daclatasvir was further loaded in the hydrophobic domains of the micelles with the extraction method described in 4.2.2. The encapsulation of p41 was monitored by size exclusion chromatography (SEC) using an ÄKTA FPLC (Amersham Biosciences). Samples (100 µl containing 150 µg on peptide basis) were applied on a Superose 12 10/300 GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). PBS (pH 7.4, 0.14 M NaCl) was used as an eluent at the flow rate of 0.8 ml/min. Both the triblock copolymer and peptide were detected by refractive index (Knauer RI 2300) and UV (280 nm) at room temperature. The loading of daclatasvir was quantified via reverse-phase HPLC with the method described in 4.2.2.

4.2.4 Preparation of (daclatasvir+p41)-loaded micelles of PEG-PLE-stearic acid conjugates

4.2.4.1 Synthesis of PEG-PLE-stearic acid conjugates

The PEG-PLE-stearic acid conjugates were synthesized using the following steps (Scheme 4.1):
4.2.4.1.1 Synthesis of Stearic acid $N$-hydroxysuccinimide ester

The solution of stearic acid (0.2 g, 0.7 mmol), EDC (202.1 mg, 1.5 eq), and NHS (121.4 mg, 1.5 eq) in DMSO was stirred overnight. The reaction mixture was precipitated in water and the filtered product was dried under vacuum. The structure was confirmed with $^1$H-NMR [Bruker AVANCE III instrument (400 MHz)] (Scheme 4.2).

Scheme 4.1 Synthesis of PEG-PLE-stearic acid conjugates.

Scheme 4.2 Synthesis of Stearic acid $N$-hydroxysuccinimide ester.
4.2.4.1.2 Modification of poly(ethylene glycol)-block-poly(L-glutamic acid γ-benzyl ester) (PEG-PBLE) with lysine

The propargyl-PEG-PBLE was synthesized as described in Chapter III. The mixture of propargyl-PEG-PLE (9.9 mg), Fmoc-Lys(Fmoc)-OH (5.5 mg, 10 eq), and DIPEA (3.245 µl, 20 eq) in DMSO, HBTU (3.53 mg, 10 eq) was added dropwise and the mixture was stirred overnight at room temperature, followed by precipitation in diethyl ether to remove excess lysine and side products and drying the product under vacuum. Standard 20% v/v piperidine/DMSO solution was used to remove the Fmoc-protecting group.

4.2.4.1.3 Synthesis of PEG-PLE-stearic acid conjugates

Stearic acid NHS ester (0.31 mg, 2 eq of polymer) was added to polymer (4.31 mg) in DMSO containing TEA (1.5 eq) and stirred overnight at room temperature. The glutamate residues were deprotected by adding 2 N NaOH. After stirring for 4 h at 40°C, water was added to reaction mixture and the pH was adjusted to neutral. The solution was dialyzed against distilled water (MWCO 3.5 kDa) for 48 h, and lyophilized to obtain PEG-PLE-stearic acid. The structure of the final product was confirmed by 1H-NMR.

4.2.4.2 Drug loading

The p41 and daclatasvir were loaded into the micelles formed by PEG-PLE-stearic acid sequentially, with the procedure described in 4.2.2 and 4.2.3. Briefly, the buffered solution of p41 (PBS, pH 7.4) was mixed with micellar solution, followed by using the extraction method to encapsulate the hydrophobic daclatasvir. The loading capacity of daclatasvir was quantified via reverse-phase HPLC with the method described in 4.2.2. The size distribution was analyzed using
the DLS.

4.2.5 *In vitro* anti-HCV effect of PEG-PLE-stearic acid/(p41+daclatasvir)

To study the intracellular effect of PEG-PLE-stearic acid/(p41+daclatasvir), cells were pre-treated with corresponding formulations (5 µM on peptide basis and 0.49 µM on daclatasvir basis) for 5 h before overnight infection with JFH-1 virus (MOI 0.1). After 48 h, HCV RNA replication was quantified by real time qPCR with details described in Chapter III.

4.2.6 Synthesis and characterization of mPEG-PLE-g-lomibuvir

As shown in Scheme 4.3, DCC (0.23 mg, 5 eq of polymer carboxyl group) and DMAP (4.47 µg, 3%) were added to the acidified mPEG-PLE (2 mg, 0.23 µmol) in anhydrous DMSO to activate the carboxyl groups. After 5 h stirring at room temperature, VX-222 (0.5 mg, 20% grafting) was added and the reaction was allowed to proceed for another 72 h. The product was dialyzed against the distilled water (MWCO 2 kDa) for 48 h and lyophilized to obtain the product. The structure of the final product was confirmed by $^1$H-NMR. The size distribution of the micelles of PEG-PLE-g-lomibuvir was determined by DLS.
Scheme 4.3 Synthetic scheme of PEG-PLE-g-lomibuvir.

4.3 Result and discussion

4.3.1 Preparation and characterization of PEG-PLE/(p41+daclatasvir)

The hydrophobic daclatasvir was solubilized in the cores of the APN (PEG-PLE/p41), which was formed due to the neutralization of polyion charges. This was achieved using the micelle extraction method. We hypothesized that hydrophobic domains formed by hydrophobic residues of the p41 incorporated into the APN cores may serve as reservoirs for solubilization of daclatasvir. As quantified by RP-HPLC, the daclatasvir loading capacity, which was defined as the net amount of drug loaded into the carrier, was about 10 w/w% weight. After the incorporation of daclatasvir, the effective diameter of the micelles increased from 42.4 nm to 104.0 nm, along with the higher polydispersity (bimodal, PDI 0.33) (Table 4.1), which implied that the loading of hydrophobic small molecules might affect the dispersion stability of the preformed micelles. This might be addressed by adjusting the feed ratio of daclatasvir to the APN.
In addition, the more stable micelles are formed by introducing the additional hydrophobic segment, which can serve as a separate compartment for daclatasvir loading.

Table 4.1 Physicochemical characteristics PEG-PLE/(p41+daclatasvir)

<table>
<thead>
<tr>
<th>Samples</th>
<th>D_{eff} (nm)(^a)</th>
<th>PDI(^a)</th>
<th>LC (w/w%)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-PLE/p41</td>
<td>42.4 ± 2.5</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>PEG-PLE/(p41+daclatasvir)</td>
<td>104 ± 14</td>
<td>0.33</td>
<td>10</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (n = 3).

\(^a\) Effective diameter (D_{eff}) and polydispersity index (PDI) were determined by DLS.

\(^b\) Daclatasvir content was determined by HPLC. Loading capacity (LC) is expressed as the mass of incorporated drug per mass of drug loaded micelles (w/w%).

4.3.2 Preparation and characterization of PEG-PLE-PLF/(p41+daclatasvir)

As discussed above, the micelles based on triblock copolymer PEG-PLE-PLF were used to load peptide p41 and daclatasvir. The presence of the hydrophobic PLF block confers amphiphilic properties and facilitates the formation of micellar aggregates in an aqueous medium: the formation of nanosized (effective diameter of approximately 75 nm) and narrowly distributed (PDI 0.16) particles were observed. Cationic p41 binds to the intermediate anionic PLE layer due to the electrostatic interaction (Z_{+/-}=1), while the hydrophobic core formed by PLF serves as a reservoir for the hydrophobic small molecules. Increased effective diameter was observed after the electrostatic coupling with p41, while after loading of daclatasvir the size is comparable to the empty micelles (Table 4.2). Overall, the macroscopic characteristics of the pre-formed micelles were maintained after the drug loading. Unexpectedly, the loading capacity of daclatasvir was around 6 w/w%, which is lower than the PEG-PLE/(p41+daclatasvir) APN. The loading of p41
into the PEG-PEL-PLF micelles was further confirmed using the size exclusion chromatography.

The chromatogram of PEG-PEL-PLF/p41 ($Z_{\infty}$ =1) showed a peak at 9.8 min (Figure 4.2 A), which corresponded to the micelles loaded with p41 with a large molecular weight, while the peptide alone eluted at 24.2 min (Figure 4.2 B). The PEG-PEL-PLF alone eluted at 10.4 min (Figure 4.2 C).

**Table 4.2** Physicochemical characteristics PEG-PEL-PLF/(p41+daclatasvir)

<table>
<thead>
<tr>
<th>Samples</th>
<th>$D_{\text{eff}}$ (nm)$^a$</th>
<th>PDI$^b$</th>
<th>LC (w/w%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-PEL-PLF micelles</td>
<td>74.7</td>
<td>0.16</td>
<td>-</td>
</tr>
<tr>
<td>PEG-PEL-PLF/p41</td>
<td>97.4 ± 3.9</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>PEG-PEL-PLF/(p41+daclatasvir)</td>
<td>65.8 ± 2.4</td>
<td>0.24</td>
<td>6</td>
</tr>
</tbody>
</table>

$^a$ Effective diameter ($D_{\text{eff}}$) and polydispersity index (PDI) were determined by DLS.

$^b$ Daclatasvir content was determined by HPLC. Loading capacity (LC) is expressed as the mass of incorporated drug per mass of drug loaded micelles (w/w%).
Figure 4.2 Size exclusion chromatography analysis with UV 280 nm (blue) and RI (red) signal. (A) PEG-PLE-PLF/p41; (B) p41; (C) PEG-PLE-PLF.
4.3.3 Micelles based on PEG-PLE-stearic acid loaded with (p41+daclatasvir)

The synthesis of PEG-PLE-stearic acid copolymer is illustrated in Scheme 4.1. The block copolymer PEG-PLE was synthesized via the ring-opening polymerization of BLE-NCA monomer, which was described in Chapter III. First, the Fmoc-Lys(Fmoc)-OH were attached to the terminal amino group of PLE block using coupling agents. Subsequently, two C18 alkyl chains were conjugated to the deprotected lysine amino residue with the stearic acid NHS ester. The structure of the final product was confirmed by $^1$H-NMR (Figure 4.3) with characteristic peaks: $\delta$ 1.3 ppm was assigned to $\text{CH}_3\text{C}_{12}\text{H}_{30}\text{CH}_2\text{-}$, and the peak at $\delta$ 0.87 ppm was assigned to $\text{CH}_3\text{C}_{16}\text{H}_{32}\text{CO}\text{-}$. The assay to quantify the C18 chains conjugation efficiency is greatly needed since the NMR results were not accurate enough by calculating the peak intensity ratios of the methylene protons of PEG to the terminal methyl protons of C18 chains.

The synthesized PEG-PLE-stearic acid was used to load the p41 and daclatasvir. Large aggregation was observed for the aqueous solution of PEG-PLE-stearic acid alone. However, the addition of cationic peptide p41 led to the formation of narrowly distributed nanosized particles in PBS, pH 7.4 (Table 4.3). Similarly, the daclatasvir was loaded into the hydrophobic domains formed by C18 alkyl chains by the extraction method. The increase of both effective diameter and PDI was observed, and the loading capacity of daclatasvir was determined to be approximately 9.4 w/w% by HPLC.
Figure 4.3 The $^1$H-NMR spectra (DMSO-$d_6$, 80°C) of PEG-PLE-stearic acid.

Table 4.3 Physicochemical characteristics PEG-PLE-stearic acid/(p41+daclatasvir)

<table>
<thead>
<tr>
<th>Samples</th>
<th>$D_{\text{eff}}$ (nm)$^a$</th>
<th>PDI$^a$</th>
<th>LC (w/w%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-PLE-stearic acid/p41</td>
<td>161.3</td>
<td>0.14</td>
<td>-</td>
</tr>
<tr>
<td>PEG-PLE-stearic acid/(p41+daclatasvir)</td>
<td>231</td>
<td>0.29</td>
<td>9.4</td>
</tr>
</tbody>
</table>

$^a$Effective diameter ($D_{\text{eff}}$) and polydispersity index (PDI) were determined by DLS.

$^b$Daclatasvir content was determined by HPLC. Loading capacity (LC) is expressed as the mass of incorporated drug per mass of drug-loaded micelles (w/w%).
The anti-HCV activity of PEG-PLE-stearic acid/(p41+daclatasvir) formulation was further evaluated in infected hepatoma Huh 7.5 cells. In this experiment, cells were pre-treated with corresponding formulations at 5 µM on peptide basis and 0.49 µM on daclatasvir basis for 5 h before 48 h infection with genotype 2a JFH-1 virus (MOI 0.1). Therefore, the intracellular antiviral effect of PEG-PLE-stearic acid/(p41+daclatasvir) was studied in this scenario. Since the picomolar potency of daclatasvir dramatically dominated the activity of p41 with submicromolar potency, higher than 99% viral replication was inhibited by both daclatasvir alone and p41+daclatasvir combination with no significant difference (Figure 4.4). Nevertheless, the complementary action of p41 is expected when the sensitivity to DAA is decreased or lost with the emergence of resistant HCV virus such as genotype 1a [14]. It has been reported that the mutation in genotype 1a NS5A led to much higher level of resistance to daclatasvir in in vitro replicon system compared with the genotype 1b [7, 15]. To test this hypothesis, the mutant virus is needed. Besides, the daclatasvir in combination with other DAA have been approved for genotype 3, which presents the greatest challenge among all genotypes for the treatment. Notably, daclatasvir is not effective if it is taken alone, which allows us to hypothesize that p41, whose activity is non-specific to genotypes, may also potentiate the effect of daclatasvir to treat genotype 3. This remains to be tested in vivo with the lack of cell culture models for genotype 3.
Figure 4.4 The intracellular anti-HCV activity of p41 and daclatasvir combination was studied. Cells were pre-treated with PEG-PLE-stearic acid/(p41+daclatasvir), p41, daclatasvir, and p41+daclatasvir for 5 h before overnight infection (MOI 0.1). In 48 h, HCV RNA expression was determined by RT-qPCR and normalized to GAPDH mRNA levels. Data are presented as %control ± SD in comparison to HCV RNA level in non-treated infected cells (100%); *** p < 0.001, ** p < 0.01, NS - not significant.
4.3.4 Preparation and characterization of mPEG-PLE-g-lomibuvir

The lomibuvir was selected as an alternative candidate sharing the equivalent potency with p41. By using DCC/DMAP as coupling agent, 20% of glutamic acid units of mPEG-PLE diblock copolymer were grafted with lomibuvir through the ester linkage. The structure of the product was characterized by $^1$H-NMR (Figure 4.5). As shown from the NMR spectra, the characteristic resonances at δ 1.3 ppm and 0.75 ppm were assigned to the protons of tert-butyl ((CH$_3$)$_3$-C-) and methyl (CH$_3$-cyclohexyl) groups of lomibuvir, respectively, which indicates the successful grafting of lomibuvir. In aqueous solution, the hydrophobically modified block copolymer self-assembles into the micelles with the effective diameter of ca. 90 nm with narrow distribution (PDI 0.1-0.2) as analyzed by DLS. The controlled release of lomibuvir is expected through the cleavage of ester bond. With this platform developed, we expected to further load the cationic p41 peptide through the electrostatic coupling with the unmodified anionic glutamic acid. More studies are greatly needed to characterize and optimize the formulations (e.g. anionic block length, degree of DAA grafting, ratio of DAA to p41). The synergistic effect of p41 and lomibuvir also remains to be tested both in vitro and in vivo.
Figure 4.5 The $^1$H-NMR spectra (DMSO-$d_6$, 25°C) of mPEG-PLE-g-lomibuvir.
4.4 Conclusion

In the present study, various poly(amino acid)-based micellar carriers were synthesized and characterized to load DAA (daclatasvir and lomibuvir) and antiviral peptide p41 simultaneously. The anti-HCV efficacy of PEG-PLE-stearic acid/(p41+daclatasvir) was tested in vitro. Due to the huge difference in potency (picomolar vs. submicromolar), the superior anti-HCV efficacy of p41+daclatasvir was not observed. Nevertheless, these platforms provide potential opportunities to encapsulate other hydrophobic DAA instead with relevant potency as p41. In addition, the block copolymer grafted with lomibuvir was synthesized, which self-assemble into micelles in the aqueous solution. More studies in vitro and in vivo are needed to further evaluate and screen the peptide and DAA combination as synergistic therapy for HCV.

4.5 References


CHAPTER V

DEVELOPMENT OF POLYMERIC MICELLES AS CARRIERS FOR PARENTAL ANTIVIRAL PEPTIDE DELIVERY

5.1 Introduction

As was demonstrated in Chapter II and III, the APN approach as a platform for the delivery of cationic antiviral peptide p41 has been successfully developed and validated [1, 2]. The parental peptide C5A (abbreviated as p1 in this study) shared the equivalent submicromolar level of antiviral potency (L-isomer IC₅₀: 0.79 µM) and mechanism of action with the p41 [3, 4]. In contrast to p41, p1 displays much lower cytotoxicity with the net charge of -2. Therefore, the toxicity-related side effects for peptide delivery can be abated. With this advantage, it was selected as an alternative candidate for the development of antiviral formulation.

Due to the amphiphatic nature of p1, the self-assembled polymeric micelles represent as attractive class of vehicles for the delivery of p1. Its incorporation within the inner hydrophobic core or the palisade layer of micelles, depending on the peptide property, results in the stabilization of peptide against proteolytic degradation with no need of chemical modification [5]. The hydrophilic shell serves as a stabilizing interface between the hydrophobic core and external milieu by inhibiting protein binding and opsonization during systemic administration, which leads to extended circulation half-life by evading the mononuclear phagocytic system. Among all the amphiphilic block or graft copolymers, the biocompatible and biodegradable PEG-phospholipid conjugate (PEG-DSPE) has been extensively explored to incorporate various therapeutic peptides
With the phospholipid moieties as hydrophobic blocks, they impart a very high stability with low critical micelle concentration value \((10^{-5} \text{ M range})\) because of very strong hydrophobic interactions between double acyl chains of the phospholipid residues [10]. The loading of amphipathic peptide into micelles is driven by the decrease of free energy in the system because of the removal of hydrophobic fragments from the aqueous environment and the re-establishing of hydrogen bond network in water. Additional energy gain results from formation of van der Waals bonds between hydrophobic blocks in the core of the formed micelles [11].

In the present study, the PEG-DSPE micelles loaded with the native form of the parental peptide p1 were prepared, characterized, and evaluated preliminarily as potential therapeutics for HIV/HCV infections. We anticipated that the challenges associated with the peptide delivery (e.g., inactivation by proteases, rapid elimination) can be overcome through its immobilization into the polymeric micelles, so that the overall therapeutic outcome will be improved.

### 5.2 Materials and methods

#### 5.2.1 Materials

Peptide SWLRDIWDWICEVLSDFK (p1) was custom synthesized by AnaSpec (Fremont, CA). N-(carbonyl-methoxypolyethyleneglycol2000)-1,2-sn-glycero-3-phosphoethanolamine (PEG\(_{2k}\)-DSPE) was purchased from CORDEN PHARMA (Switzerland). Anhydrous ethanol was obtained from the Fisher Scientific.

#### 5.2.2 Preparation of p1-loaded PEG\(_{2k}\)-DSPE micelles
The p1-loaded PEG\textsubscript{2k}-DSPE micelles were prepared using the film hydration method as reported before (Scheme 5.1) [10]. Predetermined amounts of PEG\textsubscript{2k}-DSPE and p1 peptide stock solutions in ethanol were mixed well, and the composition of the mixtures was defined as the weight percentage (wt\%) of peptide in polymer excipients. As an example, the micelles of 2 wt\% are prepared by mixing 50 µl of PEG\textsubscript{2k}-DSPE (1 mg/ml) with 2.02 µl of p1 (0.5 mg/ml) in ethanol. Following removal of ethanol with the air flow at room temperature, the formed thin film was further dried under vacuum to remove residual solvent. The dried film was subsequently redispersed with appropriate amounts of 10 mM phosphate buffer saline (pH 7.4).
Scheme 5.1 Preparation of p1-loaded PEG<sub>2k</sub>-DSPE micelles via the thin film hydration method.
5.2.3 Dynamic Light Scattering (DLS)

The size and size distribution of micelles with different compositions were measured by DLS using Nano ZS Zetasizer (Malvern Instruments, UK) as described in Chapter II.

5.2.4 Circular Dichroism (CD) spectroscopy

The secondary structure of p1 before and after its encapsulation into the PEG-lipid micelles was studied by measuring the CD spectra. The detailed procedure has been described in Chapter II. The samples (free p1, p1/PEG2k-DSPE, and PEG2k-DSPE) were prepared in 10 mM phosphate buffer saline (pH 7.4) at the concentration of 100 µM on peptide basis. The final spectral data were converted to the mean molar ellipticities.

5.2.5 Fluorescence spectroscopy

The fluorescence emission spectra of PEG2k-DSPE/p1 (2 wt%) and free p1 were measured using a spectrofluorometer system (Fluorolog®, HORIBA Jobin Yvon Inc., NJ, USA). The emission spectrum (300-400nm) of each sample was collected at 25°C in 10 mM phosphate buffer (pH 7.0) with excitation wavelength of 270 nm. All measurements were recorded with the bandwidth of 5 nm for excitation and emission. The peptide concentration was 50 µM in all samples.

5.2.6 Trypsin digestion

The stability of PEG2k-DSPE/p1 (2 wt%) against the proteolytic digestion was studied using the trypsin spin columns as previously described in Chapter II. The buffered solution of p1 and corresponding PEG2k-DSPE/p1 (30 µg based on peptide equivalents in 150 µl) were subjected to the trypsin digestion for 13 min followed by MALDI-TOF MS analysis.
5.2.7 *In vitro* anti-HIV-1 activity

The anti-HIV efficacy of the p1-loaded PEG<sub>2k</sub>-DSPE micelles was tested in human monocyte-derived macrophages (MDM). The culture condition of MDM was described in **Chapter II**. Cells were pre-treated for 2 h with p1 or PEG<sub>2k</sub>-DSPE/p1 (2 wt%) at the concentrations of 5 µM on peptide basis before overnight infection with HIV-1<sub>ADA</sub> (MOI 0.01). The supernatants were collected at days 3 and 6 post-infection. HIV-1 replication in MDM was estimated by measuring reverse transcriptase (RT) activity and adjusted to the cell viability determined by the standard tetrazolium dye method (MTT assay).

5.2.8 *In vitro* anti-HCV activity

The Huh 7.5 cells which support the replication of full-length genotype 2a HCV clone JFH-1 was used to examine the anti-HCV efficacy of p1 and PEG<sub>2k</sub>-DSPE/p1 (1 wt%). Cell culture condition has been described in **Chapter II** and **Chapter III**. The cells were inoculated with the mixture of JFH-1 (MOI 0.1) with p1 or PEG<sub>2k</sub>-DSPE/p1 at the concentration of 5 µM on peptide basis for overnight. Next day, the cells were washed and replenished with fresh medium for another 48 h culture. Total cellular RNA was extracted and was converted to cDNA, followed by HCV RNA quantification via the real-time qPCR.

5.3 Result and discussion

5.3.1 PEG<sub>2k</sub>-DSPE/p1 synthesis and characterization

A series of PEG<sub>2k</sub>-DSPE micelles loaded with different amounts of p1 was prepared by the thin film hydration method (from ethanol). The amphipathic peptide p1 was spontaneously
entrapped into the formed micelles upon hydration of film with aqueous buffered solutions. Driven by the amphipathic nature of p1, it is presumably to be encapsulated in the palisade layer of the micelles as reported [8, 11]. With the formulation prepared, the peptide–micelle association was characterized by various biophysical techniques.

First, the size distribution of p1-loaded micelles was measured using the DLS. Compared to the large and heterogeneous aggregates formed by p1 alone, the micelles loaded with 1-2 wt% p1 were small (average diameter of ~15 nm) and uniform (monomodal, polydispersity indices (PDI) of 0.1-0.2), which agrees well with the previously reported data (Table 5.1) [7, 10, 12]. When p1 content is above 2 wt%, the formation of big aggregates was determined by DLS, which implied the existence of unloaded free peptide. Therefore, the micelles loaded with 1-2 wt% p1 were selected for the following studies.

Since the antiviral function of p1 correlates with the α-helical structure [3], its secondary conformation before and after the encapsulation in the micelles was measured by CD (Figure 5.1). The characteristic double minima at ~208 and 222 nm and maximum at 195 nm of free p1 revealed the signature features of α-helices. The association of p1 with the PEG2k-DSPE micelles does not alter the inherent propensity for p1 to form the α-helical structure, which is known to be essential for its antiviral activity.
Table 5.1 Physicochemical characteristics of p1/PEG_{2k}-DSPE

<table>
<thead>
<tr>
<th>Composition (wt%)</th>
<th>D_{eff} (nm)^{b}</th>
<th>PDI^{b}</th>
<th>Particle Size Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% (p1 alone)</td>
<td>480 ± 120</td>
<td>0.50</td>
<td>Multimodal</td>
</tr>
<tr>
<td>1%</td>
<td>14.8 ± 2.7</td>
<td>0.15</td>
<td>Monomodal</td>
</tr>
<tr>
<td>2%</td>
<td>17.7 ± 0.2</td>
<td>0.18</td>
<td>Monomodal</td>
</tr>
<tr>
<td>3%</td>
<td>26.1 ± 2.3</td>
<td>0.46</td>
<td>Bimodal</td>
</tr>
<tr>
<td>5%</td>
<td>22.6 ± 0.9</td>
<td>0.36</td>
<td>Multimodal</td>
</tr>
</tbody>
</table>

^{a} Samples were prepared in PBS (pH 7.4, 0.14 M NaCl).
^{b} Effective diameter (D_{eff}) and polydispersity indices (PDI) were determined by DLS at pH 7.4, 25°C (n = 3).
Figure 5.1 CD spectra of free p1 (black line) and p1/PEG$_{2k}$-DSPE (2 wt%) (blue line) recorded in phosphate buffer saline, pH 7.4, at 20°C. The peptide concentrations were 100 µM.
The formation of p1-loaded micelles was further confirmed by measuring the intrinsic fluorescence of p1 containing 3 tryptophan residues before and after the incorporation into the micelles. The emission maximum of tryptophan in water occurs near 350 nm and is highly dependent upon the polarity of local environment. The fluorescence spectrum shifts to shorter wavelength and the intensity of the fluorescence increases as the polarity of the solvent surrounding the tryptophan residue decreases. As shown in Figure 5.2, the fluorescence spectrum of free p1 in aqueous solution displayed a broad band with a maximum at 357 nm. The fluorescence intensity of p1 increased approximately 3 folds in the presence of PEG_{2k}-DSPE and was accompanied with a blue shift of fluorescence maximum to 348 nm. These data indicate the changes in the environment of p1 fluorophores from a hydrophilic environment to a relatively more hydrophobic surrounding in the micelles, which potentially suggests the association of p1 with the hydrophobic cores of PEG_{2k}-DSPE micelles. Similar behavior was previously reported for the peptides containing tryptophan residues and incorporated into the PEG_{2k}-DSPE micelles [7, 13].
Figure 5.2 Fluorescence emission spectra of p1 and PEG_{2k}-DSPE/p1 (2% wt) in phosphate buffer, pH 7.0.
5.3.2 Stability against trypsin digestion

The stability of PEG<sub>2k</sub>-DSPE/p1 (2 wt%) against the proteolytic digestion was evaluated using the Trypsin Spin Columns. By analyzing the eluents after 13 min incubation via MALDI-TOF MS, the encapsulation of p1 into the micelles led to stabilization of peptide against trypsin digestion, with the intact p1 detected at 2311 (m/z) (Figure 5.3). In addition, no digested fragment can be detected, which suggests higher stabilization effect by PEG<sub>2k</sub>-DSPE as compared to the APN (p41/PEG-b-PLD<sub>20</sub>) platform driven by electrostatic interaction (Chapter II). However, under same condition, naked p1 was completely disintegrated with only the fragment identified as DIWDVICEVLSDFK. These data demonstrate that the incorporation into the micelles protects peptide from enzymatic disintegration, and this may result in longer circulation half-life after administration.
The stability of p1 against proteolytic degradation is enhanced in the presence of PEG$_{2k}$-DSPE. MALDI-TOF spectra represent the intact p1, digested p1, digested PEG$_{2k}$-DSPE/p1 (2 wt%) and PEG$_{2k}$-DSPE alone. After 13 min incubation on trypsin column for 13 min, the p1 remained intact with the peak at 2311 (m/z) with no digested fragment detected in PEG$_{2k}$-DSPE/p1 sample, whereas naked p1 was completely degraded.
5.3.3 Anti-HIV-1 activity *in vitro*

The preliminary *in vitro* studies were carried out to evaluate the antiviral effect of PEG$_{2k}$-DSPE/p1 formulations. The anti-HIV activity of the p1, PEG$_{2k}$-DSPE alone, or PEG$_{2k}$-DSPE/p1 (2 wt%) was tested in an *in vitro* model system using infected human MDM. MDM were pre-treated with corresponding formulations at 5 µM on peptide basis for 2 h and then infected with HIV-1 (*Figure 5.4*), followed by measuring RT activity adjusted to cell viability. The suppression of the RT activity by free p1 was only seen up to 3 days post-infection. On the contrary, the incorporation of p1 into the micelles significantly potentiated and prolonged its antiviral efficacy: around 45% of the virus was inactivated at day 6, which implied the sustained release of active p1 from the micelles. Similar results has also been observed and discussed in Chapter II, which are attributed to the protection of peptide provided by the polymer against the intracellular proteases. Unexpectedly, the PEG$_{2k}$-DSPE alone exhibited antiviral activity at day 3 post-infection. More detailed studies, including different regimens and the activity of PEG$_{2k}$-DSPE, are in great need to further investigate and validate the anti-HIV efficacy of p1-loaded micelles.
Figure 5.4 PEG$_{2k}$-DSPE/p1 exhibited superior anti-HIV efficacy to free p1 *in vitro*. MDM were pre-treated with corresponding formulations at the concentration of 5 µM on peptide basis for 2 h, infected with HIV-1$_{ADA}$, and cultured for 6 days. RT activity was adjusted to MTT values/well. Data presented as mean ± SD (n = 6); ***$p < 0.001$, NS - not significant compared to HIV infected cells.
5.3.4 Anti-HCV activity in vitro

With the preliminary results shown here, the anti-HCV activity of PEG<sub>2k</sub>-DSPE/p1 and free p1 was evaluated in Huh 7.5 cells, which are permissible to the genotype 2a JFH-1 strain of HCV [14]. The cells were treated with JFH-1 virus (MOI 0.1) and corresponding formulations simultaneously for overnight, followed by another 48 h culture. As discussed in Chapter III, this treatment regimen is a combination of extracellular virocidal effect and intracellular effect against viral assembly and release [2]. Based on the analysis of HCV RNA replication using real time qPCR, approximately 50% of HCV replication was suppressed in the cells treated with free p1. In contrast, more than 99% of viral RNA was inhibited in the cells treated with p1-loaded micelles (Figure 5.5). Overall, these data provide initial evidences of the superior anti-HIV/HCV efficacy of p1-loaded micelles, which is likely to be a result of the increased stability, higher uptake, and controlled release over the time after its incorporation into the micelles. More cell studies are greatly needed to further examine the anti-HCV activity of PEG<sub>2k</sub>-DSPE/p1.
Figure 5.5 PEG2k-DSPE/p1 displayed superior anti-HCV effect compared to p1 alone. Huh 7.5 cells were treated with the mixture of JFH-1 virus (MOI 0.1) and p1 (5 µM on p1 basis) for overnight. Next day, cells were washed and replenished with fresh media for another 48 h culture. The intracellular HCV RNA level was normalized to the GAPDH mRNA levels. Data are presented as %control in comparison to HCV RNA level in non-treated infected cells (100%).
5.4 Conclusion

In conclusion, the *in vitro* studies presented here provide initial evidences that the nanosized PEG$_{2K}$-DSPE micelles can be used as a platform for the delivery of amphipathic p1, which address the concern of the cytotoxicity associated with the cationic derivatives. The incorporation of p1 into the micelles was confirmed and characterized. Its α-helical structure was preserved upon encapsulation. PEG$_{2K}$-DSPE micelles protected p1 from degradation by trypsin and could thereby prolong and improve their antiviral activity against HIV as well as HCV, which has been demonstrated in preliminary *in vitro* cell-based studies. Collectively, p1-loaded PEG$_{2K}$-DSPE micelles have demonstrated potential as therapeutics for the treatment of HIV/HCV co-infection. Its therapeutic efficacy also remains to be validated in more cell studies (e.g. different regimens, different peptide concentrations) and *in vivo* animal models.

5.5 References


CHAPTER VI

SUMMARY

Tremendous progress in HCV therapeutics has been achieved with the development of highly selective direct-acting antivirals (DAA), but their worldwide implementation is limited due in large part to the high cost, genotype dependency, and potential resistance. The peptides p1 and the cationic analog p41 with wide spectrum of antiviral activity and the distinctive mechanism of action offer novel opportunities to become a unique class of therapeutic agents against HCV with or without the accelerating cofactors. However, its successful clinical translation suffers from the instability, short half-life, potential induction of immune response, and limited ability to cross the physiological barriers. As introduced and reviewed in Chapter I, these limitations can be overcome by a variety of polymer-based nanocarriers as vehicles, which have already demonstrated their excellent potential to deliver therapeutic peptides. Owing to their highly tunable structures and physicochemical properties, polymeric materials offer multiple remarkable advantages for the delivery of peptides, including enhanced stability, sustained release, and improved pharmacokinetics/biodistribution profiles. Importantly, each of these polymer-based platforms has their own pros and cons, and their applicability for peptide delivery is determined by the nature of peptides.

In Chapter II, the cationic antiviral peptide p41 was complexed with the anionic PEG5k-b-poly-L-aspartic acid (PEG5k-b-PLD20) or PEG5k-b-poly-L-glutamic acid (PEG5k-b-PLE25)
to form the antiviral peptide nanocomplexes (APN). As measured by DLS, the well-defined APN were ca. 35 nm in size, narrowly distributed, and stable at physiological conditions. APN exhibit spherical morphology as determined by Atomic Force Microscopy. The formation of APN was further confirmed by sedimentation equilibrium analysis and size exclusion chromatography. The unaltered α-helical structure of p41 before and after complexation was measured using CD. The increased stability against trypsin digestion and reduced hemolytic activity of p41 was observed after its encapsulation into APN. Meanwhile, the anti-HIV/HCV activity of p41 in APN format remained intact as tested in HCV and HIV susceptible and transmitting cells. *In vivo*, APN were able to decrease the viral load in mice transplanted with human lymphocytes and HIV-1-infected with no signs of toxicity.

Since hepatocytes are the primary site of HCV infection, the liver-targeting approach of APN was explored in **Chapter III**. This was achieved by modifying APN surface with hepatocytes-specific ligand galactose (Gal-APN). By using Cy5-labeled p41, the favorable galactose receptor-mediated uptake of Gal-APN was observed in hepatoma cell lines (HepG2 and Huh 7 cells) as well as in primary human hepatocytes. Even though liver-targeted and non-targeted APN displayed comparable activity against HCV RNA replication in Huh 7.5 cells, Gal-APN demonstrated a significantly stronger ability to prevent intracellular binding of HCV core and NS5A proteins to lipid droplets, which is known to be an essential step for viral assembly and release. By using the radiolabelled p41, we were able to show in the biodistribution studies the preferential liver accumulation of Gal-APN that increase the effectiveness of the targeting strategy for the delivery of antiviral peptides to the sites of viral replication.
As shown in Chapter IV, a series of poly(amino acid)-based nanocarriers were prepared to load antiviral peptide p41 and hydrophobic DAA (daclatasvir and lomibuvir) combination to test the feasibility of synergistic anti-HCV effect. First, p41 and daclatasvir were loaded in micelles formed by PEG-PLE as well as its hydrophobically modified derivatives of PEG-PLE-PLF and PEG-PLE-stearic acid. The p41 was complexed with the negatively charged PLE block driven by electrostatic interaction, followed by entrapment of daclatasvir into the hydrophobic domains of the micelles. As shown in in vitro models, the sensitivity of unmodified JFH-1 virus to daclatasvir was extremely high and the synergistic anti-HCV efficacy of PEG-PLE-stearic acid/(p41+daclatasvir) was not observed, mainly due to the dominant picomolar activity of daclatasvir. Moreover, the hydrophobic lomibuvir moieties were attached to approximately 20% of the glutamic acid residues of PEG-PLE block copolymer, and the structure of the product was confirmed by $^1$H-NMR. In aqueous solution, the block copolymer hydrophobized with lomibuvir self-assembled into the narrowly distributed micelles as analyzed by DLS. More studies in vitro and in vivo are greatly needed to load p41 through electrostatic coupling with unmodified PLE and to further validate the p41 and lomibuvir combination as synergistic therapy for HCV.

The PEG$_{2k}$-DSPE micelles loaded with the native form of the non-cytotoxic parental peptide p1 (PEG$_{2k}$-DSPE/p1) was described Chapter V. The encapsulation of p1 into the micelles was confirmed by the change of fluorescence emission spectra of p1 tryptophan residues. The $\alpha$-helical structure of p1 remained unaffected after its loading in micelles. The stability of p1 in micelles against proteolytic digestion was enhanced; thereby the anti-HIV/HCV activity was preserved as shown from preliminary data in cell culture systems, which remained to be further
evaluated and validated both \textit{in vitro} and \textit{in vivo}.

Overall, the data obtained from these studies supports our hypothesis that the immobilization of antiviral peptides into self-assembled polymeric micelles increases peptides stability, improves intracellular delivery, provides opportunities for the targeted delivery, and reduces the toxicity, if any, without the loss of therapeutic efficacy for the treatment of viral diseases. Altogether, these studies demonstrate a fundamental possibility for peptide delivery using polymeric micelles and open a new prospect for clinical development of such nanoformulations.

\textbf{Future studies}

1) Evaluation of therapeutic efficacy of APN and Gal-APN against HCV \textit{in vivo}. The highly immunodeficient NOG mice with the liver expressing a herpes simplex virus type 1 thymidine kinase (HSVtk) transgene (TK-NOG) with reconstituted human livers will be used as the animal models to study the anti-HCV efficacy \cite{1}, which is in the process of development in our group. Eight weeks after hepatocytes transplantation or when human serum albumin (HSA) levels exceed 2 mg/ml, mice will be intravenously injected with 100 µl of 1b HCV-positive human serum samples. The mouse serum samples will be obtained every 2 weeks after HCV infection, and HSA and HCV RNA levels will be measured. Allocation of mice with titers above $2 \times 10^4$ copies/ml to experimental groups will be done to balance for HCV titers, HSA levels, and weight with decreasing priority. Treatment with APNs will start when the mice develop stable viremia.

2) The activity of APN against HCV in the presence of alcohol. Since the severity of HCV-infection is accelerated by alcohol consumption \cite{2}, the potential of APN, which have
unique antiviral mechanism, to treat HCV-infected alcoholic patients will be tested. To serve this purpose, the Huh 7.5 CYP2E1+ cells with an acetaldehyde generating system as ethanol metabolite will be used [3]. Different regimens of APN treatment will be applied to examine the anti-HCV activity in the presence of ethanol metabolite. Following the in vitro study, the therapeutic efficacy of APN will be tested in corresponding animal models, which is under development in our group now.

3) Optimization of liver-specific delivery of APN by using N-Acetylgalactosamine (GalNAc) as targeting ligand. The binding affinity of GalNAc to ASGP-R was found to be 50 fold higher than that of Gal [4]. Therefore, the superior liver-targeting effect is anticipated using this strategy. The APN modified with GalNAc can be prepared by following the same method as Gal-APN synthesis. Subsequently, the liver-specific delivery of p41 will be examined in vitro and in vivo.

4) The DAA and antiviral peptide combination as synergistic therapy against HCV. As discussed in Chapter IV, p41 might complement the antiviral action of daclatasvir in the presence of mutant HCV genotype 1a, which have been reported to confer resistance to daclatasvir in in vitro models [5]. This will be tested in the cell lines harboring mutant genotype 1a replicons with specific NS5A amino acid substitutions. Moreover, further optimization and characterization studies about the PEG-PLE-g-lombuvir are needed, including the optimal anionic block length, optimal grafting degree, grafting efficiency, release of lombuvir from the nanocarrier, etc. The self-assembled micelles will also be loaded with p41 and characterized. To evaluate the anti-HCV efficacy of p41/PEG-PLE-g-lombuvir, the Huh 7.5 cells expressing genotype 1a replicons need to be established [6, 7]. Last but not least, these established
BIC-based nanocarriers can be used to encapsulate more DAA together with p41 to be tested and screened for the anti-HCV synergy.

References


