Development of cell specific aptamer conjugated nanoliposomes for the delivery of apigenin to target hepatocellular carcinoma

Thesis submitted

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"Statement of Originality"

I, Moumita Dhara registered on 30th November 2016, do hereby declare that this thesis entitled **"Development of cell specific aptamer conjugated nanoliposomes for the delivery of apigenin to target hepatocellular carcinoma"** contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies. All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work. I also declare that I have checked this thesis as per the "Policy on Anti Plagiarism, Jadavpur University, 2019", and the level of similarity as checked by I Thenticate software is 7%.

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CERTIFICATE FROM T HE SUPERVISOR

This is to certify that the thesis entitled **"Development of cell specific aptamer conjugated nanoliposomes for the delivery of apigenin to target hepatocellular carcinoma"** submitted by Mrs. Moumita Dhara, who got her name registered on 30th November 2016 for the award of Ph. D. (Pharmacy) degree of Jadavpur University is absolutely based upon his/her own work under the supervision of **Professor (Dr.) Biswajit Mukherjee,** Department of Pharmaceutical Technology, Jadavpur University, Kolkata and that neither his/her thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

15/5/2023

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Moumita Dhara.

Place: Jadavpur University (Moumita Dhara)

15/5/2023.

I am dedicating this thesis
To my mentor
Professor Biswajit Mukherjee,
and to my
Husband, daughter, parents,
and in-laws

PREFACE

Hepatocellular carcinoma (HCC) is a leading cause of death globally and worst reported diseases for survival as it is poorly responsive to both conventional chemotherapy and mechanism directed chemotherapy. This issue is due to the lack of therapeutic concentration in the tumor tissue coupled with the highly toxic side effects exerted by this compound along with concomitant drug resistance towards tumor heterogenicity. Consequently, the best packaging for the therapy of the HCC involves three components: A potent therapeutic drug, a rationally designed drug delivery vehicles to enrich the target side with optimum concentration of the drug and a surface ligand that can lead to a greater possibility for internalization by tumor cells compared to the normal parenchyma.

The effectiveness of flavonoids (apigenin) would improve using in nanoformulations as per literature. Amphiphilic liposomes protect sensitive flavonoid compounds from denaturing and improve the absorption of the encapsulated molecules. Aptamers are new class of small multifunctional ligands, comprising short single stranded oligonucleotides about 30-80 bases in length with high affinity and specificity for their targets. They are developed from RNA or ssDNA libraries via an experimental directed process referred to as systematics evaluation of ligands by exponential enrichment (SELEX).

This thesis provides an insight into the development of tumour-sensing Phosphorothioated and amino-modified aptamer (AS1411)-conjugated stealth nanoliposomes, encapsulating with apigenin for precise and significant biodistribution of apigenin into the target tumour to exploit maximum bio-therapeutic assistances. The stable aptamer functionalized PEGylated nanoliposomes (Apt-NLCs) had an average vesicle size of 100–150 nm, a smooth surface, and an intact lamellarity, as ensured by advanced microscopic studies. This study has specified in vitro process of optimum drug (apigenin) extrusion into the cancer cells by nucleolin receptor-mediated cellular internalization when delivered through modified AS1411 functionalized PEGylated nanoliposomes and ensured irreversible DNA damage in HCC. Significant improvement in cancer cell apoptosis in animal models, due to reduced clearance and higher intratumor drug accumulation along with almost nominal toxic effect in liver, strongly supports the therapeutic potential of aptamer-conjugated PEGylated nanoliposomes compared to the nonconjugated formulations in HCC. The study has established a robust superiority of modified AS1411 functionalized PEGylated nanoliposomes as an alternative drug delivery approach with momentous reduction of HCC tumour incidences.

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Chapter 1

Introduction

1. Introduction

1.1. Liver & its functional units.

Liver is an ecosystem which regulates plethora of essential bio-chemical and physiological functions for maintaining the homeostasis in the living organism. It generally comprises around 2-3% of an adult's total body weight with hexagonal functional unit lobules. The lobules comprise unique dual blood supply with the portal vein and the hepatic artery. These portal triad (portal vein, hepatic artery, bile duct), is rich in nutrients and oxygen, enters the sinusoids, whereas it drains into the central vein.

These lobules are composed of choanocytes, hepatocytes, sinusoidal-endothelial cells, natural killer cells, Kupffer cells, and some hepatic stellate cells. All they have distinct physiological structure with basolateral and apical membranes. Based on blood perfusion in different region of liver, they have classified for three functional zone.

Zone I is the periportal part of liver and due to their proximity towards portal vain, it is the best perfused and oxygenated hepatic region with multi -nutrients supply. Therefore, zone I exhibits its crucial role on oxidative metabolisms, gluconeogenesis, beta-oxidation, amino acid catabolism along with formation of bile, cholesterol other essential enzymes.

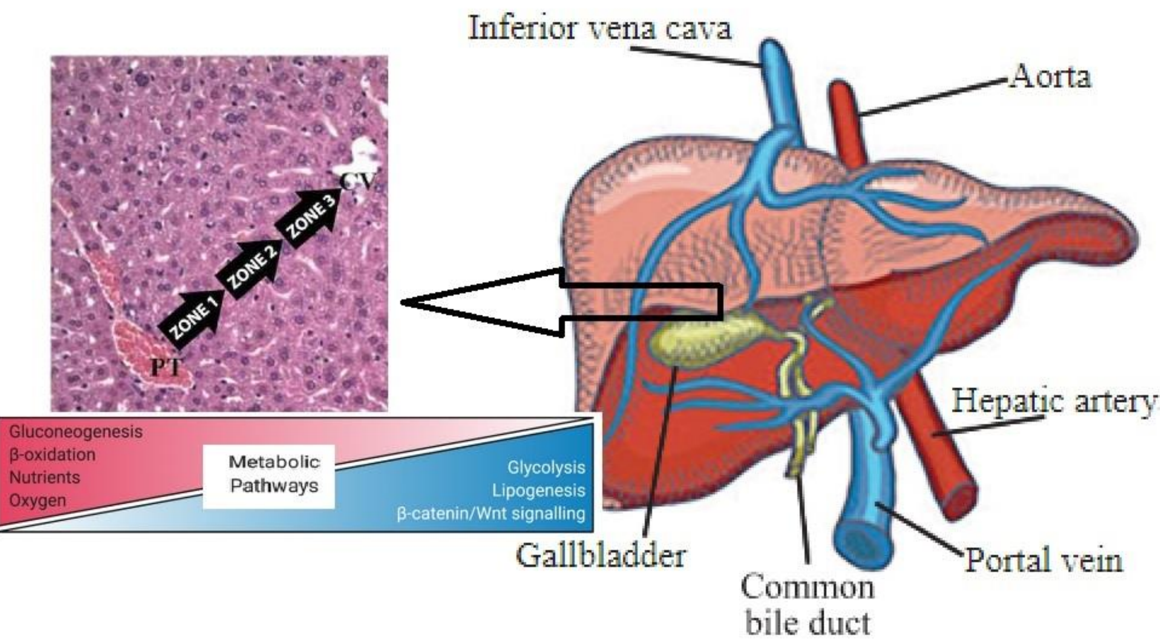


Figure 1.1 Cellular architecture of liver

Zone II is distinguished as the pericentral hepatic area located between zones I region and III region.

Zone III of hepatic region has the poorest perfusion due to its distance from the portal triad. It plays the largest role in detoxification, biotransformation of drugs, ketogenesis, glycolysis, lipogenesis, glycogen synthesis, and glutamine formation.

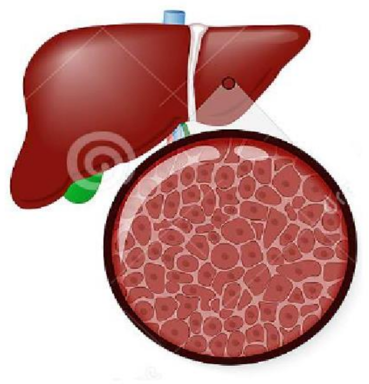
Thus, liver contributes for numerous crucial roles in body.

- Storage: In the storage and usage of glucose along with storage of vitamins and minerals for future use.
- Synthesis: Essential proteins, enzymes, bile acids, hormones are generated in body.
- Metabolism: Regulates metabolism of nutrients components (carbohydrates, lipids/fatty acids, proteins) with all the xenobiotics in body.
- Detoxification: neutralization of foreign antigens and microbes from the gut are performed by different types of liver cells including hepatocytes, choanocytes, sinusoidal endothelial cells, Kupffer cells, natural killer cells, and hepatic stellate cells.

1.2.Liver Injury and related disorders

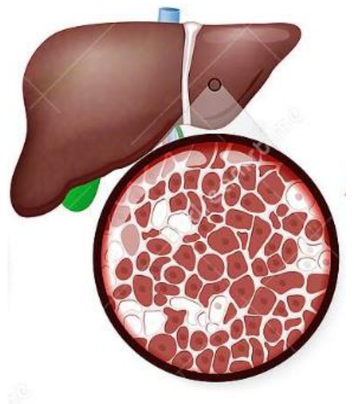
Along with portal triad arteries and central vein, the basic structural mass in liver is encompassed with numerous types of cellular units. They comprise of both parenchymal (hepatocytes cells, cholangiocytes cells) and nonparenchymal tissue structures (stellate cells, fibroblasts, Kupffer cells, and sinusoidal endothelial cells) (Stranger et. al. 2015). These organized and well-balanced cellular features with their ideal characteristic contribute for a performing healthy liver. As liver implicates all the vital metabolic processes in body, its failure persuades consequences for faulty metabolism, poor immune response, lack of detoxification and insufficient microbial defences. (Losser et al, 2008) Liver injury is very complex mechanism where it involves with numerous interrelated events of structural, physiochemical, and immunogenic, genetic, or epigenetic alterations. The metabolism processes concern majorly chemicals that require in detoxification. Faulty metabolites confer **fatty acid deposition** along with other enzymatic alteration could damage plasma membrane, cellular organelle; mitochondria, and hinder in intracellular homeostasis by changing of their pathologic/genetic/cell signalling process. Also, Inflammations in liver aggravates different cytokines release, nitric oxide storage, and complementary metabolic damage that contributes for acute to chronic liver injury as **liver fibrosis/cirrhosis**. Finally, influences molecular

microenvironment of hepatocytes in changing of their whole functional and structural panache through abnormal cell proliferation (Severi et al., 2010) and develop **carcinogenic tumour growth (hepatocellular carcinoma, HCC) in liver**.

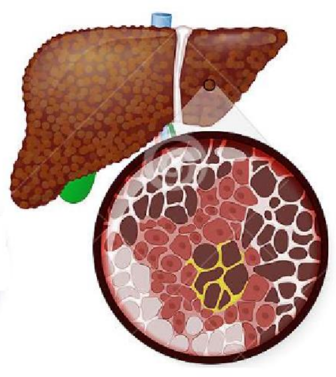


(a) Normal liver (healthy hepatocytes)

(b) Fatty liver (deposition of excess fat)



(c) Fibrosis (cellular deformities)



(d) Cirrhosis & Liver carcinomas (formation of nodular structure among tissue deformities

Figure 1.2 Different kind of liver disorder (a, b, c & d)

1.3. Types of primary Liver Cancer

Hepatocellular Carcinoma (HCC)

HCC as primary liver cancer originates from hepatocytes and frequently diagnosed liver cancer, a thorium dioxide accounts for approximately 80-90 percent of overall adults' liver cancer. (Chen et al., 2019). Also, metastasizes to other organs, the lungs, pancreas, bone marrow, brain, and other parts of digestive organs in later stage.

Intrahepatic Cholangiocarcinoma (ICC)

Intrahepatic cholangiocarcinomas initiates near small bile duct region at epithelial cell-lining arising primarily within the liver. Records show 10-20% of primary liver cancers comprises of ICC. (Gupta and Dixon 2017).

Angiosarcoma and Hemangiosarcoma

They are very rare, but aggressive forms of primary liver carcinoma. They initiate in the endothelial cell lining on the blood vessels of hepatic arteries and veins due to prolonged exposure of some harmful chemicals such as vinyl chloride, thorium dioxide, arsenics, pesticides, and androgenic steroids. (Molina and Hernandez 2003, Bolt 2005). They account for 2% of overall primary liver cancer, yet associated with very poor therapeutic outcome.

Hepatoblastoma

Unifocal immature lethal precursor liver hepatocytes seem to be considered as the source of infrequent hepatoblastoma. Even though this rare hepatoblastomas have been seen in very early stage of life (among infants / children), they may spread up to eight to nine years. and metastasize. (Wang et al., 2013).

Secondary Liver Cancer

Liver cancer at advanced stage is considered as secondary liver carcinoma, where it spreads up to the bowel section. Blood supply in liver (portal veins) is well-connected to the other parts of body. Thus, stomach, oesophagus, pancreas, ovary, kidney, melanoma, breast, and lung cancer usually reach to the hepatocytes in liver. These types of deadly liver cancer are frequently noticed in Australian content to a great extent.

1.4.Hepatocellular Carcinoma (HCC)

1.4.1. HCC prevalence

The incidence of liver cancer is increasing in many countries including India with the ranks of 5th and 7th most common cancer among men and women. Both incidences and mortality of liver cancer rising. More than 80% of liver cancer are attributed to hepatocellular carcinoma (HCC), which does not respond to most chemotherapy drugs. The estimated number of new cases annually is over 500,000 and HCC results in between 250,000 and one million deaths globally per annum. Once diagnosed with the disease, only 30-40% of the patients are deemed eligible for curative intention with treatment modalities including surgical resection, liver transplantation, and chemoembolization. Thus, hepatocellular carcinoma (HCC) is still a global health issue, and the shocking fact is future estimated incidences of hepatic malignancy that could cross one million by 2025 (Llovet, et al. 2021).

1.4.2. Aetiology & risk factors in HCC

Aetiology:

Many research studies are there for the present hypothesis, that hepatocytes and cholangiocytes both originates from a similar progenitor cell (hepatoblasts). Sequential genomic alterations finally convert them into neoplastic cells, but could be distinguished specifically with characterized histopathological and functional, signalling pathways along with some the bio-markers among the healthy progenitor vs the cancerous stem cells. (Tanimizu et al., 2013; Guest et al. 2014).

Along with portal triad arteries and central vein, the main structural mass in liver is made of different types of cellular blocks. They comprise of both parenchymal (hepatocytes cells, cholangiocytes cells) and nonparenchymal tissue structures (stellate cells, fibroblasts, Kupffer cells, and sinusoidal endothelial cells) (Stranger 2015). These organized and well-balanced cellular features with their ideal characteristic contribute for a performing healthy liver, while the process of initiation or development of any deformity (liver stenosis/fibrosis/cirrhosis/carcinomas) are tried to be figured through identifying their altered histopathological features and presence of specific molecular biomarkers. (Sia et al., 2017). Sufficient evidences are identified and reported in the literature that suggests mutation in genetic and epigenetic substances in liver take places through simultaneous alteration of the parenchymal and non-parenchymal features in liver. (Severi et al., 2010)

Liver insult through exposure of harmful toxic substances such as food toxins, alcoholic abuse or by virus and any other pathogenic spell could activate immune response and develop various macrophages (via Kupffer cells and hepatic stellate cells), leukocytes, pericytes, neutrophils, fibroblasts, HSCs, endothelial cells, and dendritic cells (Wu et al., 2012). which allows for triggering inflammations and evolving other hepatocytic growth factors in the liver. Thus, release of cytokines, interleukin-(IL-6/8), ligand 12 (CXCL12), chemokine (C-X-C motif), matrix metalloproteinase-(MMP-3/ MMP-9), TNF-α (Tumour necrosis factors) along with some other cancer-associated fibroblast (CAFs) in the extracellular matrix (ECM) damage CD8+ cytotoxic mediated T-lymphocyte (CTL) cells. As the process continues, liver fibrosis and cirrhosis may occur both in reversable and irreversible manner (Severi et al., 2010). Finally, carcinogenic characteristics appears in hepatocytes Also, develop a few of permanent damage in the hepatocyte parenchymatic architecture, appearing neoplastic lesions, nodules, and abnormal blood flow in liver. (Roskams and Kojiro, 2010).

Risk factors:

There are several risk factors which are responsible to initiation and development of HCC. Pre-existing or chronic liver diseases such as liver cirrhosis is a major contributor to HCC. (Balogh et al., 2016.). Further it is reported that, the key reason for cirrhosis/ primary liver cancer (HCC) includes viral insult in liver with hepatitis B and C virus (HBV/ HCV), excessive alcohol intake, and some metabolic health related disorder like NAFLD (non-alcoholic fatty liver diseases). Other causative factors for HCC occurrence are some hereditary genetic hemochromatosis, primary inflammation at biliary duct as cholangitis (PBC), heavy metal accumulation in liver, Wilson's disease etc. (Balogh et al., 2016).

Viral hepatitis is a critical contributor for HCC due to rapid HBV and HCV exposure, accounts for almost 70-80% of HCC cases globally with remarkably high incidences in East part of Asia and major topical parts of African region. (Park et al., 2015). The statistical epidemiology on HCC has been predicted around 257 million people could develop viral hepatitis (acute /chronic HBV infection) that may land on cirrhosis or HCC to many patients, following the consequences, more than 5 million mortality cases must be reported during the years of 2015 to 2030 globally. (Yang et al., 2019).

HBV comprehends for a unique double-stranded spherical DNA molecule with eight (A-H) kind of genotypes varies for different geographic distribution. For instance, the most predominant genotypes that studied in the Middle East and some parts of Europe are A and D.

In contrast, C and B are popularly predominant genotypes in Asia. It was observed and reported that chances of developing HCC are remarkably higher on C genotype associated viral exposure than the others genotypes (Balogh et al., 2016; Marrero et al., 2018). The HBV whole genome holds for four major overlying transcription elements which translate the nucleocapsid, the key core protein that made of surface antigen (HBcAg), known as hepatitis B antigen. This antigen (HBsAg), and the X protein (HBx), taking the major role on the HCC development through altering of oncogenic cell signalling pathways. (Yoon et al. 2018).

HCV associated HCC is mostly prevalent in central part of Asia, Middle East in Europe, and North America. (Yoon et al. 2018). The HCV characterised for a small RNA virus (single-stranded) with enormous genetic variability. The genome in HCV is 9.6-kb lengthy, and encoding some polyprotein etc. (Rusyn and Lemon 2014). During outcomes of many epidemiologic in different times, it revealed that HCV is accounted for one of the leading hazard factors in the development of primary liver cancer (Lerat et al., 2011). Plenty of incidences are reported that revealed massive impact of HCV to initiate malignant transformation, through activating STAT3 signalling pathway (IL-6 – mediated autocrine pathway), leading to heightening telomerase activity (Tacke et al., 2011) in the hepatocytes. Here, elevates hepatocytes' proliferation through upregulating phosphorylation in regulating extracellular kinases (ERKs) with inhibiting p53-facilitated apoptosis and p21 influenced activity (Yu et al., 2012). They also include as follows: (a) oxidative stress in cells ensuing in mutagenesis of DNA, (b) inflammation endorsed by NF-κβ, (c) alteration in tumour suppressor genomes, (d) direct inflection of the potent wnt/β-catenin pathway, and (e) blockade of cell TGF-β signalling pathways.

NASH (Non-alcoholic steatohepatitis), majorly categorise as NAFLD (non-alcoholic fatty liver diseases) sometimes comes out with advanced fibrosis/HCC and theses are frequently noticed with the patients having chronic metabolic disorder such as fatty liver, obesity, and diabetes over the years. NASH's progression to HCC occurs at a proportion of 0.5% on Yearly basis. (Younossi et al., 2016a). Along with some adipose tissues and muscles, majorly liver as crucial organ ensues glucose metabolism utilizing insulin and maintain normal glucose level in body. But in case of diabetes mellitus and obesity, body and specifically hepatocytes develop insulin resistance because of fatty acid accumulation, inflammatory hepatic injury, and hepatic fibrosis with simultaneous declining of β-oxidation in mitochondria of the hepatocytes. Sufficient instances are there in the literature (Huang et al., 2018) have indicated deregulation of fatty acid signalling pathways, anti-inflammatory pathway cascade elevates

hyperinosaemia/insulin resistance that finally loses hold on normal cellular activity. Here, during hyperinosaemia/insulin resistance, phosphorylation of insulin receptor substrate 1 (IRS-1) takes place in presence of higher amount of free fatty acid along with their metabolites. (Schinner et al. 2005) This phenomenon triggers the activity of phosphatidylinositol- 3' kinase and reduces glucose transporter-4 (GLUT4) translocation to the hepatic and muscular cell-membrane and develop inulin-like growth factor (IGF-1) that influence cellular proliferation and downregulation of apoptosis. (Yoshimoto et al., 2013). Thus, diabetes, obesity associated hepatobiliary metabolic disorder like hepatic steatosis, NAFLD, and cryptogenic chronic cirrhosis, may lead malignant transformation in the hepatocytes and initiation of HCC takes place. (Reddy and Rao, 2006). Diabetes mellites already indicated as an independent risk factor for HCC, which can boost the risk of HCC by 2 to 3 folds (El-Serag et al., 2006), whereas obesity alone increases the risk by 1.5 to 4-folds. (Larson and Wolk 2007).

Alcohol overconsumption is one of the prime global issues which may lead the serious concern of developing fatty liver diseases like alcoholic steatohepatitis (ASH) to cirrhosis, and lastly, as conclusion with HCC development. (Schwartz and Reinus 2012). Excessive or recurrent abuse of alcohol induce cytochrome P450 2E1(CYP2E1) enzymes, which regulates oxidative metabolism for foreign substances, drugs, xenobiotics, and alcohol in liver. But exotic activity of cytochrome P450 in liver elevates oxidative stress in liver. Thus, creates hepatotoxicity and initiate HCC. (Neuman et al., 2015). During alcohol metabolism aldehydes are formed as by product which is a vital substance for creating a oxidative stress in liver and subsequent damage in hepatocytes (Yoon 2018). As per reported data, 20-25% of total HCC diagnosed annually has background of chronic alcoholic liver disease (ALD) associating with 1.3 -3% of cirrhotic liver, additionally, sex and race contribute as significant inconstant risk in developing HCC. (Massarweh and El-Serag 2017).

Aflatoxin, a mycotoxin frequently found in varieties of oilseeds, staple and cereals has potent hepatocarcinogenic effects (Yang et al., 2019; Gouas et al., 2009) could exhibits incidences of developing HCC during recurrent consumption of contaminated food staples. Many times, a faulty harvesting is the original reason for exposure to aflatoxin in food staff, where the population is commonly found in African countries rather than the developed Western countries. The most pathogenic form is aflatoxin B1, produced from Aspergillus sp. is responsible for mutating of the tumour suppressor, TP53 gene as a result of substituting of arginine with serine (R249S) in the genome in oncogenesis and substitution of more than 50%

of total TP53 genome is predominant in the liver cancer patients exposed to aflatoxin. (Weng et al., 2017).

1.4.3. Molecular pathophysiology of HCC referring related signalling pathways

The commonly observed cellular deformities during the development of HCC are pseudo glandular, spongy, trabecular, and solid mases in liver structures. Coexistence of any of these patterns may be spotted in the similar lesion or in scattered manner. Moreover, the cells accruing ability to undergo abrupt transition in their overall structural, functional, and genetic features. Considerable alterations have been studied in neoplastic cellular structures as follow: (i) accretion of fat, (ii) disseminated distinction in cytoplasm, ground glass incidence, (iii) combination with Mallory–Denk figures, and oncogenesis spotting (Ziol et al., 2018; Salomao et al., 2010). Thus, heterogeneity in HCC tumour is common at intra- and/ inter hepatic sites. Stem cell-like properties also have been noticed roughly in different HCC-subtypes and (iCCA) intrahepatic cholangiocarcinoma (iCCA) with mutation of CK19-positive cells (Singh et al., 2013).

Studies and reports on molecular mutagenic transformation at genetic level (through modulation of tumour suppressors oncogenes including TP53, PTEN, CDKN2A, or CDKN2B), epigenetic level during gene transcription (through methylation and histone or chromatin remodelling), and at transcriptomic levels that indicted an exhaustive assessment about the major signalling pathways supporting for neoplastic development in HCC. In addition, adoption of recent advancement in NGS (next generation sequencing) method through analysing the samples collected from HCC patient provide a comprehensive molecular landscape in development of HCC. (Daniel et al. 2016). All these molecular signalling pathways also play critical roles for adapting the microenvironmental conditions which favour tumour propagation. Here, we are briefing some major signalling pathways associated with HCC.

- **Wnt/β-catenin:** This pathway is responsible for tissue regeneration, cellular migration, and differentiation during cell developmental process, thus have crucial role in overall survival of the carcinogenic hepatocytes (Saito-Diaz et al. 2013). The pathway follows a cascade with activation and accumulation of β catenin (through binding of the Wnt-ligands at Frizzled cell surface receptors) at cell membrane and translocated towards

nucleus. Specified oncogenes like c-myc, servivin, and cyclin D are often found here as regulatory genetic materials. (Saito-Diaz et al. 2013).

- **PI3K/Akt/mTOR:** This pathway plays an important role in cell growth and proliferation, thus controlling carcinogenesis, also for HCC progression. (Bunney and Katan 2010). Activation of PI3K, Akt (modulating EGF/IGF signalling cascade), is responsible for destroying key functional role for tumour suppressor (mainly PTEN) gene, by somatic mutations and epigenetic gene silencing (Schmitz et al. 2008). Studies indicated mTOR as a key regulating co-factor in PI3K-Akt molecular pathway, that allows cell progression from G1 to S phase in process of cell cycle. (Schmitz et al. 2008).
- **RAS/MAPK:** The aberrant amplification in endothelial growth factors (EGFR/ IGF signalling), directly relates to pacifying the effect of tumour suppressor oncogenes (DNA methylation or other genetic mutation), and these are regulated by activating intracellular signalling via MAP kinase pathways (MEK 1), where Ras importantly noticed for extracellular activation in (ERK1/2) kinase signalling pathways regulated by oncogenic gene phosphorylation (Min et al. 2011).
- **Notch / Hedgehog signalling pathways:** Both these pathways are involved as fate determination during cell proliferation, migration, and apoptosis in HCC, where mutation at hedgehog receptors (PTCH1) and its respective ligand are responsible for cellular differentiation, cell regeneration, unlike stem cell biologics. (Lee et al. 2016).
- **p53/p21 and RB1:** The retinoblastoma (RB) protein and the p53 proteins often found inactivated in different types of tumours and execute as a crucial transcription factor that have inhibitory roles in tumour suppressions. They are often found inactivated in various tumor types. Both proteins play central roles in regulating the cell division cycle. RB forms complexes with the E2F family of transcription factors and downregulates numerous genes. Among the RB-E2F target genes, a large number code for key cell cycle regulators. Their transcriptional repression by the RB-E2F complex is released through phosphorylation of RB, leading to expression of the cell cycle regulators. The release from repression can be prevented by the cyclin-dependent kinase inhibitor p21/CDKN1A. The CDKN1A gene is transcriptionally activated by p53. Taken together, these elements constitute the p53-p21-RB signalling pathway. (Engeland k. 2022).
- **VEGF/VEGFR, PDGF/PDGFR and FGF/FGFR signalling pathways:** The vascular endothelial growth factor (VEGF)/ receptor (VEGFR) is related angiogenesis, by

supplying blood into the proliferating tissues, thus providing huge demand of oxygen and other nutrients to the carcinogenic tumour tissues. (Daniel et al. 2016).

- PDGF/PDGFR is helps in promoting tumour growth related to TAM-Tumour Associate Microphages.
- FGF/FGFR pathways is upregulated during abnormal increase in cell growth and their survival during chemotherapeutic drug resistance (MDR).

1.4.4. Staging System in HCC

As described by previous hypothesis that molecular pathogenesis in HCC is a very complex process and there are numerous stepwise underlying genetic and epigenetic features are responsible for development of neoplasia in liver from normal hepatocytes. HCC includes of heterogenous, yet exceptionally unique cellular characteristics. Thus, prediction of stages in HCC are acknowledged by standardized graded histopathological changes along with developmental tumour- sizes and overall patient condition based on the data reported among different clinical reviews (Marrero et al., 2010; Yang et al., 2019), it helps to manage the overall treatment plan in HCC therapy.

In therapeutic oncology, different staging system are applied as key to unlock the prognosis status in HCC therapy, but with limitation of one common worldwide acceptance (Marrero et al., 2018).

- The prognosis of HCC is most frequently determined or measured by widely accepted TNM staging process, which is approved by (AJCC) American Joint Committee on Cancer.
- Europe generally follow (BCLC) system the Barcelona-clinic liver cancers staging system; a most popularly applied staging system.
- (CLIP) system are sometimes applied as Italian liver Cancer Program;
- (JIS) are well recognized in Japan integrated cancer staging system;
- The Okuda staging system;
- The French classification;
- The Chinese University Prognostic Index (CUPI) model was described by the investigators of Hong Kong.

TNM scoring system

TNM staging process is based on the number of tumour/ nodules and their sizes with pathogenic condition; **T denotes for tumour**, **N for nodes (lymphatic expansion) or nodules and M encodes metastasis (spreading carcinogenesis to distant parts of body)**. In TNM staging system all the stages have their own distinguishable features with the letter “X” and grading number (0-4); where these numbers explain their severity and severity. (Vauthey et al., 2002). For example, T4 tumour scoring indicates an advance oversized tumour in comparison with the T0 < T1< T2< T3 score. TNM staging system has been applied as an excellent diagnostic tool to predict HCC staging with significant level of performance in patients undergoing surgery than the whole liver transplantation. Currently, a more updated and detailed TNM version has now been adopted for integrating tumour staging vs fibrosis on the HCC treatment process. (Karademir et al. 2018).

BCLC (Barcelona-Clínic Liver) Cancer system

This system has been explored extensive way during relevant clinical trials and cohort studies and likely to be classification feature wise than a graded or scoring system (Former et al., 2018; Yang et al., 2019). Here, they have included a no of variables such as liver functional status, tumour stage, overall hepatic physiology, and carcinogenic symptoms. (Chen et al., 2009). As covering most of the variability, its global acceptability among the other staging system in the treatment of HCC. (Yang et al., 2019). Depending on the observed variability during different stages of HCC prognosis, BCLC system classified HCC patients among five subtypes, namely, 0, A, (preliminary stages) and B, C, D (mild and advance stages). (Chen et al., 2009).

Following are the highlighting features for describing various categories in BCLC staging system:

- Stage 0: It is classification under very early stage with no faceable abnormalities or normal liver function (named also Child-Pugh A), may contain tiny tumour < 2cm in size without vascular incursion. Here, the recommended therapy is mostly resection.
- Stage A: Here, patients could bear a single or up to three tumours with size < 3cm, generally named Child-Pugh grading A/B assigned this category and considered as early stage. The treatment approach like comprising resection, radical method, transplantation or adjuvant resection, sometimes percutaneous therapy is suggested as optimal depends on succuss on RCT studies as mentioned in the published literature (Cillo et al., 2006).

- Stage B: Again, Child-Pugh grading A/B, bearing multiple tumours, but without causing extrahepatic metastases considered to be on intermediate stage. Chemoembolization could be the ideal choice for these stage B patients.
- Stage C: An advance stage that refer extrahepatic metastases with numerous tumours and loss of complete hepatic function with Child-Pugh grading A/B. Patients here recommended for systemic therapy (newer molecular drug agents or targeted chemotherapy based on potential findings from RCTs.)
- Stage D: This is called terminal phase where patients having Child-Pugh C category with any number of tumour but below the par PS is >2. Here palliative treatment is sometimes providing some benefits as symptomatic relievers. (Chen et al., 2009).

Unlike these two systems, the French staging, CLIP and CUPI systems also have been developed to identify prognosis in patients specially at advanced stages. In case of CLIP scoring system, the total percentage of developing tumour burden are involved with liver impairment. Thus, prediction of early stage is a complicated process. Thus, many times therapy outcome is unpredictable. Present understandable fact is that identified molecular signature biomarkers in HCC could provide potential treatment relevance and extensive research is required to obtain necessary biological information which will show the corrective path to categorise the HCC patients. (Pons et. al. 2005).

1.4.5. Treatment strategies ((current and Investigational)

Depending on the magnitude of liver impairment that generally marked as staging or different grades in HCC, clinicians choose a beneficial treatment protocol for HCC patients with aiming of two distinctive purposes; curative and palliative care. Appropriate screening could bring about early-stage diagnosis of HCC may offer hepatic functional restoration through liver transplantation, surgical resection, percutaneous ablation, trans-arterial chemoembolization process (TACE), along with radioembolization, and from time to time are followed by systemic therapy during intermediate of advanced HCC, (Kumari et al., 2018). In most of the cases when dragonized at advance or last stage HCC patients recommended for palliative or symptomatic treatment, sometimes with systemic chemotherapy.

I) Curative Treatments

Surgical Resection

Surgical resection covers the highest successes rate (5-year survival that ranges between 41%-74% of treated patients) among the other treatment approaches in HCC and depending on the

considerable factors of basal hepatic activities, tumour size and positioning along with healthy functional tumour free liver mass or volumes vs tumour proportion. We can say patients bearing a solitary primary tumour without any kind of vascular invasion in liver could be an ideal candidate here and after a partial hepatectomy could afford good faith. Although practically, patients with oversized tumours are also attempted for a major hepatectomy with consequences of severe infection and incision in other related organs. Additionally, surgical resection cannot be achieved for cirrhotic patients where multiple tumours are aligning with extra-hepatic areas, such as bile duct, portal veins etc and can cause haemorrhage. (Balogh et al., 2016; Marrero et al., 2018; Yang et al., 2019).

Anatomical Resection

In HCC during metastatic spreading carcinogenicity invade into the portal vein from adjacent tissues, but widespread resection of liver mases along with major circulatory system is practically troublesome. In 2008 Makuuchi et al. introduced anatomic sub segmentectomy, where ultrasound guided intraoperative injecting of indigo carmine (a kind of biocompatible dye) is given to portal vein for marking tumours in specific hepatic locations, after that stained areas (determining location and tumour sizes) are spotted applying electrocautery and required resections are conducted. A postoperative prognosis was noticed after applying anatomic resection approach. (T. Kamiyama et al., 2010).

Laparoscopic Resection in HCC

Laparoscopic liver surgery nowadays has been increased from last few decades because of its minimal invasive process especially for cirrhotic liver condition in HCC patients. Although currency and recurrence from the perspective oncological still require more evidences to establish it as standard approach. (Buell et al., 2009).

Transplantation

Following the “Milan criteria” generally helps in selection of HCC candidates may requires liver transplantation as a better survival rate are reported here. (Kishi et al., 2011). Although, by restrictive manner like tumours smaller than 5 cm, but larger than 3 cm could should be included for reasonable survival success.

Ablation

Percutaneous ablation is a frequently used local therapeutic approach for HCC associated with radiofrequency or microwave assistance. The basic mechanism behind the ablation approach is inducing necrosis through supplying heat directly to the tumour. Additional to the microwave or radiofrequency ablation, ethanol injection by local precancerous route also has been employed frequently. The ablation approach is considered as bridge treatment (Yang et al., 2019) for coming up transplantation to avert the HCC tumour's progression.

II) Palliative Treatment

Embolizing Therapies in HCC

Likely to other vascular carcinomas HCC also shows a durable dependency for its cell proliferation to the blood supply in its surrounded arteries (75-80%) as compared to the normal liver. (Kumari et al., 2018). That is why, embolization also is one of the processes to hinder the blood flow into proliferating cancer cells ensuing induction of hypoxia at tumour site and subsequent necrosis events. Embolization has an effective role in sole therapeutic approach or as combined approach with either chemotherapy or radiotherapy.

Transarterial Chemoembolization, TACE

This is a procedure techninuecally executed on direct administration or embolization of chemotherapeutic drugs to the groin tumour region of liver through an adjacent hepatic artery under the supervision of local aesthesis. Again, embolization could be done as temporarily with gelatin sponge or with autologous blood clotting agents, and as permanent embolizing agents steel coils, polyvinyl alcohol, sometimes microspheres are used. (Wang et al., 2014). This technique generates a natural like drug-eluting bead (DEB) for TACE, which employs embolizing elements as vehicles for chemotherapeutic drugs, cisplatin and doxorubicin are most frequently used drug here. (Luz et al. 2017).

However, TACE is suitable for selective HCC patients, depending on factors like the size, location, and invasiveness of tumour, along with the overall pathophysiological condition of HCC patient as per medical guidelines. Generally, this process is recommended for HCC patients who are between moderate to advance stages (B/C stage) according to BCLC staging categories to avoid liver transplantation/ surgery and help in supressing the disease progression by, thus improving overall survival. (Otto et al., 2013).

Radio Therapy

Through high dose of radiation, radiotherapy destroys cancer & reduce tumours. It is one of the most powerful treatment processes. Through high-energy waves of particles, e.g., gamma rays, X rays, electron beams or proton, radiotherapy destroys or damages cancer cells. To prevent cancer cells' growth & division & causing their death, these DNA splits plays a vital role. 50% of people, during their cancer treatment, receives radiotherapy. Factors such as stages and types of cancer, size of the tumour, the position of the tumour within the body, closeness between tumour and normal tissues which are susceptible to radiation health status, and medical history of the patients, namely, age and whether patients receive other types of cancer treatment at the time of radiotherapy are critical factors. The external and internal beams are the fundamental modes of radiotherapy.

External Beam Radiotherapy

This is most frequently used radiation to treat cancer. From outside the body, a high-energy beam targets the tumours, without making any contact it moves around the body from multiple directions with a minimal impact on normal cells.

Internal Beam Radiotherapy

In this process, either solid or liquid radiation source put inside the body. When solid source is used, it is called Brachytherapy, where a high dose of radiation directly targets the tumour of a particular part of the body as a localized treatment through seed, ribbons or capsules and helps nearby spare tissue. Liquid source, also known as systemic therapy, treatment travels in the whole body through blood and identify the cancer cells to destroy them.

Radiotherapy Modalities

Over a past few decades through advance technology and imaging, radiotherapy has become one the potent therapeutic option to treat HCC which is one of the leading causes of death worldwide. In unresectable HCC, which requires significant vascular involvement, radiotherapy offers better local rates. Radiotherapy can provide improved palliation in metastasis. The applications of the following radioisotopes have been reported for HCC.

- Holmium -199 (Ho^{199}), a beta and a minor gamma emitter isotope, has a half-life of 26.8 h. The chitosan complex's potential (Ho^{199}) has been explored either through intratumorally or trans arterial approaches. In another phase II study, the authors reported that in the treatment of HCC, trans arterial administration of the ^{166}Ho-chitosan complex was highly effective, especially for the patients with 3-5 cm tumours

(Sohn et al. 2009). Kim et al., 2006 reported that in a phase IIb clinical trial, 91.7% response rate of percutaneous ^{166}Ho-chitosan injection showed complete tumour necrosis (tumours < 2 cm).

- Iodine -131 (I^{131}), which has a half-life of 8 days, is most prevalently a beta emitter accompanied with a limited potential as a gamma emitter. It has been applied in the form of I^{131}-Lipiodol (Boucher et al. 2007). It was also successfully used for the treatment of non-operable HCC (Lintia-Gaultier et al., 2013). The clinical response was in 17-92% of patients upon intra-arterial administration of ^{131}I-Lipiodol (Lambert et al., 2005).
- The starting information obtained from ^{188}Re and ^{90}Y-labelled conjugates are promising, which make them emerging radiotherapeutic modality.
- The therapeutic efficiency of ^{90}Y-labelled microspheres is quite similar compared to that with radiolabelled lipiodol (Lambert et al., 2005).

Literature revealed with evidence, the safety and efficacy of radiotherapy in the treatment of HCC. This approach is applicable for various size of tumours which are not vulnerable to percutaneous therapy and down-staging tumours before transplantation or surgery.

Three-Dimensional Conformal Radiotherapy (3DCRT)

This advance imaging technique generates three-dimensional images of tumours and nearby organs and tissues of patients. Radiotherapy delivers radical tumour doses to a well-defined liver lesion more effectively and precisely. For cirrhotic HCC patients 3DCRT has been explored as substitute therapy. Unlike different curative approaches such as surgical resection of tumours, liver transplantation, and radiofrequency ablation, 3DRCT exhibits limited potential.

In an early investigation at (Robertson et al. 1997), it is found out that combined conformal radiotherapy (RT) when administered with fluorodeoxyuridine (FdUrd) through hepatic artery significantly halted the HCC progression. It improves survival in patients with unresectable, no diffuse primary hepatobiliary malignancies (Robertson et al., 1997).

Trans arterial Radioembolization (TARE)

As per (Sacco et al., 2016) in this technique, intra-arterial delivery of Yttrium (Y90) is given with microsphere or lipiodol with rhenium or iodine for same type of patients as trans arterial chemoembolization (TACE). For HCC patients TARE is preferred because it can remove hepatic ischemia while delivering the local radiation to liver tumors (Cho et al., 2016).

III) Systemic Therapies of HCC

HCC generally associated with a long dormant period of developmental stages which practically consequence for late diagnosis, thus excluding surgical options and rendering the chemotherapy as only accessible treatment application. (Leung et al., 1999). A slight improvement of overall survival in HCC patients are reported upon application of typical standard chemotherapeutic agents like cisplatin, 5-fluorouracil, doxorubicin, and gemcitabine. (Yeo et al. 2005) Further, a few randomized trials marked a mild to moderate enhancement in terms of survival response upon administration of the combined chemotherapeutics (Mohamed et al., 2017). There are multiple biological and physiochemical limitations of this current chemotherapeutic approach which are responsible for very poor in clinic therapeutic responses with liberating potential non-compromising adverse effects.

To address the limitations associated with in clinic application of conventional chemotherapeutics, different advance molecular, immunological, and targeted therapies have been set up for exploring suitable benefits in chemotherapy.

First-line Therapy

Sorafenib

It is the first multi kinase inhibitor in the Brand Name of Nexavar®, by Bayer Pharmaceuticals, Berlin, Germany, which was approved by USFDA for the first line treatment of advance HCC by preventing the angiogenesis, cellular proliferation, and metastasis while it binds with the various extracellular tyrosine kinases such as vascular endothelial growth factor receptors (VEGFR 2, VEGFR-3, VEGFR1), platelet-derived growth factor receptor β (PDGFR β), thus reduces intracellular signaling molecules Raf kinases (B-Raf and C-Raf) and serine/threonine kinases (Al-Rajabi et al., 2015).

Lenvatinib

To treat advanced HCC, USFDA has approved Lenvatinib, the broad-spectrum oral MKI, as a first-line drug while it targets various tyrosine kinases, e.g. VEFGR 1-3, fibroblast growth factor 1-4 (FGFR1-4), PDGFR-α, c-Kit, rearranged during transfection (RET) (Kudo et al., 2018). In a trial on 954 patients randomized 1:1, it offered higher objective response rate (ORR), the progression-free survival (PFS) was more and prolonged time to progression (TPP) of the disease, as compared to sorafenib.

Second-line Therapy

Multi-Target Tyrosine Kinase Inhibitors

Regorafenib

It is recommended by the USFDA as a second-line agent for advanced HCC-patients who are tolerant to sorafenib. It inhibits multiple targets such as VEGFR1, tyrosine kinase with Ig and epidermal growth factor (EGF) homology domains-2 (TIE-2), PDGFR, fibroblast-derived growth factor receptor (FDGFR), and colony-stimulating factor 1 receptor (CSF1R**).** Regorafenib showed an increase in survival of patients compared to placebo from 7.8 months (in placebo) to 10.6 (after regorafenib treatment) in resource trial data.

Cabozantinib

By suppressing angiogenesis, metastasis, and oncogenesis (Yang. et al., 2019; Liu et al., 2019), Cabozantinib, is one of the oral multiple tyrosine kinase inhibitors. Phase III CELESTIAL trial (Abou-Alfa et al., 2018) showed that the treatment with cabozantinib resulted in significant improvement in OS as compared to placebo in patients (with advanced HCC) who were in treatment with sorafenib previously.

Ramucirumab

It suppresses the activation of VEGF receptor, by inhibiting VEGF receptor ligands' binding and acts as an antagonist of VEGFR2. This recombinant IgG1 monoclonal antibody showed superior OS in the ramucirumab group (9.2 months) than the placebo (7.8 months) in the REACH trial, a multicentre (154 centres from 27 countries) double-blind trial.

IV) Immune Checkpoint Inhibitor

There is a dynamic microenvironment at the site of tumour, which practically modulate cell immune surveillance and immune related enzymes or protein molecules also changes in their nature, now targeting these molecular events immune-cheek point inhibiting drug delivery system has been developed. (Keenan et al., 2019). A series of molecules including PD-L1 (programmed death-ligand), PD-1 (programmed cell death-1) and CTLA-4 (cytotoxic T lymphocyte-associated protein 4) over expressed in immune hamper mutated tumour cells. Thus, considering this event, researchers are being explored external complemental ligands, monoclonal antibodies, or related peptides against immunogenic overexpressed cell ligands. Different clinical trials also have been applied to evaluate their clinical efficacy and safety in various types of cancers, including HCC (Gong et al., 2018). Seeing their effectivity in HCC,

the earnest effort has been conducted to mature products like an antibody against PD-1 and PD-L1, which finally executed as market approval of nivolumab, an antibody acting as PD-1 inhibitor in HCC therapy regime with multi kinase inhibitor drugs Apart from the nivolumab, some other molecules like tislelizumab, tremelimumab, camrelizumab and their combinations are also examined (Gong et al., 2018).

V) Phytochemicals in HCC therapy

Typically, early, or intermediate stage HCC are managed through surgical resection or liver transplantation with liver ablation, while at advanced stage once tumour burden is more and extrahepatic metastatic growth occur systemic chemotherapeutics are recommended choice. However, individually they have own drawbacks with legitimated concern of recurrency (Gentile at al. 2020; Lau at al. 2001), toxic systemic adverse effects, developing drug resistance, so not justifying as much as efficacious and curative potential, with allowing marginal improvement in overall survival. (Tang et. al. 2020). In addition, imposes an enormous economic drain on patients, especially for patients from developing and poor countries. Thus, the development in a paradigm of an effective, sustainable therapeutic agents are necessary. Numerus scientific evidences are there that indicating phytochemicals have significant cancer curing potential and many of them are already on applications. Till date there are five to six major classes of phytochemicals (related to plant-derived compounds) used as anticancer agents include taxeme, vinca alkaloids, diterpenoids, camptothecin, and epipodophyllotoxin derivatives. Along with all these category, bio therapeutic properties (anticancer, anti-inflammatory and antioxidant) of flavonoids are on prime investigational interest among the researchers.

Apigenin (Flavonoid) as potent anticancer agent for HCC

Flavonoids are richly abundant in many plants, on their different parts (fruits, flowers, leaves) along with in some beverages for instance green tea, red wine, and some cocoa-based products. They are mainly secondary metabolites in plants, configurated as two benzene and there pyran ring, attached with more than one polyphenolic groups, that particularly influence on its biological or biotherapeutic behaviour, where they are generally classified into different groups: such flavanones, iso-flavonoids, flavones, flavanols, and anthocyanidins. A few of flavonoids (apigenin, quercetin, lutein) possess potent anticancer effects in many cancer types including HCC: they can manage ROS- (reactive oxygen species) -scavenging activity, cell

cycle arrest, persuade apoptosis, and autophagy role in different kinds of cancer cells, with mitigating proliferation and invasiveness. (Perez et al. 2018).

However, poor solubility, and subsequent bare minimum absorption along with rapid metabolism from body of flavonoid compounds, apigenin hinder their application as a potential pharmacological agent. (Haroon et al. 2021) Additionally, flavonoids may get precipitated (as inactive form) by making a complexation with other ingested food components besides their degradation in presence of gut microflora which badly impact on their overall bioavailability and stability (Amawi et al. 2017). Currently, few of innovative and novel approaches like isolation techniques, chemical modification, nanoencapsulations are being set up and have been explored extensively to optimize the likelihood of flavonoids as anticancer agents owing the present robust challenges.

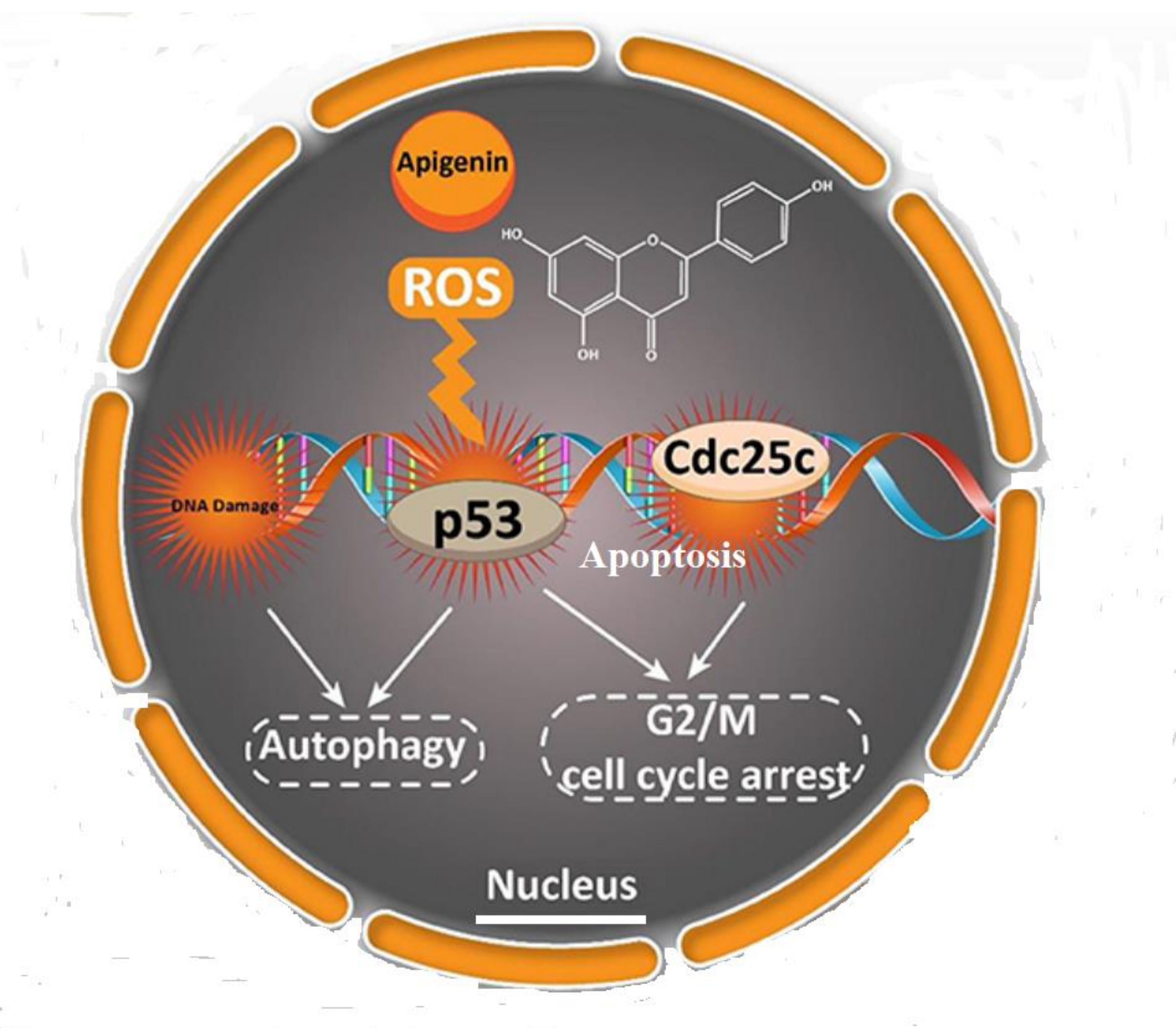


Figure 1.3. Molecular mechanism of action of apigenin as anticancer agent

VI) Nano technology-based therapeutic approach in HCC

Most of the chemotherapeutic and phytochemicals compounds employed in cancer treatment including HCC either have a bioavailibity or drug targetabilities issues or have limited application due to severe systemic side effects. Here, development of some novel approaches like nanotechnology-based drug delivery has already opened ocean of scope in therapeutic field. Nanomedicines are composed of either lipidic or polymeric composites to improve pharmacokinetic parameters for delivering the therapeutics to the target organ in predicted and controlled manner. Numerous types of nanocarriers have been developed through modifying their compositions and altering the surface characteristics, they are **liposomes, micelles, nanoparticles, cubosomes, nanotubes, quantum dots**, etc. which has been studied as drug delivery tools in the HCC therapy. These nanocarrier based chemotherapeutics have enormous prospect to fetch the therapeutic agents decidedly effective during their in-clinic use.

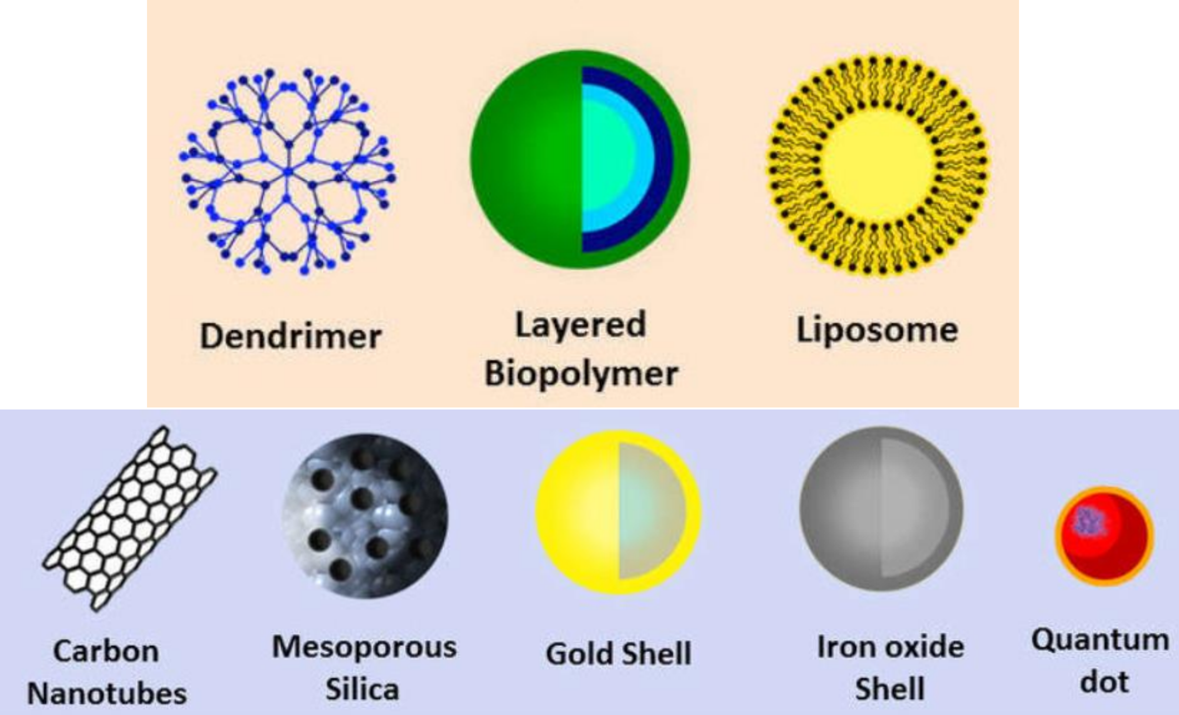


Figure 1.4. Different types of nanoparticles

Chapter 2

Literature review

1. Literature review

2.1. Drug delivery strategies in HCC applying nanotechnology

Despite of current advances in molecular targeted therapy and immune checkpoint inhibiting therapeutics, chemotherapy still is on choice for treating cancer patients to evade the economic burden on society. (**Llovet et al. 2008**). Here, landmark advances in nanotechnology enables the researchers and clinicians for more efficacious and convenient applications of chemotherapeutics along with some. Additionally, fore front use of some phytochemicals as anticancer agent in an encapsulated form via different kind of nano formulations are identified as safe and sustainable paradigm.

Nanotechnology, the emerging and latest approach, is based on its excellent structural characteristics with tiny sizes ranging from a few nanometres (nm) to 100-200 nm in solid or colloidal state. Nanomaterials also can exhibit some unique properties like opacity, thermal and pH resistant, superior pharmacokinetic mechanics both in vivo and invitro with favourable surface characteristics (**Grzelczak et al. 2008; Peng et al. 2013**). Thus, they have demonstrated some extraordinary potential in precise tumour therapy and their early diagnosis. (Farokhzad et al.2009). Generally, active therapeutics are pouring inside the nanoparticles (NPs) via dissolution or encapsulation techniques, when grafted on the nanoparticle surface by adsorption and coupling. As compared to traditional chemotherapeutic, the nano formulated drugs are intended to exhibit a sustained or controlled drug release pattern optimizing in vivo stability and tumour uptake of the therapeutics. (**Pan et al.2018; Almeida et al. 2007; Cho et al.2007**).

In a successful HCC-targeted novel nano-drug delivery approach, there are some major factors need to be addressed,

- Understanding of physiochemical and molecular nature of the heterogenous tumour microenvironment in HCC;
- Predicting precise and optimum therapeutic drug accumulation at tumour site in HCC.

Tumour microenvironment in HCC

Pathophysiology of HCC directs that there is a rapid inflammatory discharge in tumour microenvironment tissues (TME) with alteration of stromal cells with activated HSC (hepatic stella cells), CAFs (carcinogen-associated fibroblast, myofibroblasts are collectively, and immune cells (regulatory and cytotoxic T- cells, tumour-associated macrophages (TAMs), etc. The downregulated function of immune cells in the TME promotes the angiogenesis,

progression, and metastasis of tumours (**Zhou et al. 2016; Quail et al. 2013; Huang et al.2013**). These stromal cells and the surrounding tumour stroma, consisting of extracellular matrix (ECM) proteins, tumour growth factors (TGFs), chemokines, and some stromal degrading enzymes, constitute the whole tumour (**Rupaimoole et al.2016; Antsiferova et al.2011; McAllister et al. 2014)** The important roles of stromal cells in tumours include continuous proliferation, avoidance of growth inhibition, resistance to cell death, initiation of permanent replication, angiogenesis, activation of invasion and metastasis, reprogrammed metabolism, and evasion of immune destruction.

TME related attributes in tumour region

TME comprises a variety of distinctive characteristics than the normal tissue. It is commonly observed that acidic pH (6.5-6.9) at extracellular matrix the TME favour tumour growth, where the normal physiological pH (7.2-7.5) differs from this. (**Bailey et al. 2014**). Practically, the acidic TME condition develop in cancer cell site through translating glucose into LA, lactic acid as more glycolysis provides higher essential energy for tumour survival (**Du et al.2015**). One more unique characteristic of TME is hypoxia, as cells are in deeply located inside the tumour, thus they deprived of oxygen due to their extremely irregular vasculature matrix inside the tumour mass (**Wilson et al. 2011; Jain et al.2005**). These abnormal hypoxic condition in the tumours cells made them drug resistant, and less susceptible on conventional chemotherapy. Most of overexpressed enzymes at the TME region belongs to either protease family (such as MMPs, membrane metalloproteinases), or from lipase family (such as phospholipase A2). (**Radisky et al. 2017, Kessenbrock et al. 2010; Jiang et al.2004**). Tumour cells at the TME region also experience oxidative stresses due to increased levels of free radicals (hydrogen peroxide, superoxide anion, and hydroxyl radicals) (**Wang et al.2017**). To have a control on this oxidative stress tumour cells modulate their reduction potential by generating redox species (such as SOD, superoxide dismutase and GSH, glutathione). This upregulated redox level in TME, increase the overall stress (oxidative/reductive) at the tumour region. In addition, tumour cells own elevated levels of ROS, reactive oxygen species as compared to healthy cells as a result of more aerobic metabolism during oncogenic transformation process. (**Mo et al.2016**). All these distinctive attributes of TME due to endogenous alterations at tumour sites offer an extremely great opportunity in the development stimuli responsive nano formulations.

Targeting strategies of NPs

Passive Targeting

Passive targeting relies on vascular permeability and retention of tiny nanosized particles into the intended tumour cells passing through the specified damaged leaky vasculatures of endothelial structures which is a unique structural characteristic of tumorous tissues, thus minimizing non-specified distribution of drugs to other normal tissues. This is generally described as endothelial permeability and drug retention (EPR) effects which was first time reported by Maeda and his colleagues where they showed 10-100-fold of more accumulations of nanosized polymeric drug conjugated as compared to crude drugs. **(Vlerken et al.2007**). Optimum drug accumulation and their retention at the therapeutic sites also depends on prolong circulation vs leaser drug clearance time in body which practically a resultant effects of particle sizes, shapes, and overall physiochemical properties of nano formulations. Once nanosized drug formulation diffuses into the body along with nanosized advantages, morphology or shapes of the nano formulation influences their availability of uptake by different kind of body cells, and finally targeting capability relies on adapting tumour microenvironmental barriers. (**Davis et al. 2009; Alexis et al. 2008**) Here, physiochemical surface character of nano formulations are the key factors. The cellular internalization procedure is mediated through different cell transport mechanism like phagocytosis or micropinocytosis, and caveolar/ clathrin dependent endocytosis **(Rejman et al.2004**). As surface characteristics play a crucial role that could determines the degree of internalization of the nano formulations into the cells, a lot of research work has been demonstrated in this field. The physiochemical properties of surface can be manipulated using polymers or copolymers varying composition, thus governing a favourable presence of hydrophobicity or hydrophilicity and ionic condition for formulated nano system. Polyethylene Glycol (PEG) coating has been recognized to shield the nano formulations defending subsequent clearance at RES, the Reticuloendothelial system. (**Alexis et al. 2008**). A few higher molecular weight polymer compounds composed by incremental adding of aliphatic side chains in the PEG core has been reported for increasing the circulation time or higher half-life. Modulation of ionic nature by adding co-polymer is also familiar now a days.

Some the major limitation of practical application of passive targeting is non specificity, irregular therapeutic distribution affecting in distinguishing the tumour tissues areas from the normal healthy tissue. It was also reported that the uptake of nanoparticles in tumour cells was

limited to 0.7% of the injected therapeutics (**Wilhelm et al.2016**), unless some surface modification techniques have been imposed to drug nano carriers.

Active targeting and applied strategies

Active targeting enables the nano formulations in target specific drug delivery towards the desired cancer tissues through functionalizing the nano system by a ligand that can proceeds its delivery against target organ due to theirs binding specificity towards the complementary proteins or receptors overexpressed on respective cancer cells. (**Su et al.2018**). Thus, surface engineered HCC specific ligand functionalized nano formulations direct precise delivery of the therapeutic to the tumour region in liver exclusively, without effecting the surrounding normal tissues or cells, and increased drug uptake by neoplastic cells with providing optimum curative effects. (**Zhou et al.2020; Elnaggar et al.2021**). Here, concerned challenges are identifying most suitable biomarkers on targeting cancer cells and their complementary ligands for successfully precise delivery of nano system to the cancerous tissue with avoiding other systemic toxicity. Additionally, drug uptake in to the target cancer cells (through receptor mediated endocytosis/ micropinocytosis) also depends on the affinity of ligands functionalized nano formulation towards the complementary target receptors. Therefore, proper recognition of biomarkers in cancer cells and establishing their complementary targeting ligands are necessary. Several studies have been conducted to identify and understand about the antibodies, transferrin, polysaccharides, peptides, nucleic acids, small molecules (folate, anisamide, etc.), and aptamer moieties as ligands that could bind to their corresponding tumour cell specific biomarkers overexpressed in HCC. (**Yoo et al.2019**).

1.2. Ligand-based nano -medicines in HCC and targeting biomarkers (active ligands|)

Active targeting based nano drug carriers in HCC could disperse the medicine in to the tumour site accurately. The characteristic fenestration of hepatic arteries allowing EPR responses and interaction of tumour cell biomarkers with the corresponding ligand functionalized nano formulation allowing optimal therapeutic accumulation and their internalization into the HCC cells exclusively. Here, we are applying strategies like precise receptor-mediated endocytosis/ micropinocytosis of ligand conjugated nano system for targeting drug accumulations. (**Li et al.2016**) Several Ligands (**polysaccharides, peptides, antibody, and aptamer**) functionalized nano medicines for HCC therapy which are based on their complementary tumour biomarkers has been exploded and some are at investigational stage that are described in Table 2.1

Table 2.1. A brief summary on ligands applied in nanodrug delivery

Ligands	**Corresponding HCC Biomarkers**	**Nano drug delivery system**	**Active therapeutics**	**Ref**
Galactose	ASGPR	Liposomes PEGylated nano composite	Mitoxantrone Plasmid pXL3031	**Zhang et al.2012** **Frisch et al.2004**
Lactobionic acid	ASGPR	Chitosan polymeric nanoparticles	Paclitaxel Doxorubicin	**Yang et al.2011** **Zhong et al. 2013**
Galactosamine	ASGPR	Polymeric nanoparticles	Paclitaxel Doxorubicin	**Ma et al. 2010** **Xue et al.2016**
Pectin	ASGPR	Pectin nano composites	Methotrexate 5-Fluorouracil	**Chittasupho et al.2013** **Yu et al.2014**
Hyaluronic acid	CD44	Gold nanoparticles	Metformin	**Kumar et al.2015**
Biotin	Biotin receptor	Pullulan acetate	Adriamycin	**Hong et al.2009**
HAb18 mAb F(ab’)	HAb18G/CD147	Pegylated nanocomposites	Doxorubicin	**Jin et al.2010**
CD147 mAb	CD147	Chitosan nanoparticles	α-Hederin	**Zhu et al.2015**
EGFR mAb	EGFR	Polymeric nanoparticles		**Liu et al. 2010**
CD44 Ab	CD44	Liposomal nanoparticles	Doxorubicin or triple fusion plasmid	**Wang et al.2012**
GPC3 mAb	Glypican-3	Chitosan lipid nanoparticles	siRNA	**Wang et al. 2016**
EPAP, RNA Aptamer	EpCAM, cancer stem cell biomarkers	Aptamer- Gene chemical ligation	-	**Xiao *et al.*, 2017**
TLS11a, DNA Aptamer	HCC cell surface receptors	PLGA-nanoparticles	Pacretaxel	**Chakraborty et al. 2021**
AS1411, DNA Aptamer	Nucleolin, Cancer cell surface receptors	Nanoparticles Nanoliposomes	Doxorubicin Apigenin (flavonoid)	**Trinh *et al.,* 2015** **Dhara et al. 2023**

Cell surface receptors/proteins as biomarkers in HCC

Biomarkers (generally identified in form of receptors, cell adhesion moiety, cell transporters, and proteins in body) are overviewed through proteins expression studies in the cancer cells upon their associated molecular signalling pathways in HCC. Here, we are mentioning about some potential biomarkers have been studied extensively in developing targeted nano drug delivery system.

Asialoglycoprotein receptor

Baenziger and Maynard first identified Asialoglycoprotein receptor, ASGP-R present abundantly on the cell surface of malignant hepatocyte having binding affinity with a galactose compound and galactosamines (**Elanggar *et al.* 2019**). Finding of Baenziger was further explained by **Trere *et al.*** during1999 that 80% ASGP-R was expressed, well-differentiated and characterized in HCC, where 20% was poorly-differentiated. Additionally, reported that ASGP-R are located at rapid DNA synthesizing neoplastic cells surface in HCC. This potential biomarker (ASGP-R) has been used as tool to recognize the HCC tumour cells and explored extensively for targeted delivery of chemotherapeutics in HCC (**Thapa *et al.* 2015**).

Transferrin receptor

Transferrin receptor, Tf-R is a glycoprotein type transmembrane receptor generally acknowledged as CD71 which is composed of two homodimers having molecular weight of 90 kDa, and joined together through a disulphide bonds. The characteristics of the overexpression of Tf-R in HCC unlike other malignant cells, as compared to non-malignant cells has been applied in the development of ligand (transferrin specific) targeted nano drug delivery system in HCC (**Tortorella and Karagiannis 2014**).

Epidermal Growth Factor Receptor (EGF-R)

It is tyrosine kinase family receptors made off with one single chain of glycoproteins and frequently observed in the epithelial layer at tumour cell sites. Ito et al. showed that EGFR remain overexpressed in almost 68% cases of HCC. This clinical finding opens a promising and alternative ligand (antibody or aptamer, peptide) mediated drug therapy for HCC with providing a more effective treatment options as compared to conventional chemotherapy or surgery (**Song et al.2017**).

Frizzled Receptors (FZD-R)

Frizzled (FZD) proteins receptors are a sub class of GPCR family, composed by ten members of frizzled (FZD1-10) tangled with Wnt/β-catenin molecular signalling pathway, frequently triggered in HCC. (**Merle et al.2004**).

Cluster of Differentiation 44 (CD44)

CD44 is an exclusive transmembrane glycoprotein type of receptor can do a favourable attachment with several ligands, such as monoclonal antibodies, hyaluronic acid (HA), collagens in presence of co-receptor EGFR also c-Met. CD44 generally is expressed in the immunogenic progenitor myeloid cells of liver Kupffer cells under healthy circumstances in body. (**Flanagan et al.1989**). However, in case of malignant condition, CD44 is overexpressed as cancer biomarker in the progenitor HCC cells (HcPCs), categorised as cancer stem cells some times. (He et al.2013; Dhar et al. 2018). Further, the presence of EpCAM (epithelial cell adhesion) molecules, CD90, CD133, CD13, and CD24 are considered as cancer stem cell (CSC) biomarker in HCC also. (**Yamashita et al.2013**). In numerous studies they have been explored as ligand guided biomarkers for targeted nano drug delivery approach in HCC.

Endoglin

Endoglin also recognized as CD105, a transmembrane type of cluster differentiating glycoprotein overexpressed in hyperactive endothelial level of proliferating cancer cell surface with co-relating the growth factor, TGF-β in HCC and explored as targeting biomarkers (**Kasprzak et al**.)

Integrins

This is an essential membrane glycoprotein consisting of two subunits (α- and β), noncovalently attach with each other with overall multi heterodimers composites. Integrins majorly responsible for two biochemical functions in body: one of them is acting as adhesion molecules located on cell surface towards the ECM, (extracellular matrix) and triggering proliferating cell signalling pathways (**Sökeland et al. 2019**). Thus, excess presence of the β1 integrin could be corelated with cancer progression and targeting biomarker-based HCC therapy. (**Tian et al.2018**).

Glypican-3

Glypican-3 or GPC−3 is a glycosylphosphatidylinositol-anchored protein attached on the cell surface. (**Filmus et al.2001**). This protein is generally expressed in embryonic tissues, placenta,

adult ovary, mesothelium, and in mammary glands, but not in healthy liver tissues or early-phasic cirrhotic liver lesions. On contrast, numerous reports are there that demonstrate presence of GPC−3 related mRNAs and overexpressed protein in neoplastic hepatic cells (**Hsu et al. 1997; Capurro et al.2003**). Recently, in some meta-analysis it was indicated that overexpression of GPC−3 is directly related with restricted overall survival (OS), along with disease-free survival (DFS) in HCC patients. GPC−3 encouraged the proliferation in hepatocytes through activating the Wnt molecular signaling pathway (**Capurro et al.2005; Zittermann et al.2010**). Another study reported that ERK pathway is also associated with GPC-3 mediated proliferation in cancer cells, and finally metastasis (Wu et al.2015). As GPC−3 is exclusively expressed in carcinogenic hepatic tissues locating at their cell surfaces, it has been recognized as potential biomarkers in HCC targeting therapy by using nanomedicines. **(Ishiguro et al.2008; Feng et al.2013**).

Nucleolin

Nucleolin is a multifunctional nucleolar protein, present in genome of many species, starting from yeast to plants, animals, and higher species like humans also. Orrick and his colleagues (**Orrick et al.1793**) first time separated and identified this nucleolar type of proteins in rat liver and named C23 protein, locating at healthy nucleus. In Human, the same nucleolin protein encoded as NCL gene present in the shorter arm of chromosome (Chromatin-2) in nucleus majorly. Nucleolin protein is generally related to miscellaneous cellular processes in normal physiological conditions, which involve in typical cell cycle regulation, cell proliferation, angiogenesis, and apoptosis evasion, whereas, in carcinogenic condition over upregulation of these process are hallmark characteristics of tumour cells. Further, it was observed that nucleolin is also overexpressed in cancer proliferating cells (**Derenzin et al.1985**). endorsing antiapoptotic mRNAs and blocking the proapoptotic FAS receptor (Wang et al.2014, Wise et al.2013). with relocating at the carcinogenic cell surface membrane through a shuttling mechanism (**Ginisty et al.1999**). Here, heat shock cognate 70 (Hsc70) is the concern protein moiety that is responsible for the translocation of nucleolin towards the cell surface by providing its stability associating with MyH9 protein. (**Ding et al. 2012**) Thus, cell surface nucleolin has been acknowledged as typical functional biomarkers for recognizing cancer cells. Being present more actively in the cancer cells including HCC, nucleolin can form a linkage with the specified ligands (complementary antibodies or aptamers) and mediate the receptor specific internalization of ligand functionalized nano formulations (**Hovanessian et al.2000**).

Vitamin-based active targeting

Vitamins are essential in every cell of the body for their growth and survival. Again, in case of rapid proliferating tumour cells this vitamin requirement is too high and that can be related with surplus number of certain vitamins such as biotin, folate, and retinoic acid (RA), along with presence of alpha-fetoproteins (AFP) and dehydroascorbic acid (DHAA) to endure their rapid tumorigenic development. Thus, receptors accompanying the utilization of these vitamins are basically upregulated at tumour cell. Consequently, these vitamin receptors assist to target oncogenic tissue substrates during tumour-targeted nano drug delivery. (**Niu et al. 2011; Na et al.2003**)

Other potential markers

Some cell transporter, such as annexins, Solute Carrier Transporters (SLCs), mucins are frequently active in proliferating cells including HCC and involve in apoptosis related signalling activations. These transporters are also some times choice of interest for identifying the tumour cells among mass tissues or organs in body. Thus, it is another opportunity in biomarker based targeted drug delivery approach. (**Siracusano et al.2020**)

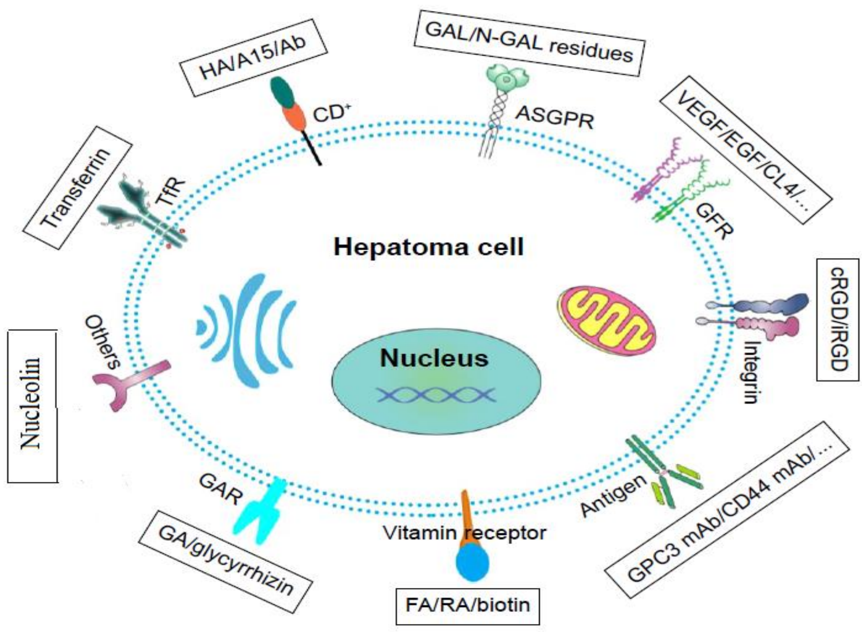


Figure 2.1. Surface biomarkers in HCC

1.3. Aptamer based nanomedicines in HCC

Aptamers

The therapeutic approach on antibodies mediated targeted drug deliveries and related nanomachines has been explored extensively and some of them are either in on clinical trial or already in clinical application (**Köhler et al.1975**). Although they can selectively recognize their biological target (tumour or apoptosis associated proteins) and can exert desired physiological responses, yet in clinic experience of these medicines are not up to the mark because of their high immunogenicity, low stability and of curse huge production cost (**Bradbury et al.2011; Chames et al.2009**). In this present sicario oligonucleotide-based aptamer guided nanomedicine has been considered as an alternative smart choice for not only in therapeutic field, but also as a powerful toll in cancer diagnosis. In case of HCC both the applications have been demonstrated with promising outcome. Aptamer based nanomedicine could predicted a higher upshot in their clinical acceptance in terms of their undoubted specificity towards target, low immunogenicity in a very cost-effective manner. In January 2017, the Food and Drug Administration also approved the usage of oligonucleotides after the appraisal through a stringently controlled trial (**Stein et al.2017**). Aptamers are known as single-stranded RNA or DNA oligonucleotides molecules possessing low molecular weight (6-30 kDa) that can bind specifically to their target molecule with predicting their extremely high affinity (**Hicke et al.2006; Lundin et al.2015**). Aptamer or aptamer complexes have very specific and flexible configurations that allow them to bind with their intracellular or extracellular target with high affinity and specificity even at picomolar to nanomolar range them ideal as suitable tool for their applications. (**Hermann et al.2000**). They have very low dissociation constants (picomolar to nanomolar) because of simple interconnected hydrogen bonding (**Nimjee et al.2005; Sun et al.2015**).

The aptamer can be synthesized by using a technique commonly known as SELEX, "Systematic Evolution of Ligands by Exponential Enrichment" process was first demonstrated by Tuerk and Ellington (**Tuerk et al.1990; Ellington et al.1990**). The process consisting of a continuous rapid selection of DNA from a pool of DNA (through binding, partitioning, and elution phase in presence of the target proteins), then amplification, and conditioning (**Stoltenburg et al.2007**). But, in current era, numerous modifications have been initiated in the SELEX method to improve its specificity as well as their commercial applications. A list is given below in Table 2.2.

Table 2: modified process in selection of aptamer

Modified SELEX process	**Basic Principle**	**Ref**
In vivo	Localizing target molecules inside a living cell	**König et al.2007**
One-round step	One selection rounds of aptamer generation	**Arnold et al.2012**
Capillary electrophoresis	Electrophoretic mobility-based separation	**Mendonsa et al.2004**
High-throughput sequencing-based	High-throughput sequencing and bioinformatics analysis	**Hervas et al.2016**
Isogenic cell Isogenic	cell line application in counter selection step	**Takahashi et al.2016**
Cell-based	Targeting the whole live cell	**Kim et al.2014**
In silico	Computational docking technology	**Chushak et al. 2009**
Magnetic	bead-based Magnetic beads immobilization	**Duan et al.2016**
Ligand-guided	selection Using specific antibody to compete with the target molecule	**Zumrut et al.2016**
Quantitative parallel aptamer selection system	Combination of microfluidic, next generation sequencing, and in situ-synthesized arrays	**Cho et al.2013**

Chapter 3

Objective & Plan of work

Objective & Plan of work

Despite some landmark development in chemotherapy, molecular or immunotherapy, treating or curing hepatocellular carcinoma (HCC) is still a global health issue, and the shocking fact is future estimated incidences of hepatic malignancy that could cross one million by 2025 (Llovet et al. 2021). The development of more and more potential, alternative, and cost-effective therapeutics is on-demand to combat this dynamic challenge. Naturally occurring flavonoids, such as apigenin, quercetin, and many having enormous potential as anticancer agents, have already been tried to formulate by encapsulating in nanoparticles owing to their poor solubility and bioavailability (Oiseth et al.2017). But in clinics, therapeutic challenges such as lack of specificity towards the target organ and desired curing response along with bare minimum side effects to the normal or healthy cells are yet to be adequately addressed (Yan et al.2019). Optimizing precise therapeutic accumulation to the target site with a ligand functionalized nanoformulations could minimize the drug resistance and improve the therapeutic tolerances (Dou et al. 2018). Thus, it can accelerate their scope for preclinical to clinical translation (Belfiore et al.2018). Apigenin has been well demonstrated both *in vitro* and *in vivo*, promoting apoptosis, inducing cell cycle arrest, suppressing the cancer cell invasion along with its autophagy and immunogenic activities (Yan et al,2017). Apigenin encapsulated nanoparticles were already applied effectively for delaying the progression of different types of cancer, including HCC (Bhattacharya et al.2018). However, along with refining solubility and bio-availability, precise delivery through ligand functionalized lipid nanocarriers could improve the apoptotic potential of apigenin in a great extent (Mahmoudi et al.2019). Aptamer fabricated nano liposomal drug delivery is already on prime focus relating to advanced targeted drug delivery for cancer (Moosavian et al. 2019). Further, apigenin-loaded nano liposomes modified with ligand targeted drug delivery system has not been explored yet, for HCC.

Formulation optimizations, process of incremental biotherapeutic accumulation in tumor cells and related apoptosis still need scientific clarity. We hypothesize that the therapeutic threshold of apigenin might be uniquely upgraded by precise, predictable, and effective drug accumulation to the target, cancerous hepatocytes, applying stable, aptamer functionalized PEGylated nanoliposomes.

AS1411 is a non-immunogenic, thermostable 26-mer guanine-rich DNA aptamer with quadruplex structural advantages, which provides exceptional affinity toward the nucleolin proteins overexpressed on the surface of HCC cells (Yazdian et al.2020). Customized phosphorothioate backbone modification in amino-modified DNA aptamer (AS1411) offers superior pharmacological stability to the aptamer and is used for the effective target-specific nano-drug delivery system (Volk et al.2017). DSPE-PEG intended to provide a longer half-life in vivo with reduced clearance due to its stealthy coating over the nano liposomal surface (Hussain et al.2019).

The study was designed to …

I. **Develop plain nanoliposomes (NLCs), PEGylated nanoliposomes (PEG-NLCs) and aptamer conjugated PEGylated nanoliposomes (Apt-NLCs)** with physiochemical characterization (drug loading, drug release, particle size, zeta potential and surface morphology) of prepared nanoliposomes. Stability study will be conducted as per ICH guide line.

II. ***In vitro* studies: Evaluation of anticancer activity using cell-biology tools.**
a) Cell cytotoxicity assay (MTT assay), b) Apoptosis analysis (by flow cytometric annexin V/PI assay), Identify process of cellular uptake. (Both through confocal and flow cytometric process), c) Estimate molecular DNA damage estimation through analysing cell cycle process along with marking respective apoptotic marker proteins. (By flow cytometric process).

III. ***In vivo* studies: The final optimized formula will be applied on DENA (diethylenetriamine)induced HCC animal model to observe…….**

a) Pharmacokinetic studies will be conducted by iv administration of free drugs, drug loaded nanoliposomes (ligand conjugated and non-conjugated). Blood level and tissue levels of the drug will be determined by LC-MS/MS analysis. b) Biodistribution of 99mTc- labelled free drugs and their nano formulations in different organ including liver will be monitored by γ-scintigraphy analysis. c) Apoptotic potency of ligand functionalized nano-formulation estimated in comparison with non-functionalized nanoliposomes for HCC induced animals.

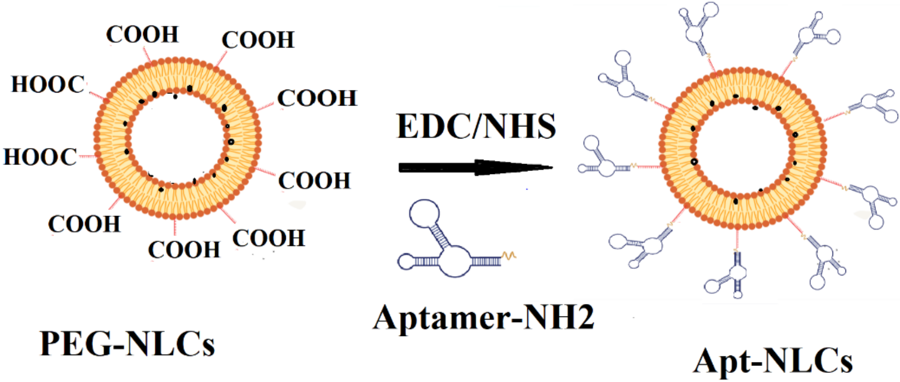


Figure 3.1 Formation of aptamer conjugated PEGylated nanoliposomes

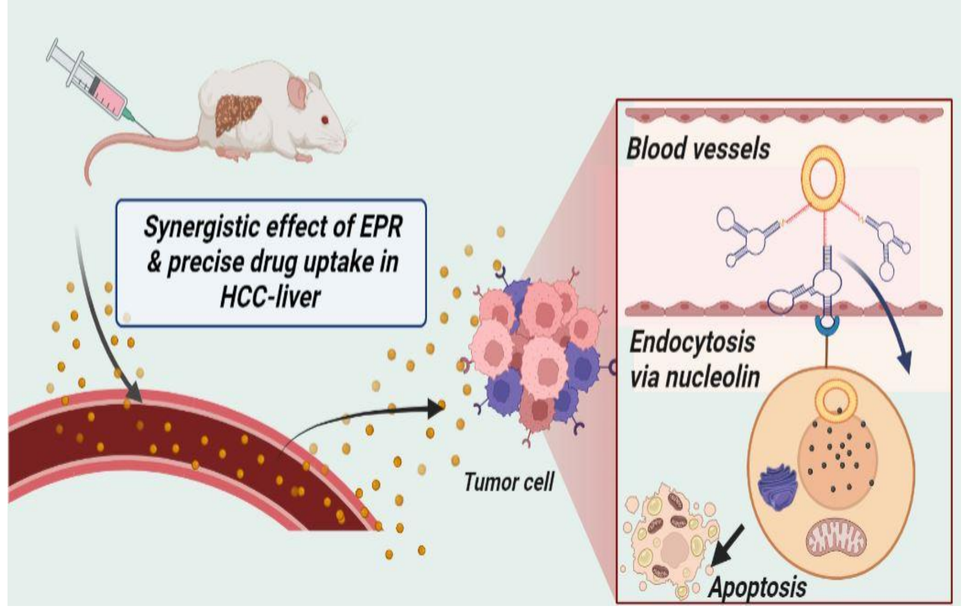


Figure 3.2 Delivery of aptamer functionalized nanoliposomes s to HCC through nucleolin mediated endocytosis

Chapter 4

Materials and Methodologies

1. Materials

1.1. *Chemicals*

Apigenin was obtained from Sigma–Aldrich Lab. (MO, USA). Carboxymethyl-PEG2000-1,2-Distearoyl-sn-glycero-3-phosphoethanolamine or (DSPE-PEG2000-COOH) was purchased from Laysan Bio Inc. (AL, USA).1-Ethyl-3-(3-dimethylamino propyl) carbodiimide, chloride (EDC, HCL), N-hydroxy succinimide (NHS), cholesterol, dimethyl sulfoxide, butylated hydroxytoluene (BHT), ethanol, chloroform, sodium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphate were procured from E Merck Specialties Ltd. (Mumbai, India). Fluorescein isothiocyanate (FITC) and Soya-l-α-lecithin (SPC) were acquired from Hi-Media Lab. Pvt. Ltd. (Mumbai, India).

The 26-mer- AS1411 DNA aptamer, having sequence GT GGT GGT GGT TGT GGT GGT GGT GG (molecular weight: 8674.3 g/mole) was custom-synthesized as phosphorothioate backbone and 3´- amino-modified form by GCC Biotech (Kolkata, India).

1.2. *Cell lines*

All the in vitro cell based studies were conducted on human liver cancer cell lines, Hep-G2 / Huh-7 cell lines purchased from NCCS (National Centre for Cell Science, Pune, India). The cell lines were sub-cultured in DMEM media, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution and maintained at 5% CO_2 containing humidified air at 37°C.

1.3. *Animals, Sprague Dawley (SD) rats for hepatocellular carcinoma (HCC) model*

Sprague Dawley (SD) rats (120-150 g) were procured from NIN (National Institution of Nutrition), Hyderabad, India, and animal studies, conducted here were approved by the Institutional Animal Ethical Committee (AEC), Jadavpur University on submission of proper

experimental protocol plan following guidelines of the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2. Methodologies

2.1. *Pre-formulation studies (FTIR-Fourier -transform infrared spectrometry)*

Fourier -transform infrared spectrometric (FTIR) analysis was applied to examine if there was any molecular interaction between the drug and the excipients used during the formulation development (17). FTIR spectroscopy was carried out with the drug (pure apigenin, Api), drug excipients (SLY, CHC, DSPE-PEG, BHT) individually along with their physical mixture, formulated plain nanoliposome (NLCs) with or without the drug, PEG-NLCs and finally aptamer conjugated PEGylated nanoliposomes, Apt-NLCs within the wave number array of 4000–400 cm^{-1} under inert atmospheric conditioning KBr palate by an FTIR spectrophotometer (Bruker-(Alpha), Ettlingen, Germany) and were analyzed.

2.2. *Preparation of apigenin (Api) loaded nanoliposomes (NLCs, PEG-NLCs) and aptamer functionalized PEGylated nanoliposomes (Apt-NLCs):*

Apigenin encapsulated plain nanoliposomes (NLCs) and PEGylated NLCs were prepared by the thin-film hydration method (Dey et al. 2016). For subsequent preparation of the aptamer functionalized PEGylated nanoliposomes (Apt-NLCs), we have used the PEGylated NLCs (PEG-NLCs). The coupling of aptamer on the liposomal surface was accomplished by a covalent attachment of the amino group of the aptamer (NH_2-modified AS1411) with the carboxyl group of PEG-DSPE-COOH in the PEG-NLCS (Mashreghi et al.2020). Surface grafting of amino-modified AS1411 aptamer on the prepared pegylated NLCs was conducted through the activation carboxyl group of PEG component (PEGylated-1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (PEG-DSPE-COOH). The pegylated NLCs were suspended in a mixture of 150 mM N-hydroxy succinimide (NHS) and 300mM ethyl (dimethyl amino

propyl) carbodiimide (EDC) at room temperature for 1 h by maintaining all through a neutral of 1M Tris HCI buffer (Mashreghi et al.2020; Alibolandi et al.2016). The resulting solution was added to (20-25) µl aliquot from100 µM of 3´amine-modified AS1411 aptamer stock solution (final concentration of AS1411 in experimental nanoliposomes Apt-NLCs was- 5µM) (Moosavian et al.2016). Here, the portion of the sample aptamer solution was previously arranged applying denaturation−renaturation process, so that it would interact in biochemical reaction readily. The solution mixture was kept in a shaker incubator overnight at normal room temperature to accomplish aptamer ligation on the surface of PEGylated NLCs. Finally, we removed the excess and unconjugated aptamer from aptamer-conjugated nanoliposomes (Apt-NLCs) by centrifuging and washing the mixture thrice with nuclease-free deionized water and the aptamer conjugated formulations were stored at 4°C for further analysis (Dutta et al.2018).

The blank aptamer conjugated liposomes (blank-Apt-NLCs) were also prepared following a similar process but without encapsulating apigenin in the liposomes. Both NLCs and Apt-NLCs were labeled with FITC (fluorescein isothiocyanate) dye by incorporating 10µl of FITC (0.4% w/v ethanolic solution) into the organic phase during thin layer formation of liposome preparation (Li et al.2014).

2.3. *Determination of aptamer conjugation agarose gel electrophoresis:*

To determine the successful ligation of aptamer AS1411 with the PEGylated liposomes (PEG-NLCs), aptamer conjugated nanoliposome (Apt-NLCs), free aptamer (AS1411), and a commercial 50 bp DNA ladder were sequentially placed in the wells of previously prepared 1% agarose gel plate. Through running electrophoresis for 20 mins at 100 V, we observed the migration pattern for different samples as mentioned above (Dutta et al.2018, Li et al.2014)

Here, 0.5mg/ml ethidium bromide was used in the agarose gel to visualize bands in the gel electrophoresis, and gel loading dye was used for staining DNA during the electrophoresis

process. Finally, free DNA aptamer, DNA ladder, and the DNA-conjugated nanoformulations in gel plate were visualized using a UV transilluminator as reported earlier (Wang et al.2012).

To determine the conjugation of AS1411 aptamer on to PEG-NLCs quantitatively, on precedence we repetitively washed (more than three times) the Apt-PEG-NLCs solution with nuclease free water to remove all the unconjugated free aptamer available in Apt-PEG-NLCs. Finally, with proper dilution by adding nuclease free water to it we conducted spectrometric analysis. We compared the amount of AS1411 aptamer in aptamer conjugated nanoliposomes, Apt-PEG-NLCs along with 1µl of a free AS1411 (DNA aptamer) and a plain PEGylated nanoliposomes, PEG-NLCs (without aptamer) by applying nanodrop UV spectrophotometric method at 260 nm wavelength (nano 300 micro spectrophotometer, IGene Labserve Pvt Ltd. India) (Yuce et al.2017, Peng et al.2020).

2.4. ***Percentage of drug loading and drug entrapment efficiency:***

To determine drug-loading, accurately weight of lyophilized liposome (2mg), NLCs/PEG-NLCs or Apt-NLCs was dissolved in 2ml of ethanol-acetonitrile-dimethyl sulfoxide solvent mixture, at a ratio of 0.5:1:1(v/v) as the best suit solvent composition for testing drug loading of the experimental formulations. It was determined by the trial-and-error method. The resulting solution was vortex mixed for 1-2h and then centrifuged for 20 min at 10,000 rpm, clear supernatant (1ml) was collected to measure the absorbance intensity by the UV/VIS-spectrometer at the corresponding λ_{max} of apigenin, 340nm and the drug content in the test liposomes were analyzed from the corresponding standard calibration curve prepared early [Dey at al. 2018]. Thus, percentage drug-loading, and drug encapsulation efficacy were obtained using the formulae mentioned below.

% Drug loading = (Amount of drug in liposome/Amount of liposome used) X 100…. (1)

% Yield = (Weight of dry powdered liposome/Total weight of all the components used in the formulation) X 100…. (2)

2.5. ***Particle size distribution & ζ- potential measurements***

Particle size distribution and ζ-potential of NLCs, PEG-NLCs, and Apt-NLCs were analyzed by Malvern Zetasizer Nano-ZS 90; (Malvern Instruments, UK) applying dynamic light scattering (DLS) technique with sample suspension of the experimental liposomes by diluting it properly through vortex and sonication process. The results represented were the average particle size considering the standard deviation of at least three different batches of the experimental liposomes.

2.6. ***Field emission scanning electron microscopy (FESEM)***

Surface morphology of prepared test nanoliposomes (NLCs, PEG-NLCs, and Apt-NLCs) were observed under FESEM by forming a very thin layer of samples coated with platinum with a platinum coater at an accelerating voltage of 10 kV (Rudra et al.2010).

2.7. ***Atomic force microscopy (AFM)***

Three-dimensional architecture of Apt-NLCs was observed under atomic force microscopy (AFM) by placing one drop of properly diluted and air-dried formulation suspension on a mica surface coverslip plate. Thus, shapes of the Apt-NLCs were observed through AFM by fixing a resonance frequency at 150-250 KHz under ACAFM mode (Ruozi et al.2011).

2.8. ***Cryo-transmission electron microscopy (Cryo-TEM)***

The internal bilayer morphology, membrane stability and surface functionalization characteristics of the prepared experimental nanoliposome, Apt-NLCs, were determined by cryo-TEM. Here test liposomes were dispersed (100 μg/ml) in Milli Q water, and an aliquot (4

µl) of it was placed on a glow-discharged 300 mesh carbon-coated copper grid (TEM grid), and the air-dried samples were visualized under the cryo-TEM instrument (200 kV Talos-Arctica electron microscope, FEI/ Thermo Scientific) (Peretz Damari et al.2018).

2.9. *In-vitro drug release*

In vitro drug release study was conducted taking 2 ml of experimental liposomal suspensions (which were prepared by dispersing 2 mg of lyophilized NLCs, PEG-NLCs, and Apt-NLCs individually in 2 ml of buffer solutions, PBS phosphate buffer with pH 7.4 and acetate buffer, with pH-5) into a dialysis bag (Dialysis Membrane-110, Himedia, India). Then it dialyzed into 50ml of respective drug release media, PBS containing or acetic buffer containing 0.01% (w/v) β-cyclodextrin at room temperature (37°C) by placing the dialysis system on a magnetic stirrer with very slow stirring at 100 rpm. During each study, 1 ml of drug release media was collected from 50 ml of solution at predetermined intervals up to 96 h. and was replaced by 1ml of fresh media immediately (Sengupta et al.2018). Finally, all the samples were analyzed by UV-VIS spectroscopy at 340 nm wavelength against the respective medium without drug and drug concentration was determined using calibration curve prepared earlier. *In vitro* cumulative drug release data were represented through various release kinetic models such as zero order, first order, Higuchi model, Korsmeyer–Peppas and Hixson–Crowell models, and the highest R^2 (correlation coefficient) value was evaluated after plotting the released data to determine the best suit drug-release kinetic model (Dutta et.al.2018).

2.10. *Stability study*

Stability assay of the intended experimental formulation aptamer conjugated PEGylated nanoliposomes (Apt-NLCs) was performed as per ICH guidelines (Dutta et al. 2018), at the settings of storage conditions (40 ± 2°C and 75 ± 5% RH) and in the refrigerated form at (~4 °C) for 6 months, with the samples were subjected to examine for any changes in particle size

or morphology using FESEM and variation in drug content in the prepared nanoliposome (Apt-NLCs) by conducting drug loading assay.

2.11. ***MTT assay to evaluate in vitro antiproliferative activity of test liposomes***

Two different liver cancer cell lines, Hep G2 and Huh-7, were procured from the National Centre for Cell Science, Pune, India. The cells were cultured in DMEM media, maintaining all necessary conditions (Ganguly et al. 2021). The cell (taking 1 x 10^3 cell/wells) viability expressed as IC_{50} doses for the free drug (apigenin), NLCs/PEG-NLCS, Apt -NLCs and blank-Apt-NLCs (Apt -NLCs without the drug) in both the cell lines were evaluated through MTT, (4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide assay using a wide range of individual equivalent drug concentration (1µM, 10µM, 20µM, 30µM, 40µM, 50µM) following the standard protocol (Farahbakhsh et al.2020).

2.12. ***Human peripheral blood mononuclear Cell (PBMC) processing***

This process is performed by isolating peripheral blood mononuclear cells (PBMC) from anticoagulated blood. Anticoagulated blood was added with an equal volume of Ficoll-Hypaque (Histopaque-1077) and centrifuged at 400 g for 30 min to separate the cells from the whole blood. The PBMCs were collected from the interface of two liquids, washed with PBS (0.01 M, pH 7.4) twice, and resuspended in the RPMI 1640 medium (Dutta et al. 2018). The cytotoxic activity of different nanoliposomes suspended in PBS in the presence of a very minute amount of dimethyl sulfoxide, DMSO (final DMSO concentration<0.1%), was evaluated for PBMC using an MTT assay.

2.13. ***Apoptosis assay***

To verify the apoptotic potential of aptamer functionalized nanoliposome, apt-NLCs, in comparison with the nonconjugated ones (NLCs/Peg-NLCs) in Hep G2 cells, we executed a flow cytometric (BD LSRFortessa™, BD Biosciences) study using FITC-Annexin V staining

assay kit (BD Biosciences). Apoptotic activity of the experimental nanoliposomes containing an equivalent amount of active drug (considering the IC50 values from MTT) was determined upon 48 h of prior treatment and following the protocols (Paul et al.2019).

2.14. ***Determination of cell-targeting potential of the aptamer (AS1411) assessing in vitro cellular uptake of the experimental nanoliposomes***

In vitro uptake of FITC-labeled PEG-NLCs and Apt-NLCs by HepG2 cells treated with the formulations containing the equivalent amount of drug was evaluated quantitively at the three successive time points (1 h, 2h, 4h) by flow cytometric method. To confirm the receptor-mediated cellular uptake of an aptamer (AS1411) functionalized PEGylated nano-liposome (apt-NLCs) over the other non-functionalized pegylated nanoliposome (PEG-NLCs), a competitive assay was performed. In this competitive assay, Hep G2 cells were preincubated with an excess amount (0.2µg/well) of free aptamer (AS1411) for 2h to block available coupling receptors (majorly nucleolin) (Farahbakhsh et al.2020; Liao et al. 2015). After that, it was treated with apt-NLCs maintaining all other conditions the same. A quantitative comparison of *in vitro* cellular uptake between FITC-labeled formulations PEG-NLCs, Apt-NLCs in the Hep G2 cells, and FITC labeled Apt-NLCs against aptamer sensitized Hep G2 cells were estimated to verify receptor-mediated cellular uptake of aptamer conjugated experimental nanoliposomes, Apt-NLCs. FACS Diva software (BD Biosciences) was used to analyze the data (Liao et al.2015) We have also captured confocal microscopic (Olympus Fluo View FV10i, Olympus) images of Hep G2 cell lines treated with FITC-labeled peg-NLCs and apt-NLCs at 1h and 4h intervals to visualize the cellular uptake qualitatively.

2.15. ***Study of cell cycle arrest and apoptosis-related protein***

FACS (BD Biosciences, USA) analyzer was also used to analyzed different phases of cell cycle during cell propagation, referring apoptotic behavior of test nanoliposomes (an equivalent IC_{50}

dose of apigenin containing in the respective amount of NLCs, PEG-NLCs and Apt-NLCs was used to treat 1.5 × 10^5 Hep G2 cells per well in six-well plates for 24 h). After this washing in 50 µl RNase water and treating with Anexin -V, propidium iodide (PI) solution, the cells were subjected to analysis (Jin et al.2017).

Similarly, apoptosis-related signaling proteins (p53, cleaved caspases, Bcl-2) were also estimated using respective protein markers by flow cytometric assay. These proteins have a major influence on the genetic regulation process (DNA synthesis, damage, or repair) during apoptosis. The experiments were conducted in Hep G2 lines, treated with Apt-NLCs, PEG-NLCs, and NLCs taking equivalent dose of apigenin, after a prior incubation of 24 h following the published protocol and guidelines (Jin et al.2017).

2.16. ***Development of DENA-induced hepatocellular carcinoma in Sprague Dawley (SD) rats***

All the animal studies were approved by the Institutional Animal Ethical Committee, Jadavpur University. HCC hepatocellular carcinoma was developed in Sprauge Dawley rats by chemical inducing method applying initially a single intraperitoneal dose of 200 mg/kg of diethyl-nitrosamine (DENA), followed by a bi-weekly oral administration of 0.5% of w/w 2-acetylaminofluorene (2-AAF) for 16 weeks (Sarkar sarkar et al. 1994; Ghosh et al.2014). Experimental grouping was done as normal control rats, carcinogen control rats, carcinogen-treated rats received free apigenin, carcinogen-treated received NLCs, carcinogen-treated rats received PEG-NLCs, carcinogen-treated rats received Apt-NLCs, and normal rats received Apt-NLCs. All the carcinogen-treated groups were injected (through iv route) 20 mg/kg body weight of free apigenin or equivalent of the experimental formulations once a week for eight weeks after 16th weeks of HCC induction in animals (Dutta et al.1018).

2.17. ***Pharmacokinetic study***

The plasma and liver pharmacokinetic profile for free drug, NLCs, PEG-NLCs, and Apt-NLCs were evaluated in HCC induced Sprague Dawley (SD) rats (body weight,125-150mg). Plasma and liver samples were collected from the experimental animals after injecting a single i.v. bolus dosage of 2mg/kg of body weight of apigenin or corresponding amount of NLCs, PEG-NLCs, and Apt-NLCs containing equivalent apigenin, at 2, 4, 8, 12, 24, 48, 72, 96, and 120 h. The final analytical samples were prepared from the biological samples through the liquid-liquid extraction process (using TBME, tetra butyl methyl ether as a volatile solvent for drug extraction from plasma and liver homogenate) and analyzed by LC-MS/MS method applying naringenin as an internal standard (Dutta et al.2018). The experiments were repeated in triplicate.

2.18. ***Assessing biodistribution of test nanoliposomes, NLCs, PEG-NLCs, and Apt-NLCs***

Gamma scintigraphy study

To investigate the accumulation efficiency of liposomes, NLCs, PEG-NLCs and Apt-NLCs towards the target organ (liver), the formulations were radiolabeled by technetium-99m (^{99m}Tc) following the stannous chloride reduction method with a radiolabeling accuracy almost above 90% (Ganguly et al.2021). Radiolabeled formulations (equivalent drug dose) were injected through the cannulation process to carcinogen-treated rats to ascertain the pattern of bio-distribution of different test nanoliposomes (NLCs/PEG-NLCs/Apt-NLCs) at 4h and 8h after their administrations. The results were articulated as % administered dose distributed per gram (%ID/g) of tissues or organs. Gama-scintigraph images of the animals were captured at 4 and 8 h post-injection through GE Infinia γ Camera facilitated along with Xeleris Work Station, USA [Paul et al. 2019, Ganguly et al.2021].

2.19. ***In vivo hepatocyte uptake of fluorescent-labeled formulations by confocal microscopic study***

To elucidate the accumulation pattern of different test nanoliposomes in the neoplastic hepatocytes, we have injected FITC-labelled NLCs, PEG-NLCs, and Apt-NLCs into the DEN-induced HCC positive animals through the tail veins. Taking a time interval of 4h and 8h post-injection, tumor-adjacent tissues were collected by sacrificing the animals and stored at10% formaldehyde [Dutta et al.2018]. The tumor sections were fixed on slides and evaluated under confocal microscopy.

2.20. ***Assessing antitumor efficacy of the experimental nanoliposomes using a prepared animal model***

To ascertain the antitumor potency of the test nanoliposomes, we divided the animals into seven groups, Gr.A, normal (control), Gr. B, HCC positive control animals HCC, Gr.C, HCC induced animals treated with the free drug, Gr.D, HCC animals induced treated with NLCs, Gr.E, HCC induced animals treated with PEG-NLCs, Gr. F, HCC induced animals treated with Apt-NLCs, Gr G, normal animals treated with Apt-NLCs. In the case of Gr C, D, E, F, and G, we followed a treatment schedule of once-weekly i.v. dose of 20mg/kg body weight of free apigenin or equivalent amount formulations for twelve weeks. There were seven experimental animals in each group. At the end of the treatment, we collected the whole liver or liver sections from the sacrificed experimental animals to assess the degree of antitumor potency of Apt-NLCs compared to NLCs/PEG-NLCs or free drug.

2.21. ***Gross and histopathological examination for liver morphology***

We performed a gross examination of the whole livers to identify the percentage of tumor incidences. Microscopic observation of tumor tissue sections by histopathological staining (with hematoxylin and eosin, periodic acid Schiff) was conducted to evaluate the microscopic changes in the liver histopathology and to identify hepatic altered focal lesions (Bhattacharya et al.2018).

2.22. ***Identifying apoptotic gene expression level through qRT-PCR analysis***

Total RNA was extracted from frozen (stored at -70°C) liver tissue sample (from Gr B, C, D, E & F animals) with Trizol reagent and was evaluated through nanodrop (QI Aexpert) after following standard protocol. Then, with required amount of sample RNA, cDNA was prepared and using this template RT-PCR studies were performed with the aid Bio Rad SYBR green PCR master mix along with corresponding primers as per manufacturer's instructions (Mabrouk et al. 2022; He et al.2018). The specific synthetic primers (for p^{53}, F:5´-ATGTTTTGCCAACTGGCCAAG-3´, R:5´-TGAGCAGCGCTCATGGTG-3´); (for caspase 3, F: 5′-GTGGAACTGACGATGATATGGC-3′, R: 5′-CGCAAAGTGACTGGATGAACC-3′); (for Bcl-2, F:5′-TGTGGATGACTGACTACCTGAACC-3′, R:5′-CAGCCAGGAGAAATCAAACAGAGG-3′) and (for β-actin, F:5'-AAGATCCTGACCGAGCGTGG-3', R:5'-CAGCACTGTGTTGGCATAGAGG-3') were purchased from Saha Gene, Hyderabad, India and maintaining the thermo-cycling conditions per standard protocol the qRT-PCR studies were performed on Rotorgene (Qiagen) instrument. The fluorescence activity in qRT-PCR demonstrated the relative genetic expression level for corresponding target genes and it was determined by applying 2-ΔΔCt calculative process, where β-actin was taken as housekeeping gene (Wang et al.2017).

2.23. ***Hepatic function test***

Blood samples were collected from the all the groups of experimental animals (Gr A-G) and after processing as per the reported method (Ganguly et al.2021) the level of AST (aspartate aminotransferase), ALT (alanine transaminase), and ALP (alkaline phosphate) were determined using commercially available bioassay kits (Coral Clinical Systems, Goa, India) following manufacturer protocols.

2.24. ***Statistical analysis***

The data were statistically analyzed using ORIGIN 8.0 software, one-way and two-way ANOVA followed by post hoc Dunnett's test using Graph Pad Prism Software, considered 5.0. $p < 0.05$ was as a minimum level of significance.

Chapter 5

Results & Discussion

1. Results and Discussion

1.1 Physical Characterization

Pre-formulation and preparation of NLCs/PEG-NLCs/Apt-NLCs

Thin film hydration methods were followed for preparation of experimental nanoliposomes. FTIR spectra for apigenin (Api), each of the excipients (SLY, CHC, DSPE-PEG, BHT), their physical mixture with drug, along with test nanoliposomes NLCs, apt-NLCs with Apigenin (Api).(**Figure 5.1**) showed the presence of their characteristic functional groups, for Api (C=C stretching at 1556.92 $cm^{-1}$and C=O stretching at 1651.21 cm^{-1}); for SLY (C=0 stretching at 1735.99 cm^{-1} and C-H stretching at 2922.15 cm^{-1}); for CHC (C-H bending at 1459.31 $cm^{-1}$and C-O stretching at 1053.26 cm^{-1}); and for DSPE-PEG (2000)-COOH (C-N medium intensity stretching at 1104.01cm^{-1}) in their physical mixture and in the formulations as in original components referring that nanoliposomes were formulated successfully without any chemical interaction among the ingredients. However, minor shifting of bands (H−O stretching) suggests formation of weak Van der Waal interaction or weak hydrogen bond due to physical interaction during formulation development. The characteristic peak of apigenin was absent in the NLCs without the active drug (called here Blank formulation). It was also missing in the prepared test nanoformulations (both in NLCs and Apt-NLCs), indicating complete drug encapsulation. Thus, we confirmed the chemical compatibility of apigenin and other excipients using FTIR studied and successful drug encapsulation in the developed nanoliposomes (NLCs, PEG-NLCs, and Apt-NLCs).

Determination of aptamer conjugation

Successful conjugation of phosphorothioated amino-modified AS1411(DNA-aptamer) with PEG-NLCs was determined using agarose gel-electrophoresis through observing prominent fluorescence bands under UV-transilluminator. (**Figure 5.2**) depicted gel-electrophoresis assay, the migration of parallel luminating bands for 50 bp commercial DNA ladder was noticed clearly. Free DNA (AS1411) was also migrated near 25 bp position of 50 bp commercial DNA ladder, while little or no migration for aptamer-conjugated nanoliposomes (Apt-NLCs) was observed. The three stable luminating bands near the starting wells for three individual samples of Apt-NLCs from three different batches indicated successful DNA conjugation with the nanoformulations. Aptamer-conjugated DNA could not migrate through the agarose gel plate

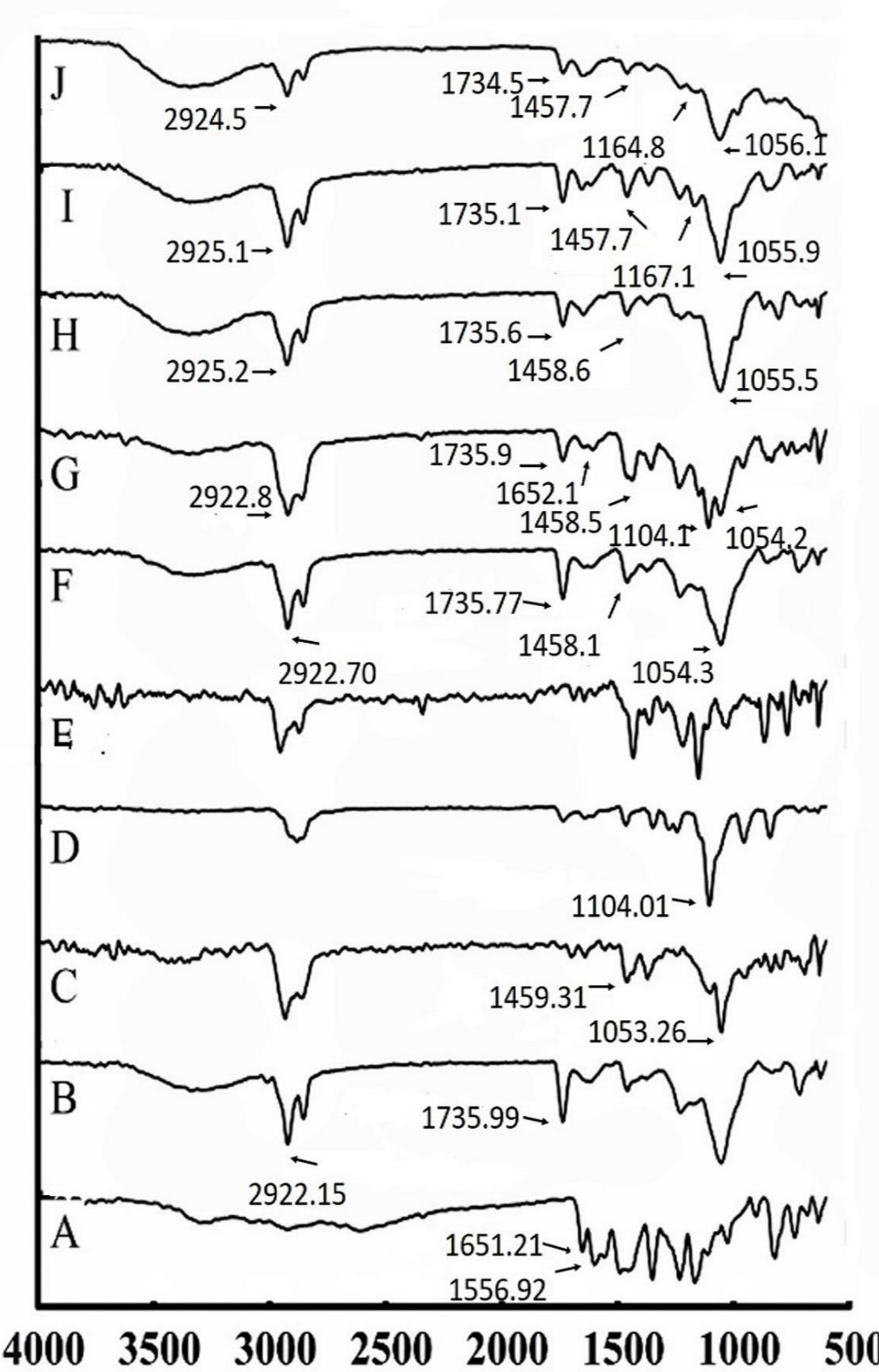


Figure 5.1. Drug-excipients interaction and aptamer conjugation on stealth nanoliposome. (a) FTIR spectra of apigenin (A), soya lecithin (B), cholesterol (C), DSPE-PEG-2000-COOH (D), BHT (E), Blank formulation (F), the physical mixture of all constituents (G), NLCs (H), PEG-NLCs (I), and Apt-NLCs (J)

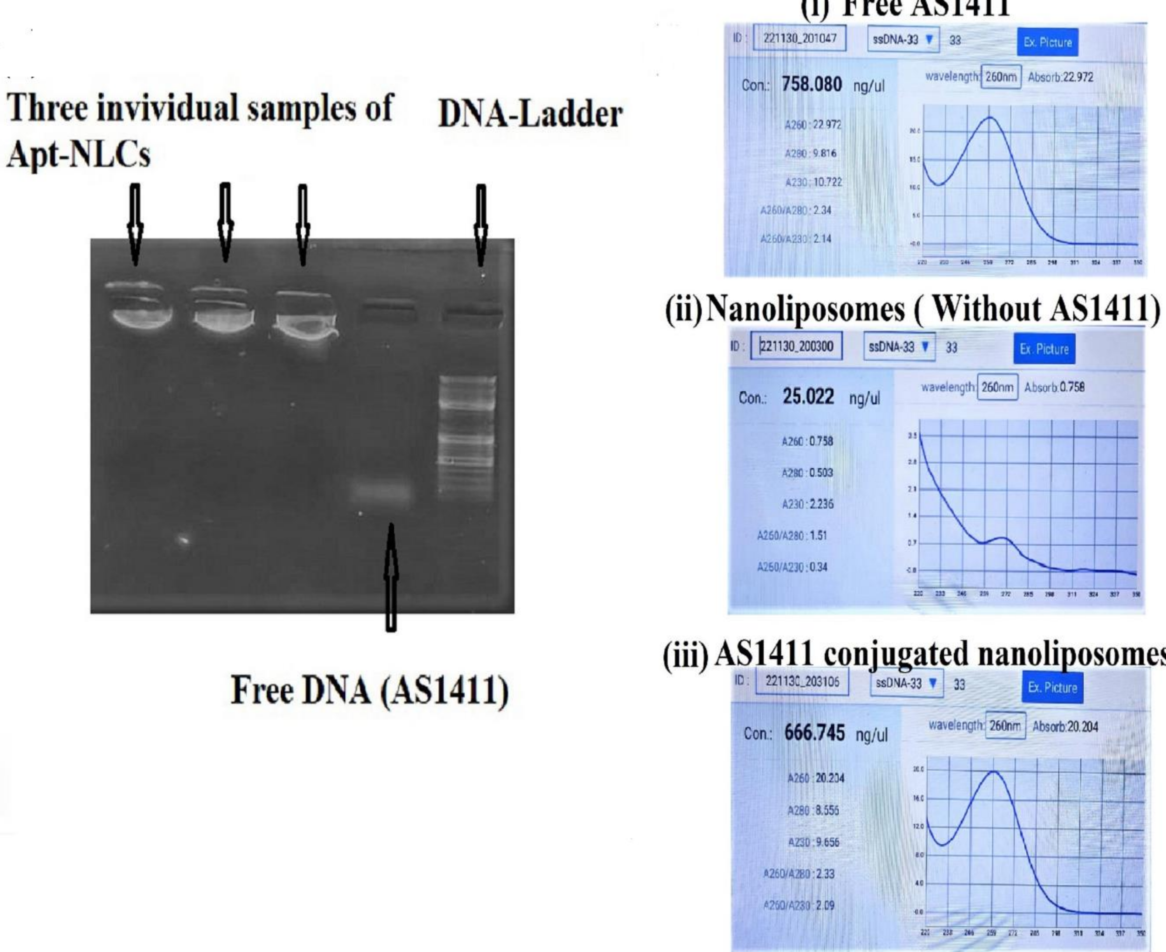


Figure. 5.2. Determination of aptamer conjugation by agarose gel. The first three lanes from the left had (Apt-NLCs) showing no sample run, the 4th lane showed free aptamer (AS1411) showing movement, and the 5th lane had DNA ladder; i, ii, iii represented spectrometric reading for free AS1411, plain nanoliposomes AS1411 conjugated nanoliposomes by nanodrop UV spectrophotometer, (experiment was conducted on triplicate set).

with the same speed of free aptamer as it was covalently attached with the bulky PEGylated nanoliposomes.

Through nanodrop UV spectrophotometer, we observed 1µl of free AS1411 contain 758.04 ± 55 ng/µl of ssDNA at 260 nm and compared this with the equivalent amount of AS1411 from the diluted solution of Apt-NLCs (we had added 20µl of 100 µM AS1411 aptamer to 20 mg of PEG-NLCs to get 5µM AS1411 in sample). The ssDNA concentration in Apt-NLCs was 666.01 ± 03 ng/µl. While, non-conjugated plain nanoliposomes (without aptamer conjugation) did not showed any significant absorbance at 260 nm range in nanodrop UV spectrophotometer. All

spectrometric readings are depicted in figure 5.2. This result indicated that more than 85% of AS1411 aptamer used for conjugation was coupled with PEG-NLCs, so can conclude that 1mg of PEG-NLCs had capacity to bind approximately 0.20μM of AS1411 of ssDNA.

Physio-chemical characterization for test nanoliposomes:

The mean particle size, PDI, ζ- potential, drug loading, and encapsulation efficacy were optimized for NLCs, PEG-NLCs, and Apt-NLCs. NLC had an average diameter size of 30 nm (Table 5.1), which increased to 140 nm and 150 nm, respectively, upon PEGylation (PEG-NLCs) and PEGylation followed by aptamer conjugation (Apt-NLCs). (Figure 5.3 & 5.4). The average surface charge (ζ-potential) was found to be 1.16mV, −55.9 mV, and -22.4 mV for NLCs, PEG-NLCs, Apt-NLCs, respectively (Table 5.1 and Figure 5.3 & 5.4.). The presence of DSPE PEG-2000-COOH in the liposome has provided a negative charge, which has been decreased for using amino terminated aptamer conjugation. PDI values (Table 5.1) for the respective formulations were 0,251, 0.256 and 0.316. referring to the formulations as uniformly distributed and could possess minimal aggregation in deionized water (Wang et al.2012). Respective drug loading for NLCs, PEG-NLCs and Apt-NLCs (4.59 ± 0.02, 4.38 ± 0.04 and 4.33 ± 0.05) along with drug loading efficacy in case of all the optimized experimental nanoliposomes (NLCs, PEG-NLCs, and Apt-NLCs) were estimated in Table 5.1. Satisfactory drug entrapment efficacy (>85%) in the experimental nanoliposomes was determined depicted in Table 5.1.

Indicated physiochemical characterizations were permeable for both in vitro and in vivo drug application, particularly for hepatocellular drug delivery (Hoshyar et al.2016). Mean zeta potential values (-22mv) in case of Apt-NLCs referred their stability when dispersed in aqueous suspension for drug administration (Soema et al.2015).

The in-vitro drug release profile of NLCs, PEG-NLCs, and Apt-NLCs with follow up kinetics:

In vitro cumulative percentage of drug release (Figure 5.3) for NLCs, PEG-NLCs and Apt-NLCs were plotted against time in PBS (7.4) containing (0.1% W/V) β-cyclodextrin. Here, we observed all the formulations initially upheld almost similar rapid drug release patterns up to 12 hours. Later phase PEG-NLCs and Apt-NLCs exhibited a prolonged drug release profile compared to NLCs, most likely because of the PEG coating present outside the test liposomes formed. NLCs took 92 hours to display 90% of cumulative drug release in β-cyclodextrin-PBS medium, whereas PEG-NLCs and Apt-NLCs took 144h for almost 90% drug release. Further,

drug release at acetate buffer media (pH 5) also showed similar pattern for PEG-NLCs and Apt-NLCs depicted in Figure 5.4

Table 5.1. Details of physicochemical characterization of experimental nanoliposomes

Formulation Type	Composition	% Of Drug Loading[a]	% Of Loading Efficiency[a]	Z-Average (nm)	Zeta potential(mV)	PDI values
NLCs	D: CHL: SPC (5:25:70)	4.59 ± 0.02	91.86%	20	1.16	0.251±1
PEG-NLCs	D: CHL: SPC: DSPE –PEG-2000 (5:25:60:10)	4.38 ± 0.04	87.6%	100	-55.9	0.262±1.5
Apt-NLCs	D: CHL: SPC: DSPE –PEG-2000 (5:25:60:10) (Functionalized with aptamer, AS1411)	4.33 ± 0.05	86..6%	155	-22.4	0.316±1.1

Note: aEach value represents mean ± SD (n=3).

Abbreviations: D: Drug (apigenin), CHL: Cholesterol, SPC: Soya lecithin.

Table 5.1. Details of physicochemical characterization of NLCs, PEG-NLCs, Apt-NLCs: Table for particle size, drug loading and zeta potential of plain nanoliposomes (NLCs), PEGylated Nanoliposomes (PEG-NLCs) and Aptamer functionalized PEGylated nanoliposomes (Apt-NLCs)

The drug release data were tested on different kinetic models. The regression coefficient (R^2) values for each Zero-order Kinetics, First-order kinetics, Korsmeyer–Peppas kinetics, Hixson–Crowell, and Higuchi kinetics were elaborated in (**Table 5.2**). The data suggest that drug release from the prepared formulations best fit with the Higuchi kinetics model (R^2= 0.9811) and specified n value in derived from Korsmeyer–Peppas kinetics equation suggested here (0.81- to 0.86) range, which indicated non-Fiskian types of diffusion and drug release pattern were maintained by all the prepared test nanoliposomes in the used media.

An extended drug release profile showed that it took almost 7 days for 90% of drug release in 1% β-cyclodextrin-PBS media (pH 7), from PEG containing nanoliposomes (with or without aptamer conjugation, PEG-NLCs/Apt-NLCs). This pattern was due to contributary stealthy

property of DSPE-PEG-2000 in the test nanoliposomes which predicted prolong circulation time. Almost similar pattern of drug release by Apt-NLCs at lower pH (pH 5) also assumed their accumulation towards target tumor (Kanamala et al.2019).

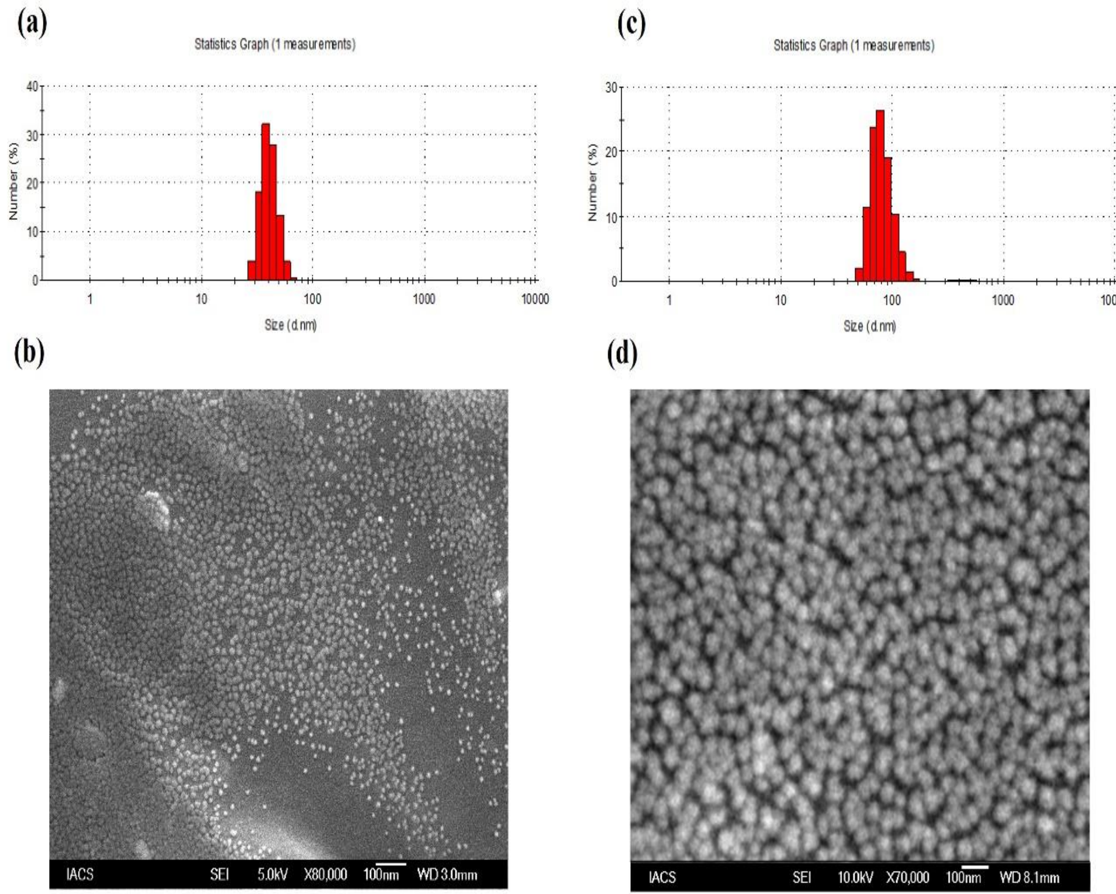


Fig 5.3.: Particle size and surface characterization of plain nanoliposomes (NLCs) and PEGylated Nanoliposomes (PEG-NLCs). (a), (c) average particle size distribution for NLCs and PEG-NLCs respectively, (b), (d) surface morphology applying FESEM images for NLCs and PEG-NLCs respectively.

Surface and internal morphology of NLCs/PEG-NLCs and Apt-NLCs

FESEM, AFM, and Cryo-TEM evaluated the surface and internal morphology of the prepared nanovesicles. FESEM observation for NLCs /PEG-NLCs (Figure 5.3) and apt-NLCs (Figure 5.4) suggested that the vesicles were nanosized (20-120 nm) with a smooth surface and homogenously well-distributed. Further, the physical architecture of the particles in a three-

dimensional mode in AFM revealed that the average mean height for Apt-NLCs was 21.20 ± 0.05 nm (Figure 5.4). The thick and intact uni- and bi-lamellar PEG-coated - oligo functionalized surface morphology for Apt-NLCs having particle size within 100 nm range was clearly visible upon Cryo-TEM observation (Figure 5.4). These types morphological characteristics ensured their stability, cellular accumulation, and sustainability during in vitro and in vivo drug application (Mashreghi et al.2020).

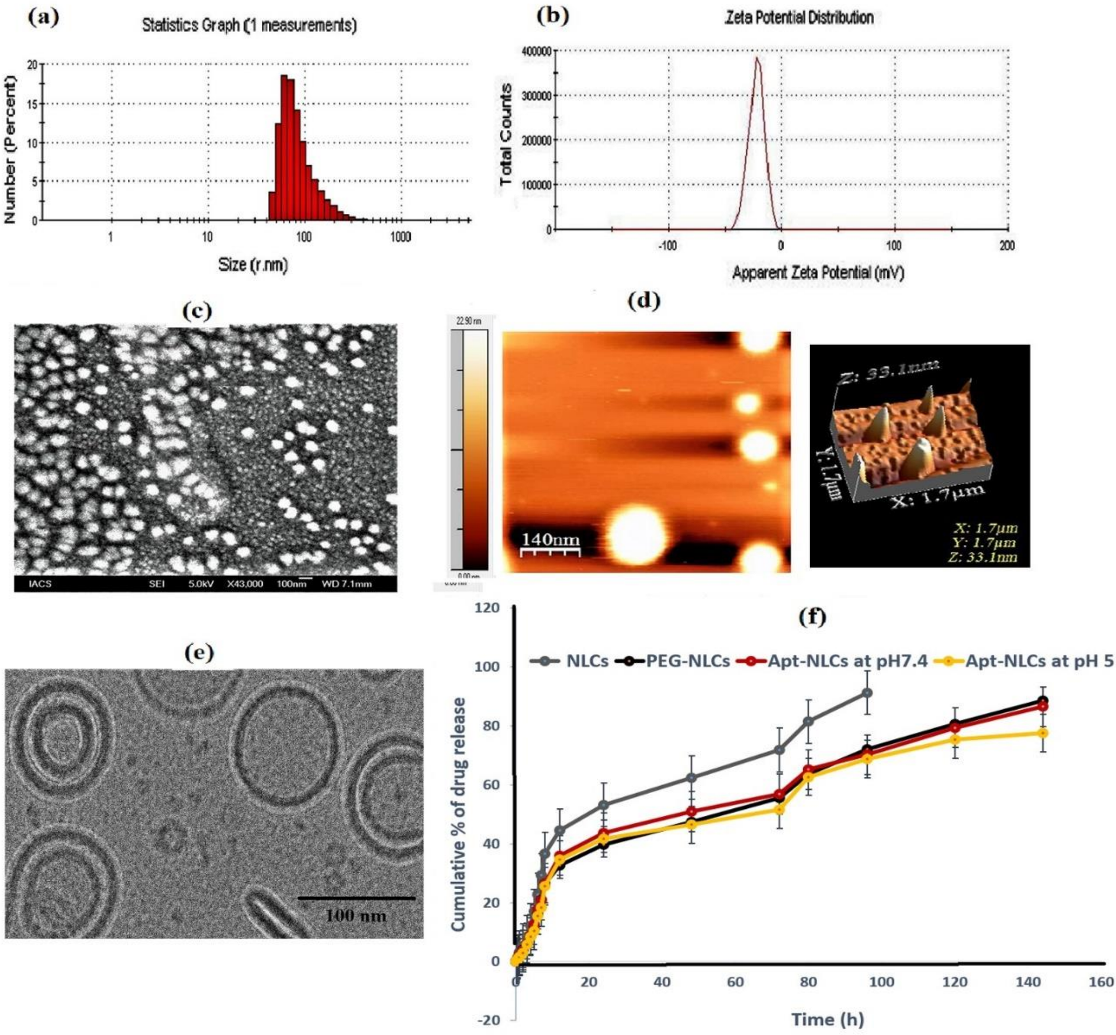


Figure 5.4. Characterization of aptamer conjugated nanoliposomes Apt-NLCs. (a) average particle size distribution, and (b) Zeta potential, (c)surface morphology applying FESEM images at 43 000 ×. (d) Data by atomic force microscopy. (e) Cryo-TEM image depicts internal morphology, (f) Cumulative % drug release against time for Apt-NLCs as compared NLCs/PEG-NLCs. Data show mean ± standard deviation applying three different experimental values.

Table 5.2. ***In vitro*** **drug release kinetics**

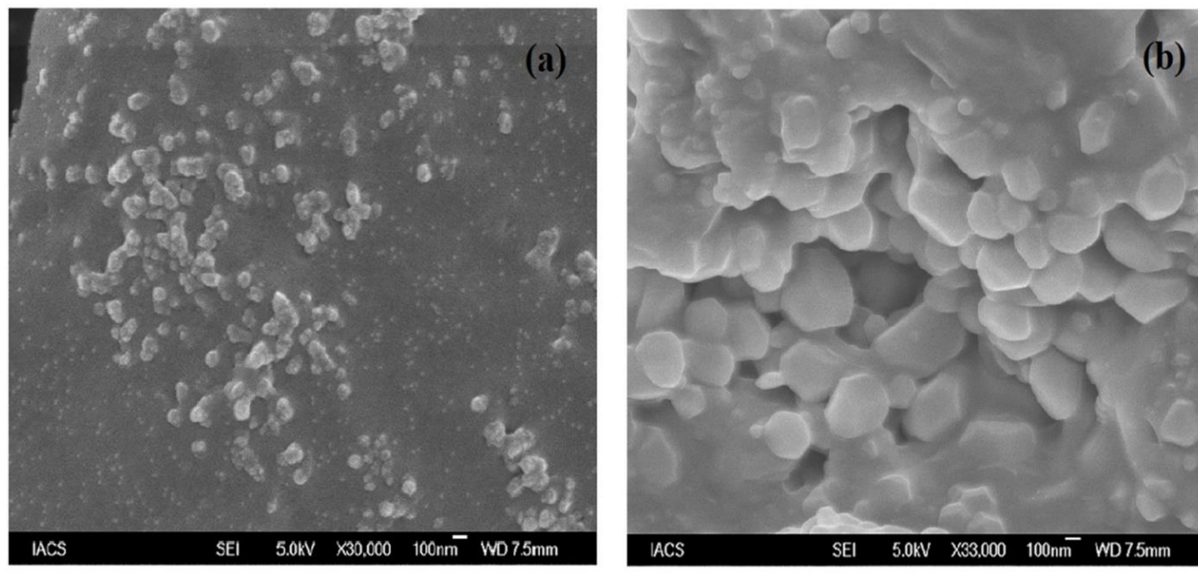
Kinetics models (in vitro release)	**NLCs**		**PEG-NLCs**		**Apt-NLCs**	
	R^2 Value	Kinetic eq^n	R^2 Value	Kinetic eq^n	R^2 Value	Kinetic eq^n
Zero-order	0.8165	y = 0.8932x +12.476	0.9191	y = 0.6166x + 9.996	0.8917	y = 0.6014x + 11.024
First-order	0.8615	y = 0.0092x + 12.476	0.9691	y = 0.0059x + 1.9764	0.9715	y = 0.0055x + 1.9649
Higuchi	0.958	y = 0.1006x + 0.3276	0.9811	y = 0.1297x + 0.407	0.9739	y = 0.1305x + 0.3145
Korsmeyer−Peppas	0.9062	y + 0.8665x + 0.4616 (*n* value 0.866)	0.9287	y = 0.8169x + 0.3617 (*n* value 0.817)	0.9211	y = 0.8185x + 0.3695 (*n* value 0.818)
Hixson−Crowell	0.9391	y = 0.0237x + 0.1705	0.9713	y = 0.0157x + 0.1239	0.9581	y = 0.0149x + 0.0152

Note: Each value represents mean ± SD (n=3), ***n*** **value indicates fickian/non-fickian diffusion.**

Table 5.2. ***In vitro*** **drug release kinetics: The kinetic equations of drug release data tested for NLCs/PEG-NLCs/Apt-NLCs on various kinetic models with corresponding** R^2 **(Regression coefficient) values were studied.**

Stability testing

FESEM studies for Apt-NLCs were conducted after six months of storage. When refrigerated at -4° C) did not portray any major distinguishable changes as compared with freshly prepared Apt-NLCs, while storing at 40 ± 2 °C and 75 ± 5% RH morphology differed (Figure 5.5 and Table 5.3). Further through estimating drug loading and zeta potentials, it revealed that the Apt-NLCs remained equally potent at refrigerated conditions on six-month storage (depicted in Table 5.3). Still, differences were present when stored at 40 ± 2 °C and 75 ± 5% RH setting. Hence, the formulations were absolutely stable over the period of six months at refrigerated condition.



differences were present when stored at 40 ± 2 °C and 75 ± 5% RH setting.

Figure 5.5. Stability studies: (a) FESEM image of APT-NLCs on (-4 °C) storage, (b) FESEM image of Apt-NLCs on (40 ± 2°C and 75 ± 5% RH).

On six months of storage, (when refrigerated at 4° C) FESEM studies did not portray abrupt distinguishable changes as compared with freshly prepared Apt-NLCs, while storing at 40 ± 2 °C and 75 ± 5% RH showed morphological disruption in test formulations depicted in Figure 5.5. Further, assay for drug content estimation (drug loading) and zeta potential at respective condition revealed that the Apt-NLCs remained equally potent at refrigerated conditions on six-month storage (depicted in appendices Figure 5.5 & Table 5.3).

Table-5.3. Drug loading and zeta potential of Apt-NLCs stored at (-4 °C) and (40 ± 2°C and 75 ± 5% RH) for six months.

Formulation Type	% Of Drug Loading[a]	Zeta potential(mV)
Apt-NLCs (-4 °C)	**4.165 ± 0.04**	**-53**
Apt-NLCs (40 ± 2°C and 75 ± 5% RH)	**0.771 ± 0.02**	**-1.6**

Note: Each value represents mean ± SD (n=3)

Thus, we confirmed the development of an aptamer conjugated stable nanoliposomes, encapsulated with apigenin that also composed with ideal physiochemical characterization as experimental Nano system. Further we conducted the other intro and in vivo experiments.

1.2. In vitro cellular studies

In vitro antiproliferative activity

We investigated *in vitro* antiproliferative profile of free drug, NLCs, PEG-NLCs, Apt-NLCs, and blank test nanoliposomes against two different types of liver cancer cell lines (Hep G2 and Huh-7 cell lines) over a range of concentrations (1−100µM) following 48 h incubation period (Figure 5.6.). We found a similar trend of cytotoxic profile in order of IC_{50} values, Apt-NLCs<PEG-NLCs<NLCs<free drug<blank in both the cell lines Figure 5.6.(a). The results reflected the highest cytotoxic potential of aptamer conjugated nanoliposomes (Apt-NLCs) toward liver cancer cells compared to free drug or other nonconjugated nanoliposomes. A negligible cytotoxic activity produced by aptamer conjugated blank nanoliposomes revealed that neither the aptamer nor the carrier nanoliposome had any interference on the cytotoxicity of Apt-NLCs. The lowest IC_{50} value, 10.1 µM, was observed for Apt-NLCs, against HepG2, while the corresponding IC_{50} values of Apt-NLCs towards Huh-7 were 17.1 µM. So, we have proceeded with Hep G2 cell lines for further *in vitro* studies to evaluate apoptotic potentialityof aptamer-conjugated nanoliposomes over nonconjugated nanoliposomes. Additionally, insignificant cytotoxicity toward normal Human peripheral blood mononuclear cells, PBMC (>50 µM) had proved Apt-NLCs were suitable to use as a nontoxic delivery system to target HCC (Depicted in Table 5.4).

Table 5.4. Cytotoxicity studies by MTT-assay

	IC50 (µM), Half maximum inhibitory concentration		
Sample	**HepG2**	**Huh7**	**PBMC**
Apigenin	**34. ± 0.16**	**40.0 ± 1.74**	**>50**
NLC	**21.5 ± 0.60**	**29.6 ± 1.37**	**>50**
PEG-NLC	**19.6 ± 0.85**	**28.4 ± 4.54**	**>50**
Apt-NLC	**10.0 ± 0.85**	**17.0 ± 3.98**	**>50**
Apt-BNLC	**>50**	**>50**	**>50**

Note: Each value represents mean ± SD (n=3)

Table 5.4. Cytotoxicity studies by MTT-assay: Table depicted respective IC50 (µM), Half maximum inhibitory concentration for apigenin, NLC, PEG-NLCs, Apt-NLSs, Apt-BNLCs in Hep G2 and Huh7.

Apoptosis

We have quantified the apoptotic potential of the experimental nanoliposomes applying Annexin V-FITC staining using flow cytometric method. Hep G2 cells were exposed to the nanoliposomes (NLCs, PEG-NLCs, and Apt-NLCs) with their equivalent IC_{50} drug doses for 48 h. The treated group with aptamer functionalized liposomes (Apt-NLCs) showed impressive improvement in the percentage of apoptosis (78.9% in early and 6.7% in late phase, total 85.6%) compared to nonconjugated groups (NLCs/PEG-NLCs) (Figure 5.6.b). NLCs and PEG-NLCs portrayed a total of 68% and 74% of apoptosis, respectively.
This indicated the antitumor potential of apigenin increased even at a low concentration (IC50 value in Hep G2) when delivered through an aptamer functionalized nanoformulations.

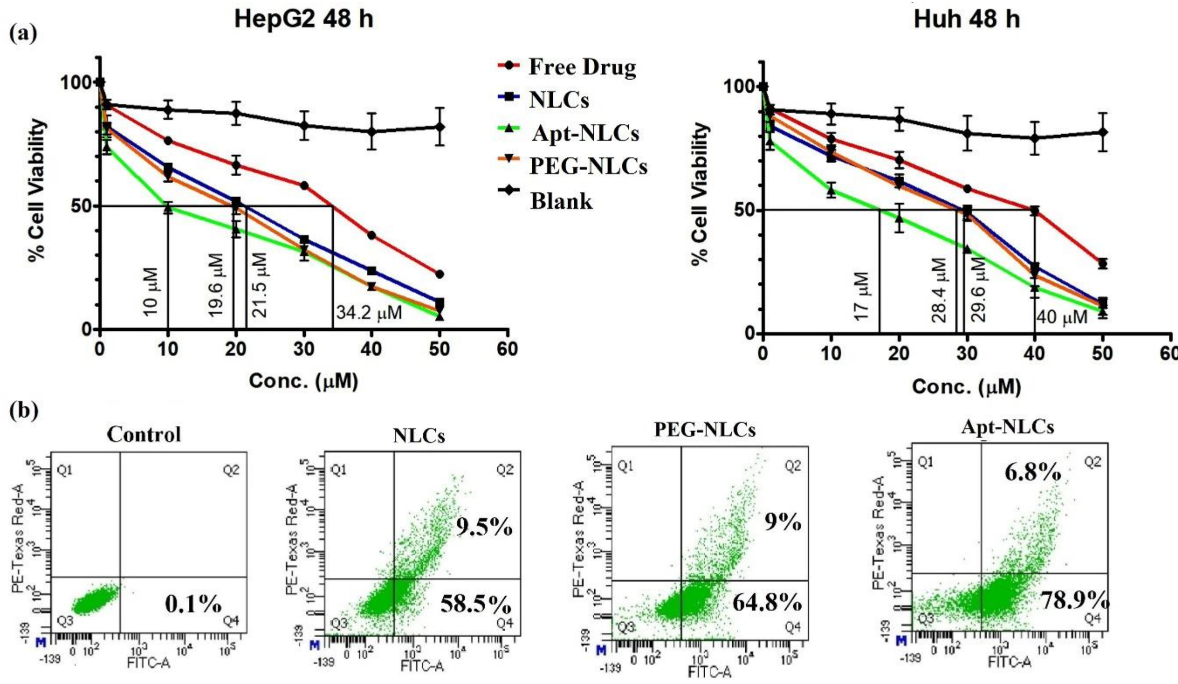


Figure 5.6. In vitro cytotoxicity and apoptosis analysis: (a) Viability assay in HepG2 and Huh-7 cells after treating them with free drug (apigenin), different experimental formulations (NLCs, PEG-NLCs, and Apt-NLCs), and AS1411 functionalized blank liposomes for 48 h with drug concentration range (10-100 µM) respectively. (b) FACS analysis of cellular apoptosis applying Annexin V-FITC staining in HepG2 cells treated with NLCs, PEG-NLCs, and Apt-NLCs with their equivalent IC50 doses of the drug at 48 h.

Receptor-mediated cellular uptake

We estimated uptake of FITC-labelled nanoliposomes, PEG-NLCs and Apt-NLCs (with their equivalent IC50 doses of the drug), in Hep G2 cells quantitatively through the flow cytometric method at different time intervals. Parallelly, we run a set with FITC-Apt-NLCs treated HepG2 cells pretreated with an excess amount of free aptamer (AS1411) denoted as receptor blocking study. There was no considerable difference of mean fluorescence intensity in the case of normal Hep G2 cells treated with PEG-NLCs and in the receptor blocked Hep G2cells (using aptamer alone) treated with Apt-NLCs. In contrast, there was a significant improvement in uptake values in the case of normal Hep G2 cells treated with Apt-NLC in a time-dependent manner (Figure 5.7. a-d). These flow cytometric analysis strongly supported our hypothesis that aptamer (AS1411) functionalized nanoliposomes (Apt-NLCs) augmented cellular uptake in comparison with the nonconjugated nanoliposome (PEG-NLCs), most likely by the nucleolin receptor-facilitated endocytosis as in the cytotoxicity and apoptosis study, we observed notable superior antiproliferative potency of aptamer-conjugated nanoliposomes (Apt-NLCs) over the nonconjugated nanoformulations. FITC-Apt-NLCs showed much superior cellular uptake in Hep G2 cells at 1h and 4h in comparison with PEG-NLCs during confocal microscopy (Figure 5.7..e and, f).

In this competitive cellular uptake study, we tried to understand the probable receptor mediated endocytosis approach for cell uptake. We observed significant improvement of in vitro uptake of Apt-NLCs (aptamer conjugated PEGylated liposomes) in Hep G2 cells as compared to uptake of Apt-NLCs in free-aptamer pre-treated HepG2 cells. Also, bio-ligand AS1411 already reported as biosensor aptamer had shown a tremendous high affinity towards the carcinogenic biomarker nucleolin receptor of HCC cells (Kanamala et al.2019). Here, AS1411 aptamer possibly facilitated nucleolin dependent superior cellular uptake of Apt-NLCs to Hep G2 cells.

Cell cycle arrest and comparative estimation of apoptosis-related proteins

Arresting cell cycle by modulating phases of cell propagation was postulated as causing DNA damage by the experimental nanoliposomes (NLCs, PEG-NLCs, Apt-NLCs) in Hep G2 cells during cellular apoptosis. After flow cytometric analysis, we found that there was a distinguishable increase in the G2/M (73.1%) phase with some changes in the S phase in the Apt-NLCs treated cells as compared with NLCs (51.5%) and PEG-NLCs (55.1%). (Figure 5.8.).

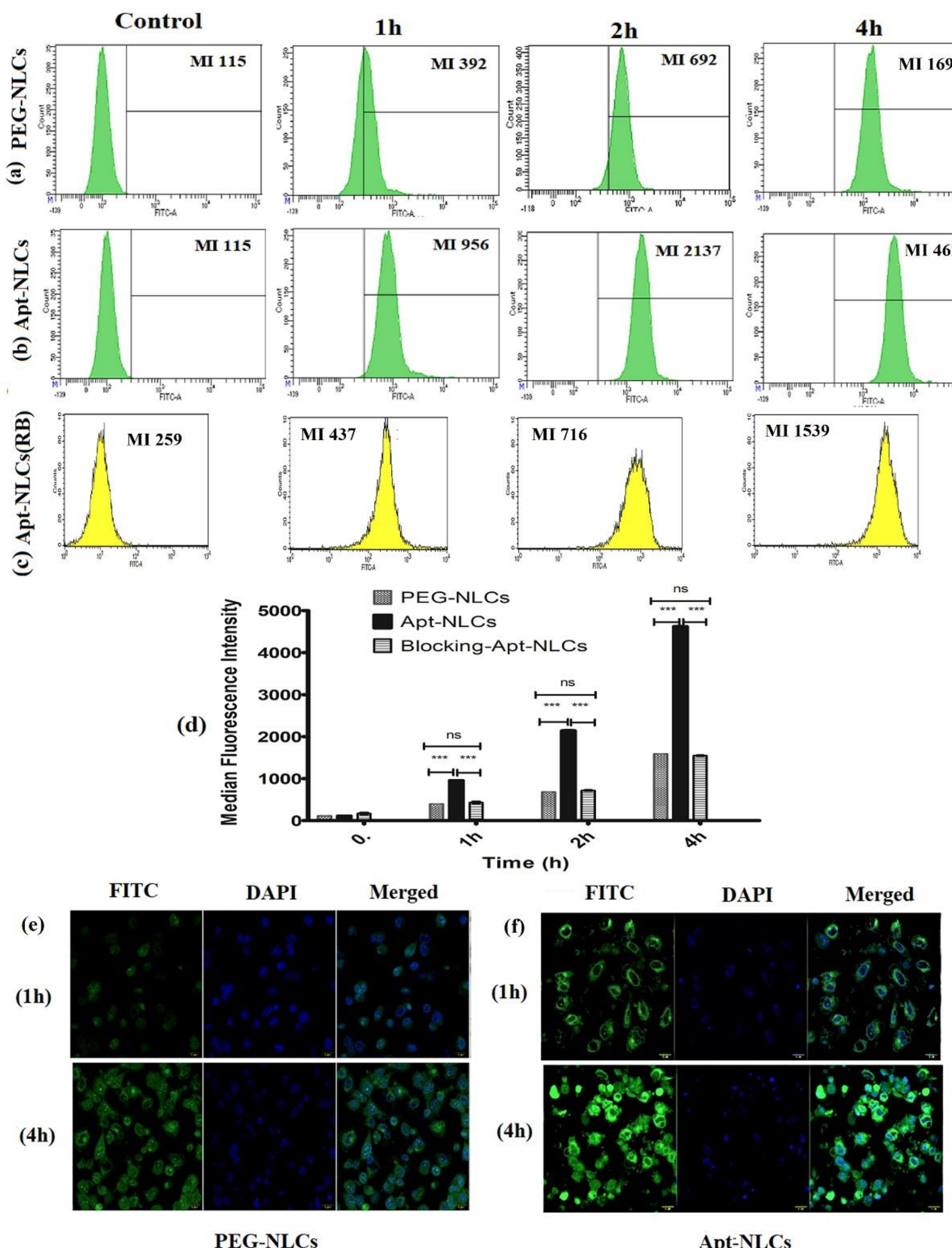


Figure 5.7. Cellular uptake studies. Flow cytometric data of cellular uptake of (a) PEG-NLCs, (b) Apt-NLCs, (c) Apt-NLCs in the presence of free AS1411 (receptor blocking condition) in Hep G2 cells at 1h, 2h, and 4h (d) Histogram representation of FACS mean fluorescence values obtained through above-mentioned uptake studies. Data represents mean ± SD, (n=3), bar (-) indicates groups between which the comparisons were made. ns: statistically insignificant, * refer statistical significance at a level of $P<0.05$. (e) & (f) Confocal microscopic images of cell uptake of PEG-NLCs and Apt-NLCs in Hep G2 at 1h and 4h (Green color shows for FITC-labelled experimental nanoliposomes, PEG-NLCs/ Apt-NLCs, and blue color indicates nucleus stained by DAPI.**

This clearly suggests the enhanced antitumor potency of apigenin while delivered through receptor-targeted drug delivery of nano-system Apt-NLCs. The damage or repair in DNA during apoptotic processes (either extrinsic or intrinsic) are closely monitored with the different apoptotic related proteins. Flow cytometric estimation of tumor suppressor proteins p53, caspase activities, and anti-apoptotic proteins, Bcl-2 activity was conducted. The study depicted the highest mean values for p53 and caspase-3 expressions and the lowest Bcl-2 values with Apt-NLCs compared to other nonconjugated formulations (NLCs/Peg-NLCs) in Hep G2 cells (Figure 5.8.). Arresting cell-cycle at G2/M phase through upregulation in p53 and caspase activities and downregulation of Bcl-2 activity strongly indicate cellular apoptotic potential of apigenin.

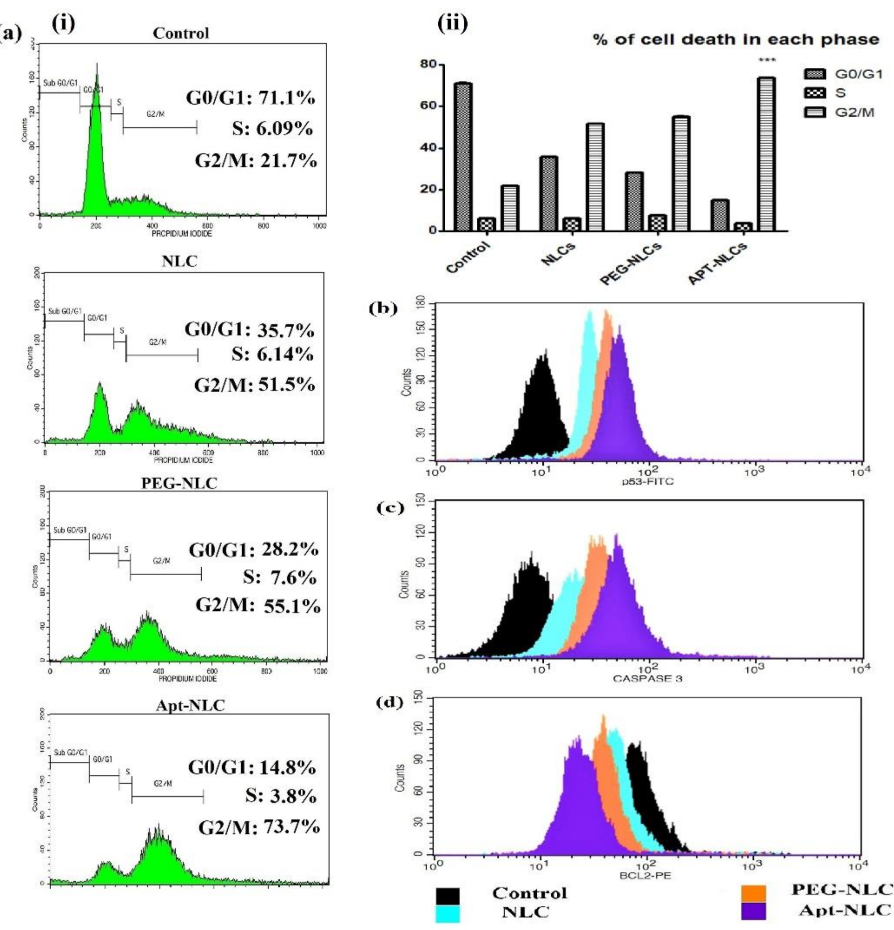


Figure 5.8. Flowcytometric representation of cell cycle analysis and apoptotic protein expressions studies in Hep G2 cells. (a), i & ii Cell cycle analysis of different experimental nanoliposomes NLCs, PEG-NLCs and Apt-NLCs (highest improvement in G2/M phase in aptamer conjugated nanoliposomes, Apt-NLCs in comparison to non-conjugated nanoliposomes, NLCs/PEG-NLCs. Data represents mean ± SD, (n=3), * refer statistical significance at a level of P<0.05. (b), (c), (d), p53, Casapae-3, and Bcl-2 protein quantifications upon NLCs, PEG-NLCs, and Apt-NLCs treatments, respectively, using FACS.**

Apigenin allowed apoptosis in neoplastic cells following several apoptotic signaling pathways (extrinsic and Intrinsic), where tumor suppressor protein p53 and anti-apoptotic proteins Bcl-2 (Bcl-XL, Bcl-W, etc.) are very much associated with initiating the apoptotic process (Belfiore et al.2018).

In contrast, caspase activation (majorly caspase-3, caspase-8, and caspase-9) and breakdown or damage in DNA irreversibly play a central role for promoting apoptosis the tumor cells (Shendge et al.2021). Here, the noticeable advanced apoptotic event of arresting p53-mediated cell cycle propagation by apigenin delivered through Apt-NLCs was observed as compared with non-functionalized plain nanoliposomes. This suggests that the modified drug delivery system (Apt-NLCs) boosted apoptotic potentiality of apigenin through significant in vitro drug accumulation in the immunomodulatory Hep G2 cells by regulating corresponding signaling pathways irreversibly, thus, strongly check apoptotic precedence of Apt-NLCs over the nonconjugated nanoliposomes.

1.3. *In vivo* studies

Plasma and liver pharmacokinetic study

Amounts of apigenin in plasma and liver at predetermined time intervals were estimated after injecting free-apigenin, NLCs, PEG-NLCs, and Apt-NLCs (2 mg apigenin or equivalent dose of formulations /kg body weight) through tail vein, and the corresponding bio-samples were collected and analyzed using LC-MS/MS method (Faghihzadeh et al. 2016). After plotting the data (representing) graphically, the pharmacokinetic parameters were derived for the experimental nanoliposomes along with the free drug. (Figure 5.9.) All the nanoformulations maintained a steady drug plasma concentration up to 96 h in contrast with a negligible amount of free-apigenin in plasma at 48h. But there were significant improvements of plasma $t_{1/2}$ (~3.5fold), AUC (~2 fold), and MRT (~ 1.2-fold) values in PEG-NLCs and Apt-NLCs treated animals compared to NLCs treated animals were observed. Plasma drug clearance was reduced up to 50% in the case of both PEG-NLCs and Apt-NLCs compared to NLCs. Further, hepatic apigenin accumulation was highest in the case of Apt-NLCs, and it was increased by 1.2-fold and 3-fold compared to PEG-NLCs and NLCs, respectively. Elevated hepatic MRT value and reduced hepatic clearance (Table 5.5) in the case of Apt-NLCs compared to PEG-NLCs and NLCs strongly suggest a greater hepatic accumulation of apigenin when administered as an encapsulated form through a target-specific aptamer mediated-PEG containing drug delivery system.

In vivo, animal models play a vital role in understanding the drug-delivery features and their efficacy in the physiological systems (Park et al.2016). Here, in vivo pharmacokinetic

Table 5.5. Plasma and liver pharmacokinetics parameters in the HCC rats treated with NLCs/PEG-NLCs/Apt-NLCs and free apigenin as iv bolus form through tail vain.

		Api	NLCs	PEG-NLCs	Apt-NLCs
***in vivo* plasma pharmacokinetic data**	Cmax (ng/ml)	15.5± 0.16	17.2±1.13	18.1±0.55	19.2±0.51
	AUC last (ng/h/ml)	125.8±52	496.4±62.9	826.4±53#	986.2±47#
	AUMC	1608±1419	15290±3089	30457±2350	37988±1994
	MRT (h)	12.81±1.58	30.83±1.09	36.94±0.41$	38.91±0.21$
	AUC 0-∞ (ng.h/ml)	130.61±53.4	539.64±79.34	1072.47±171.21*	1446.21±182.65*
	T1/2 (h)	5.25±0.25	13.51±0.2	39.02±0.2	43.02±0.25
	Clearance (L/h/Kg)	15.9±0.55	4.03±0.63	2.42±0.66	2.03±0.52
in vivo liver pharmacokinetic data	Cmax (ng/ml)	160±7.87	165.7±7.37	178.9±8.14	223.4±6.64
	Tmax (h)	1±0.15	3±0.2	6±0.22	6±0.26
	AUC last (ng.h/ml)	1828±194	4336±474	9410±553	14658±592
	AUMC	25535±3589	119549±17352	330328±2126*	582753±21258*
	MRT (h)	13.2±3.8	27.55±6.1	35.48±5.9	42±4.6
	AUC 0-∞ (ng.h/ml)	1898±197	6440±542	8961±705	12955±892
	T1/2 (h)	5.65±0.52	16±1.2	34±2	50±1.45
	Clearance (L/h/Kg)	1.01±0.01	0.461±0.033	0.213±0.006^	0.136±0.005^

Data from the three independent experiments denote mean ± standard deviation (n=3).

#, $ indicated significant ($p < 0.05$) improvement in AUC and MRT values in plasma for Apt-NLCs/PEG-NLCs treated animals in comparison to NLCs. (*) Indicated significant ($p < 0.05$) improvement in AUC in liver values in Apt-NLCs/PEG-NLCs treated animals in comparison to NLCs. ^ indicated significant ($p < 0.05$) reduction in hepatic clearance in Apt-NLCs/PEG-NLCs treated animals in comparison to NLCs.

evaluation of the nanoliposomes (NLCs/PEG-NLCs/Apt-NLCs) in HCC induced experimental animals (SD, rats) revealed Apt-NLCs carried the highest Plasma half lifr (t1/2) and AUC. Apt-NLCs also exhibited maximum hepatic drug deposition over more extended periods with the highest MRT values as compared with the nonconjugated nanoliposomes NLCs/PEG-NLCs. They endorsed the lowest drug clearance in HCC positive experimental rats. All these superior pharmacokinetics parameters directed prolong and optimum drug bioavailability for apigenin in system, as because PEGylated nanoliposomes might improve the circulation time and sustainability on tumor surrounding areas (Batra et al.2012).

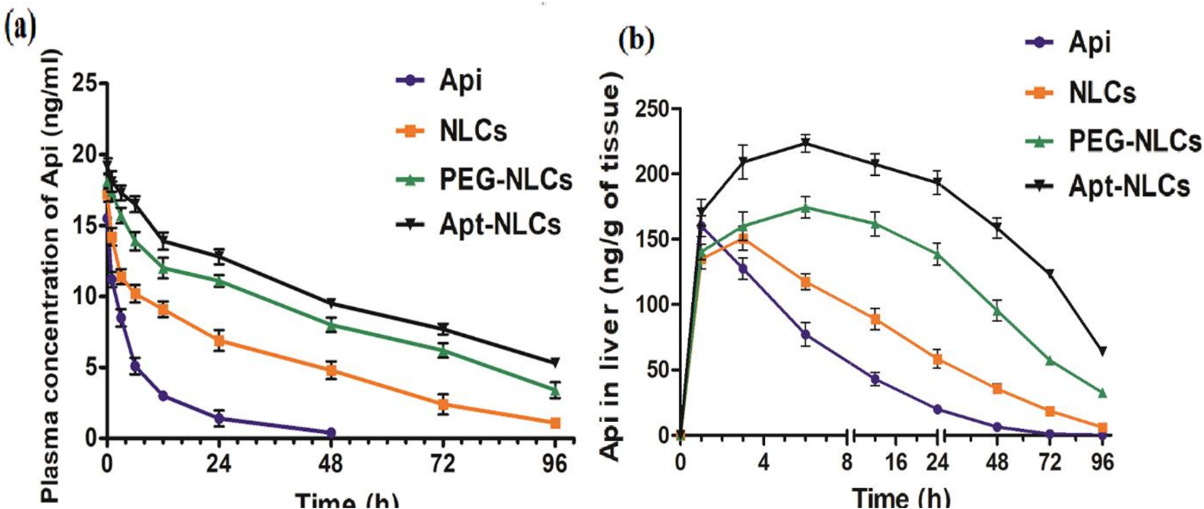


Figure 5.9. Blood and tissue drug levels, gamma-scintigraphy images, and presence of formulations in the hepatic cancerous tissues (a) Plasma concentration of apigenin vs. time, upon iv administration of NLCs, PEG-NLCs and Apt-NLCs in HCC induced rats, respectively (Data from the three independent experiments show mean ± standard deviation (n = 3 in); (b) liver concentration of apigenin vs. time curve, upon iv administration of NLCs, PEG-NLCs and Apt-NLCs in HCC induced rats, respectively.

Drug biodistribution study by γ-scintigraphy

After injecting ^{99m}Tc-NLCs/^{99m}Tc-PEG-NLCs/ ^{99m}Tc-Apt-NLCs in the carcinogen-treated animals, radiolabeled nanoliposomes accumulation were visualized through γ-scintigraphy at 4h and 8h of post-injection with estimating bio-distribution of the different experimental nanoliposomes among the different organs of the body. In γ- scintigraphy images, the radio signals were visible in most of the peritoneal region in animals treated with the experimental nanoliposomes, NLCs sand PEG-NLCs, at 4h, although exclusive and maximum hepatic signals were initiated in Apt-NLCs treated animals (60.69 ± 1.63% ID/g in liver tissue) (Table

5.6, Figure 5.10). Further, at 8h, a time-dependent drug distribution pattern was observed. Clear and predominant hepatic restricted signals were noticed in Apt-NLCs treated animals (49.51 % ID/g of tissue) (Table 5.6) whereas, NLCs/PEG-NLCs offered indistinct drug accumulation (18.89%ID/g and 33.078 % ID /g of tissue, respectively) in liver, along with considerable detectable signals in stomach, lung, kidney and to the other adjacent tissues of the body (Table 5.6). This could mention selective hepatic accumulations of aptamer functionalized nanoliposomes over nonconjugated formulations *in vivo*, most likely after following the receptor-mediated uptake of Apt-NLCs through the judicious attachment of aptamer, AS1411 to the target oncogenic biosensor nucleolin receptor overexpressed on neoplastic hepatocytes of the experimental HCC induced rats.

Table 5.6. Results of biodistribution of experimental formulations.

Organ/Tissues	Biodistribution of ^{99m}Tc-labeled experimental nanoliposomes in HCC induced rat model					
	^{99m}Tc-NLCs		**^{99m}Tc-PEG-NLCs**		**^{99m}Tc-Apt-NLCs**	
	4h	**8h**	**4h**	**8h**	**4h**	**8h**
Heart	0.587±0.043	0.347±0.065	0.489±0.047	0.309±0.028	0.199±0.026	0.155±0.007
Blood	2.538±0.067	1.484±0.091	5.8973±0.53	2.369±0.366	4.392±0.201	2.523±0.115
Liver	**37.234±0.33**	**18.899±1.25**	**47.686±1.92$^{\#}$**	**33.078±1.06**	**60.69± 1.63***	**49.51±0.478^**
Lung	3.778±0.209	2.554±0.411	2.502±0.295	2.678±0.135	1.667±0.349	2.319±0.260
Spleen	0.627±0.033	0.545±0.036	0.833±0.047	0.633±0.106	0.724±0.052	0.446±0.042
Muscle	0.163±0.013	0.142±0.013	0.1893±0.01	0.172±0.022	0.118±0.024	0.156±0.011
Intestine	13.902±0.29	8.988±0.932	17.974±0.61	14.27±0.249	5.828±0.074	3.651±0.048
Stomach	0.487±0.018	0.342±0.028	0.653±0.039	0.355±0.012	0.387±0.016	0.308±0.032
Kidney	10.033±0.71	4.461±0.465	4.475±0.276	3.254±0.127	3.631±0.406	2.591±0.268
Urine	15.616±0.14	14.706±0.24	10.702±0.82	9.755±0.769	6.929±0.851	5.654±0.650

Data are expressed in % mean of injected dose (ID) per gram of organ/tissue ± SD (n=3).

(#) Indicated significant ($p < 0.05$) amount of drug accumulation in liver in PEG-NLCs treated Gr over NLCs, while (*) indicated significant ($p < 0.05$) amount of drug accumulation in liver in Apt-PEG-NLCs treated Gr over PEG-NLCs Gr. at 4hr. (^) indicated significant ($p < 0.05$) amount of drug accumulation in liver in Apt-PEG-NLCs treated Gr over PEG-NLCs at 8hr.

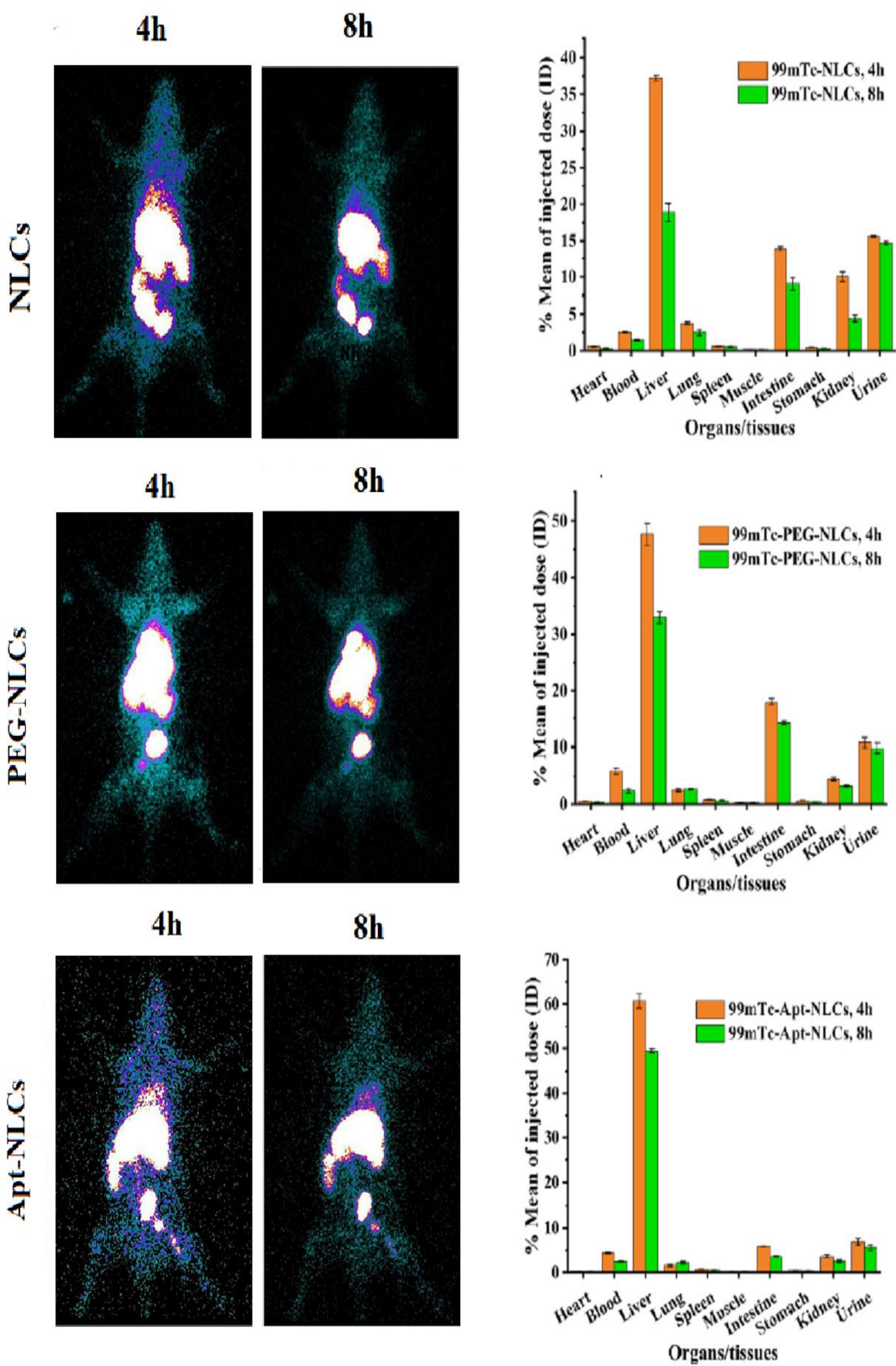


Figure 5.10. γ Scintigraphy imaginings of HCC rats at 4h and 8h after injecting 99mTc-labele– (NLCs, PEG-NLCs and Apt-NLCs) through venous cannulation process.

All these data indicated the rationally designed aptamer (AS1411) functionalized PEGylated nanoliposomes offered significant drug retention (Apt-NLCs > PEG-NLCs/ NLCs) selectively in the carcinogenic liver due to judicial uptake of aptamer (AS1411) conjugated nanoliposomes (Apt-NLCs) by the neoplastic hepatocytes. Most likely, it followed aptamer (AS1411) sensitive biomarker nucleolin receptor-mediated cellular uptake. Thus, synergic effect of precise drug accumulation and their sustainability in neoplastic hepatic tissues reinforced dominance of Apt-NLCs as finest as well as promising drug delivery vehicles in in vivo.

Intratumor deposition of FITC-labeled nanoliposomes

We investigated the neoplastic hepatic area after injecting FITC -labeled nanoliposomes into HCC positive animals to compare the uptake of different test nanoliposomes within the neoplastic tissue. We visualized a distinctive higher fluorescence in the neoplastic hepatic tissue of the apt-NLCs injected animals at different time points (Figure 5.11). At the same time, much less localized fluorescence was observed in tumors treated with the other nanoformulations than Apt-NLCs in HCC animals. It suggests the superiority and specificity of AS1411 conjugated drug delivery towards the HCC tumors.

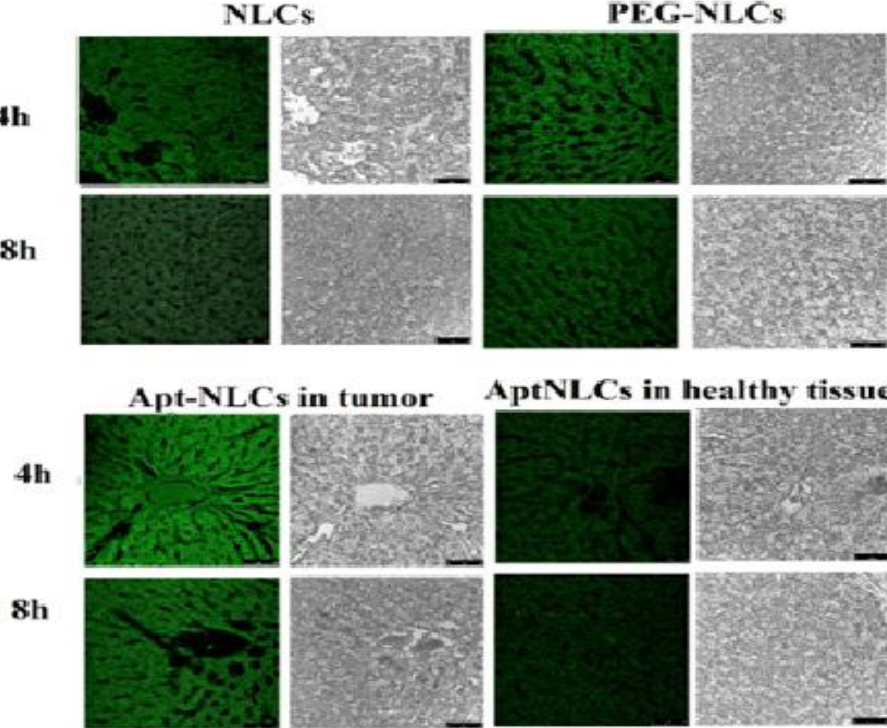


Figure 5.11. Confocal microscopic observation of tumour tissues sections of carcinogenetic rats upon the treatment of FITC-labelled – (NLCs, PEG-NLCs, and Apt-NLCs) at4h and 8 h of administration of iv injection.

Macroscopic and microscopic examinations of liver of rats treated with experimental nanoliposomes

One or multiple tumorigenic hyperplastic nodules (HN) with different sized of an average tumor volume were developed in experimental animals at different point of time receiving carcinogen and various drug formulations. Initially, before treating with test nanoliposomes, we checked the tumor incidences among various animal groups randomly. Further, on post treatment, we observed Gr-B animals (HCC control) showed enormous tumorigenic growth after following the whole treatment protocol (Figure 5.12). There was no gross improvement of tumor suppression noticed upon free apigenin treatment in Gr C animals. In the case of nonconjugated nanoliposome (NLCs/PEG-NLCs) treated groups (Gr D & E), almost 50-60% reduction in average tumor volume was detected in comparison to control group. But a significant diminishing event in tumor incidences (>90%) in the case of Gr-F animals were noticed comparing to others treated groups. The carcinogen-treated animals (Gr F) received Apt-NLCs comprised some tiny tumor lesions with an average size of <50 $mm^{3,}$ confirmed the superior therapeutic potential of Apt-NLCs as a commanding anticancer formulation.

We have done an extensive microscopic examination of hepatic tissues collected from all the treatment group animals at the end of the treatment protocol. In histopathological images (Figure 5.12.), we observed characteristic HAF lesions with lobular or spongy inflammatory ducts in the case of Gr B, carcinogen control animals. However, upon anticancer treatment with Api/NLCs/PEG-NLCs/Apt-NLCs, various degrees of improvement in reforming typical hepatocellular architecture were observed. The appearance of scattered apoptotic artifacts in the case of group D & E animals was initiated. Although, in the case of Group F animals, Apt-NLCs promoted restructuring the cellular panache almost towards normal hepatocyte. The highest degree of reduction in hepatic altered focal lesions (HAF) occurrence (Figure 5.12) was observed in the case of Apt-NLCs (Gr F), along with significant reduction of tumor volume as well as HAF area (Table 5.7) as compared to NLCs/PEG-NLCs (Gr D & Gr E) treated experimental animals also supported the optimum curative potential of aptamer-conjugated nanoliposomes rather than other normally developed nonconjugated nanoformulations (NLCs/PEG-NLCs).

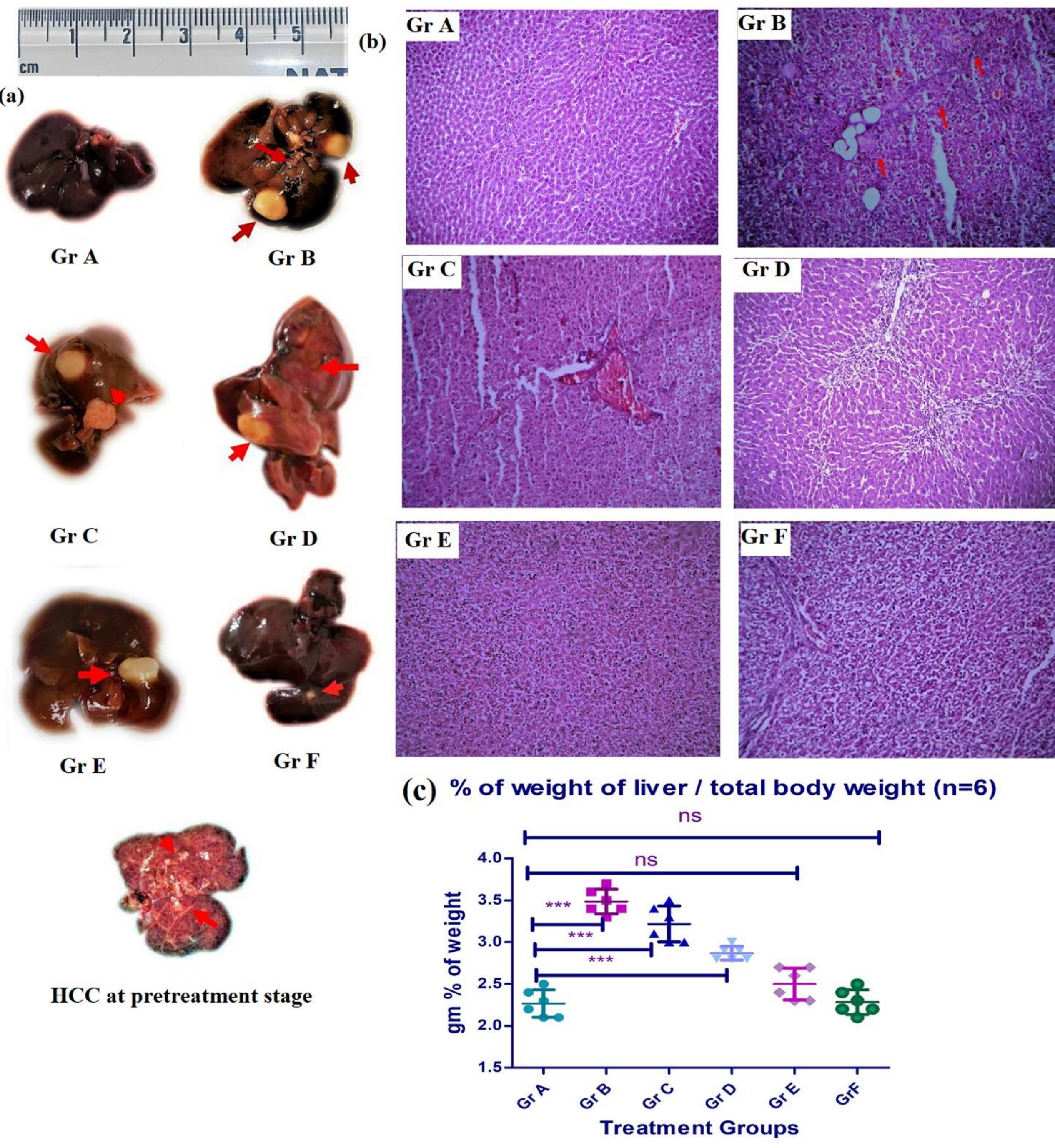


Figure 5.12. Microscopic and macroscopic hepatic analysis of the experimental rats (a) Macroscopic (in 100X magnification) liver images for the different experimental animal groups, Gr A to Gr F. (b) H & E histopathological microscopic images of liver tissue sections for various animal groups. (c) Represented % of weight of liver vs total body weight in different treatment group. (***) represent significant ($p < 0.05$) changes among group as indicated in figure and ns denoted as non-significant changes.

Table 5.7. quantitative data referring tumor development and total HAF (hepatic altered foci) area among different group of experimental animals

Treatment Groups	Number of the rat developed tumour post treatment	Average tumour volume(n=6) $(mm)^3$	Number of HAF area per cm^2 under microscopic observation on treatment
Gr A	**0/6**	**-**	**-**
Gr B	**6/6**	**1502.89 ± 9.98$^{\#}$**	**86.74 ± 5.90$^{\#}$**
Gr C	**6/6**	**1261.5 8± 8.09$^{\#\$}$**	**67.89 ± 8.09$^{\#\wedge}$**
Gr D	**5/6**	**821.08 ± 4.67$^{\#\$}$**	**56.89 ± 6.06$^{\#\wedge ns}$**
Gr E	**3/6**	**786.73 ± 5.56$^{\#\$ns}$**	**49.97 ± 0.58$^{\#\wedge **}$**
Gr F	**2/6**	**35.89 ± 7.56$^{\#\$***}$**	**12.63 ± 0.43$^{\#\wedge ***}$**
Gr G	**0/6**	**-**	**-**

 # Data represented mean ± SD (where, n=6 in each group of animals)

$ and ^ indicated significant ($p < 0.05$) reduction of tumor volume and HAF observed in (Gr C-Gr F) in comparison to positive control group Gr- B. Again, compared among the test nanoliposome (Gr D, Gr E, and Gr F), in Gr F (aptamer conjugated nanoliposome treated group) significant ($p < 0.05$) reduction of both tumor volume and HAF were observed mentioned in figure (***); while in Gr E (PEG-NLCs treated group showed non-significant (ns) amount of tumor volume reduction, (**) significant amount of HAF reduction and Gr D (NLCs treated animal group) showed non-significant (ns) amount of HAF reduction.

Assaying apoptotic related signaling proteins in the experimental rats

Histograms (Figure 5.13) representation from the data generated through RT-PCR assay with liver tissue samples obtained from different experimental group of animals expressed the maximal upregulation of p53, caspase-3 activation, and the highest downregulation in Bcl-2 in carcinogen-induced rats treated with Apt-NLCs (functionalized nanoliposomes), showed the highest degree of apoptosis caused by Apt-NLCs in comparison to non-conjugated nanoliposomes (NLCs/PEG-NLCs).

Assaying hepatic functionality in different groups of experimental animals

Serum AST, ALT, and ALP levels generally indicate normal or pathological conditions of the liver [27]. Here, serum AST, ALT, and ALT were increased in carcinogen-treated (carcinogen control) rats compared to the normal rats. Otherwise, the levels were decreased upon treatment of NLCs/Peg-NLCs and Apt-NLCs. (Table-5.8). The data showed the highest level of improvement in Gr F animals (Apt-NLCs treated carcinogenic animals). Moreover, no significant changes in normal serum AST, ALT, and ALP values in Gr G animals (normal animals treated with Apt-NLCs).

Higher bioavailability and intratumor drug delivery by an aptamer conjugated nanoliposomes, Apt-NLCs, in experimental animals predicted the greater anticancer potential of apigenin than other plain nanoliposomes (NLCs/Apt-NLCs). Likewise, predominant, and significant therapeutic progress was observed in HCC developed rats treated with Apt-NLCs by controlling tumor incidences in the liver and restoring HAF towards normal in H&E-stained liver tissue sections over the nonconjugated nanoformulations and free apigenin. Further, in vivo apoptosis-related protein expression estimation among the different groups of experimental animals using qRT-PCR revealed the highest activities in caspase-3, caspase-9, p53, and the lowest activity in Bcl-2 in Apt-NLCs treated animals (Figure 8). There were not many changes observed in liver functionality (ALT, AST, alkaline phosphate) in Apt-NLCs treated in normal animals. The findings, thus, support the remarkable opportunity of aptamer functionalized PEG-containing nanoliposomes (Apt-NLCs) as a nontoxic potent drug delivery system that could deliver chemotherapeutics, importantly active bio-flavonoids (here apigenin) to the target neoplastic hepatic region.

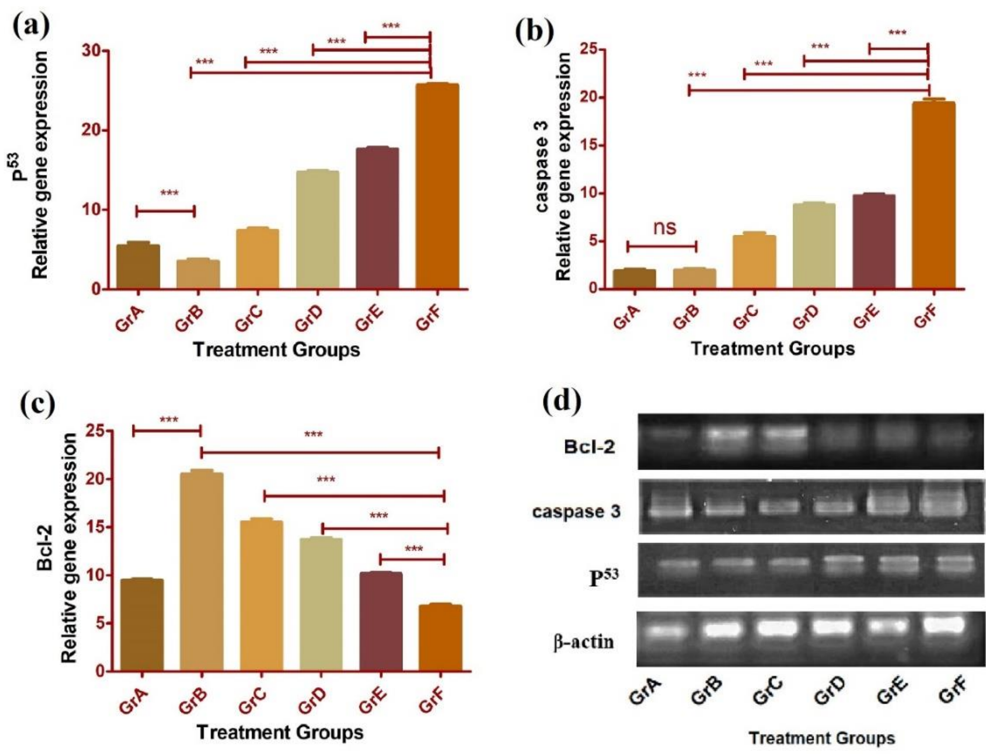


Figure 5.13. Comparative apoptotic gene expression analysis through RT-PCR with liver tissue samples from different experiential groups of animals (A-F). (a) Representing increases level of p53, (b) caspase-3, and (c) decreased level of Bcl-2 expression. Data indicated ± SD (n=3), (***) expressed significant ($p < 0.05$) upregulation or downregulation in gene, in conjugated nanoliposomes Apt-NLCs treated animals (GR F), samples in comparison to non-conjugated NLCs/ PEG-NLCs nanoliposomes (Gr D/F), free apigenin (Gr C) and carcinogen positive animals (Gr B). ns represented no significant changes among Gr A and Gr B for caspase expression assay. (d) Representing β- actin, p53, caspase -3, Bcl-2 gene expression of different experiential groups of animals in agarose gel electrophoresis study.

Table-5.8.: Assaying hepatic functionality in different groups of experimental animals:

Treatment groups	Hepatic enzyme parameters		
	AST (IU/L)	ALT (IU/L)	ALP (KA units)
Group A	75± 1.67#	26± 1.43#	60± 2.19#
Group B	182± 1.39#	68± 2.24#	154± 1.33#
Group C	167± 2.27#	53± 1.36#	139± 1.59#
Group D	132± 1.51#	41± 2.29#	117± 1.73#
Group E	129± 1.71#	34± 1.62#	98± 1.87#
Group F	72± 2.19#	25± 1.87#	59± 2.35#
Group G	69± 1.32#	22± 1.53#	54± 1.74#

Note: Each value represents mean ± SD (n=3)

Table-5.8.: Assaying hepatic functionality in different groups of experimental animals:
 # Data represented mean ± SD (where, n=6 in each group of animals). Table depicted respective AST/ALT/ALP values in all the experimental carcinogenetic animal groups treated with apigenin (Gr C), plain nanoliposomes (Gr D), PEGylated nanoliposomes (Gr E), aptamer conjugated PEGylated nanoliposomes (Gr F) along with normal animals treated with normal saline (Gr A) and normal animal treated with aptamer conjugated nanoliposomes.

Chapter 6
Conclusion

Conclusion

Achievement of any chemotherapeutic or any bioactive compounds in cancer therapy depends on them effective and rational drug delivery approach. Bioflavonoids, as a lipophilic anticancer agent, must overcome robust pharmacokinetic as well as molecular drug delivery challenges in HCC. In our present study, we have developed aptamer functionalized nanoliposomes and highlighted first time the mechanistic approach of drug uptake, accumulation, and modulation in apoptotic signalling pathways for apigenin in neoplastic hepatic cells, which was triggered by reasonably designed aptamer functionalized PEG-containing nanoliposomes. Phosphorothioated amino-modified AS1411 aptamer-conjugated apigenin-loaded PEG-NLCs successfully induced apoptosis in Hep G2 liver cancer cells and arrested cell-cycle mostly at G2/M phase by upregulation in p53 and caspase activities and downregulation of Bcl-2 activity. Accumulating the aptamer conjugated formulation (Apt-NLCs) was distinctively more in the liver than in the other tissues. Further, Apt-NLCs accumulation in tumours in the liver was predominantly greater than in the surrounding non-cancerous hepatic tissue, suggesting successful site-specific drug distribution. Apt-NLC reduced tumour incidences and neoplastic hepatic altered lesions, suggesting its potential anticancer effect in vivo. Apt-NLCs impressively targeted the neoplastic hepatic region in rats. Further, we can explore the functional efficacy of aptamer functionalized nanoliposomes in additional preclinical animal models for gathering more relevant scientific pieces of evidence. Thus, the process for translation from preclinical to clinical drug development will be narrow.

Chapter 7

Summary

Hepatocellular carcinoma (HCC) is one of the major causes of cancer related death across the globe. Even though progressive invention of some very potent therapeutics (like immunotherapy and molecular therapy) has been seen, the success is limited because of complex heterogeneous neoplastic behaviour of the malignant tumours in HCC with developing multi drug resistance (MDR), other adverse effects. Again, with a huge economic burden to the patient and their family. To combat these dynamic challenges researcher have been developed safe and sustainable alternative treatment strategies using phytochemicals as anticancer agent. Here, precise tumour targeting with ligand functionalized nano formulations have already catch immense consideration in delivering bio-therapeutics at the cancerous site of organ exclusively.

We have developed tumour-sensing phosphorothioate and amino-modified aptamer (AS1411)-conjugated stealth nanoliposomes, encapsulating with apigenin for precise and significant biodistribution of apigenin into the target tumour cell in HCC induced liver to exploit maximum bio-therapeutic assistances.

The stable aptamer functionalized PEGylated nanoliposomes (Apt-NLCs) having an average vesicle size of 100- 150 nm with a smooth surface and an intact lamellarity, as ensured by DLS, FESEM, AFM, Cryo-TEM reached in systemic circulation and showed immersive pharmacokinetic properties both in invitro and in vivo. Further, this study has specified in vitro process of optimum drug (apigenin) extrusion into the cancer cells by nucleolin receptor mediated cellular internalization, when delivered through modified AS1411 functionalized PEGylated nanoliposomes and ensured irreversible DNA damage in HCC. Significant improvement in cancer cell apoptosis in animal model, due to reduced clearance and higher intratumor drug accumulation along with almost nominal toxic effect in liver strongly supports the therapeutic potentiality of aptamer-conjugated PEGylated nanoliposomes compared to the nonconjugated formulations in HCC. The study has established a robust superiority of modified AS1411 functionalized PEGylated nanoliposomes as an alternative drug delivery approach with momentous reduction of HCC tumour incidences.

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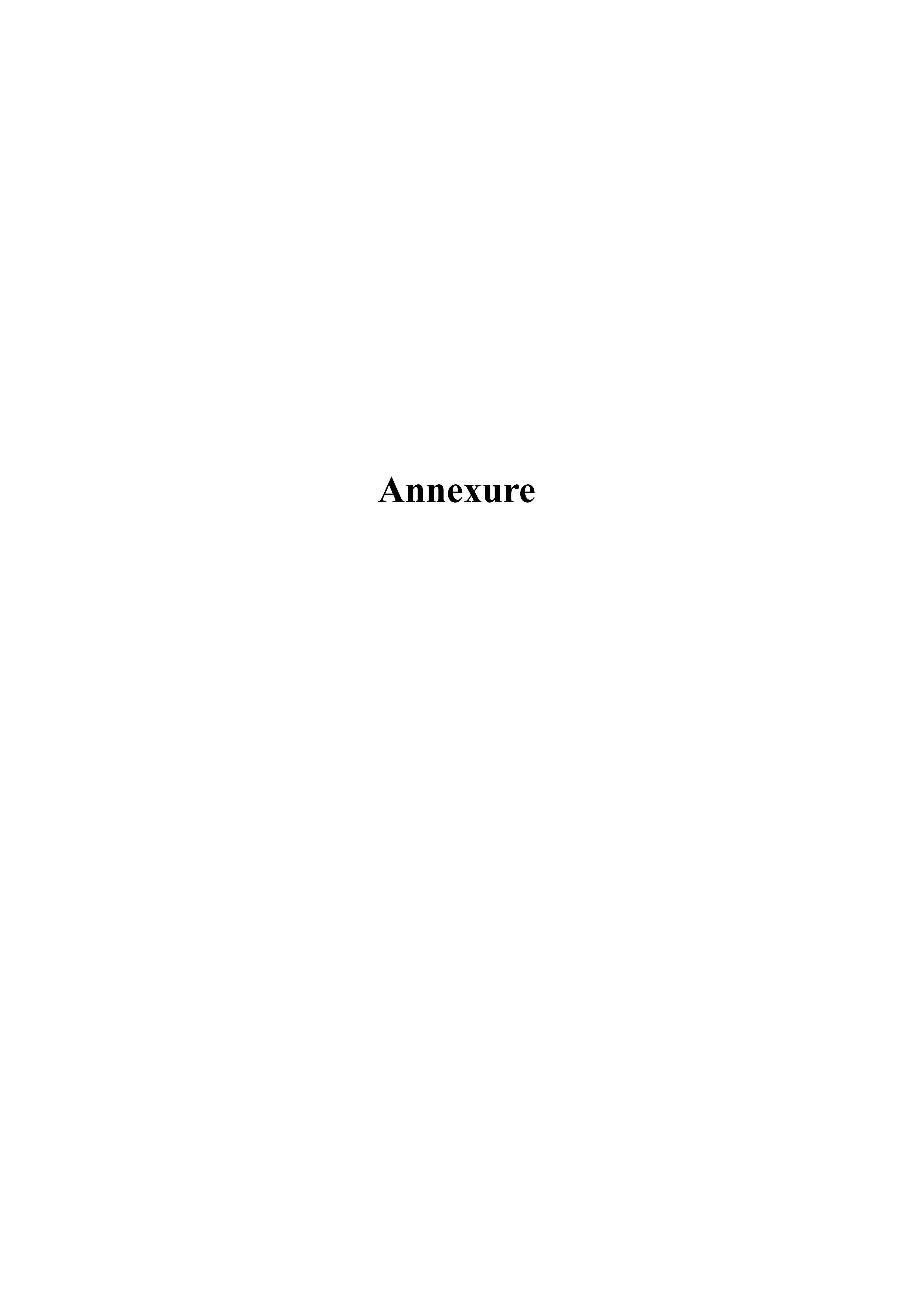

Annexure

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Dept. of Pharmaceutical,
Jadavpur University,
Kolkata-700032

Subject:- Award of Research Fellowship to **Mrs. Moumita Dhara,** SRF on the Research fellowship project entitled ***"Development of apigenin loaded ligand conjugated nanoliposomes to target liver cancer cell"***.

Sir,

The Director General, ICMR sanctions Research Fellowship to to **Mrs. Moumita Dhara, SRF** on a stipend of **Rs. 28,000/-** p.m. to carry out research on the project mentioned above, under your guidance. H.R.A. and Medical reimbursement will be paid as per rules of your University.

The award of SRF will be subject to the following terms and condition:

TENURE: It will be tenable for one year only from the date of joining duty and will be on yearly basis subject to maximum of **two years.**

Its continuance will, however, depend on the satisfactory progress of work and can be terminated at any time on a one month's notice, if the progress is not satisfactory, or on receiving adverse report from the Guide. The Fellow will be required to work on the project for a period at least one year.

The event of his/her leaving before completing one year on the fellowship, he/she may be required to refund the stipend drawn by him/her from the date of joining to the date of leaving the fellowship.

PRIVATE PRACTICE: Private practice of any kind, or taking up any appointment even in an honorary capacity during the fellowship is not permitted.
ADMINISTRATIVE CONTROL: The candidate will be under the administrative control of the Institution where he/she works, and will also be subject to the rules and regulations of the Institute.
LEAVE: Leave will be admissible according to the rules of the Institution, however in the case of female research fellows leave without stipend upto 6 months (in lieu of maternity leave) may be granted. No other kind of leave (such as sick leave) etc. will be admissible. Awardees are not entitled to vacation normally admissible to the staff of an Institution.
HRA: HRA will only be paid, if the fellow is not availing any hostel facility. A certificate to this effect should be sent along with joining report for payment of HRA.
REPORTS: The awardee shall submit 1st annual reports for the first 10 months on the prescribed standard proforma.

The first annual report should be submitted after 10 months from the date of commencement of the fellowship giving complete factual details of the research work done through the Guide alongwith his/her appraisal. Subsequent annual report should be submitted through the Guide two months before the completion of fellowship tenure. Failure to submit reports in time may lead to termination of the award. Six copies of the final report in the prescribed form clearly shall be submitted one month before the date of termination of the award.

A list of the papers published or presented at Scientific Conferences during the tenure of the fellowship should also be furnished with the annual and final reports.

PUBLICATION OF PAPERS: Prior permission for publication of papers based on the research work done during the tenure of the award should be obtained from the Council. The paper should be sent to the Council through the Guide with his/her recommendations. Due acknowledgement to the Council should be made in these papers.

PAYMENT OF FUNDS: The stipend and the funds for contingencies shall be paid as per procedure laid down in the enclosed an annexure.

CONTINGENT EXPENDITURE: An annual contingent grant of Rs. 20000/- p.a. will be admissible. The contingent grant is given to meet petty expenditure for purchase of chemicals, reagents etc. No non-expenditure article or equipment can be purchased out of the grant.

TRAVEL:-

Traveling allowance will not be admissible for joining duty or on termination of the award.

The Council may approve tours of research fellows/associate for:-

1. **Attending symposium/seminar/conference provided the fellow/associate is presenting a paper which has been accepted by the organizers of the symposium/seminar/conference.**
2. **Field work connected with research**
3. **TA/DA would be admissible as per the rules applicable to Central Government Officers with basic pay equivalent to the amount of the fellowship stipend.**

NOTE:- **The expenditure on this account will be met**

POST FELOWSHIP CARRIER:-

1. The Research Fellow can register himself/herself for postgraduate qualification and to utilize in his/her the work done by him/her during his/her fellowship tenure. A copy of these submitted for postgraduate degree will have to be sent to the Council for information and record **from the contingent grant sanctioned to the fellow.** Due acknowledgement to the ICMR should be made in the thesis by the research fellows.
2. The Research Fellow should also send to the Council for information a brief report on the post/job taken by him/her after the expiry of the fellowship.

The date indication forenoon/afternoon on which he/she the fellowship may please be intimated to this office. He/she may be asked to report for duty within a month from the date of issue of this letter failing which the award will be treated as cancelled.

Yours faithfully,

(G.S.Sandhu)
Administrative Officer
For Director-General

Copy to :- , The Registrar, Dept. of Pharmaceutical Technology, Jadavpur Univeristy, Kolkata-700032.
2 Mrs. Moumita Dhara, Dept. of Pharmaceutical Technology, Jadavpur University, Kolkata-700032.
3 Accounts Section – V, ICMR
4 IRIS Cell No. 2017-4038

Administrative Officer
For Director-General

যাদবপুর বিশ্ববিদ্যালয়
কলকাতা-৭০০০৩২, ভারত

*JADAVPUR UNIVERSITY
KOLKATA-700 032, INDIA

By Speed Post

Ref. No P-1/RS/107/16
Dated: 17/06/2016
২১.

To
Smt. Moumita Phauzdar
21/4, Kanrarpukur Lane
Howrah - 1

Madam ,

I am pleased to inform you that you have been selected as **Senior Research Fellow** to work in the **Department of Pharmaceutical Technology** under State Government Fellowships Scheme of this University.

You will be paid a Fellowship of **Rs. 20,000/= (fixed) per month**. The tenure of your fellowship is initially for two years provided you work satisfactory and exhibit good conduct.

You shall have to register your name **for the Ph.D Degree within one year from the date of your joining** the post and the registration documents to be submitted to the Research Section. You have to take classes up to six periods a week. You have to serve one month prior notice before submission of registration.

Your service will be governed by the same terms and conditions of the University Service Rules as may be applicable to the temporary staff of the university and you will be under the administrative control of undersigned.

You are requested to join the post within ten days from the date of receipt of this letter and to submit your joining report through proper channel to the undersigned with a declaration stating that you are not a recipient of any emoluments from any other source from the date of your joining the university fellowship.

The duplicate copy of this appointment letter duly signed by you should also be submitted at the time of joining.

Yours faithfully,

[signature] 17/6/16
Registrar

*Established on and from 24th December, 1955 vide Notification No.10986-Edn/IU-42/55 dated 6th December, 1955 under Jadavpur University Ac
(West Bengal Act XXXIII of 1955) followed by Jadavpur University Act,1981 (West Bengal Act XXIV of 1981)

দূরভাষ: ২৪১৪-৬৬৬৬/৬১৯৪/৬৬৪৩/৬৪৯৫/৬৪৪৩
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Dr. Biswajit Mukherjee

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To,
Prof. Biswajit Mukherjee,
Dept. of. Pharm. Tech.
Jadavpur University,
Kolkata-32, West Bengal.

Project title: Development of Apigenin Loaded Ligand Conjugated Nanolioposomes to Target Liver Cancer.

Ref No. AEC/PHARM/1502/04/2015/2018(renewed)
Date: 30.07.2018

Dear Sir/Madam,
As per the meeting of Animal Ethics Committee (AEC), Jadavpur University. I am pleased to inform that the AEC, Jadavpur University, has approved your project for fresh/renewal applied to the respective agencies.
However, you are requested to abide by the following guidelines:

1) All the animal experiments should be conducted strictly following the guidelines of CPCSEA (Govt. of India) and the UGC, New Delhi.
2) Animals are to be purchased from registered breeder only and record should be maintained and required to be submitted do the convenor,AEC, Jadavpur University.
3) Details of animal's house and sacrifice of animals being carried out should be given to the AEC time to time.

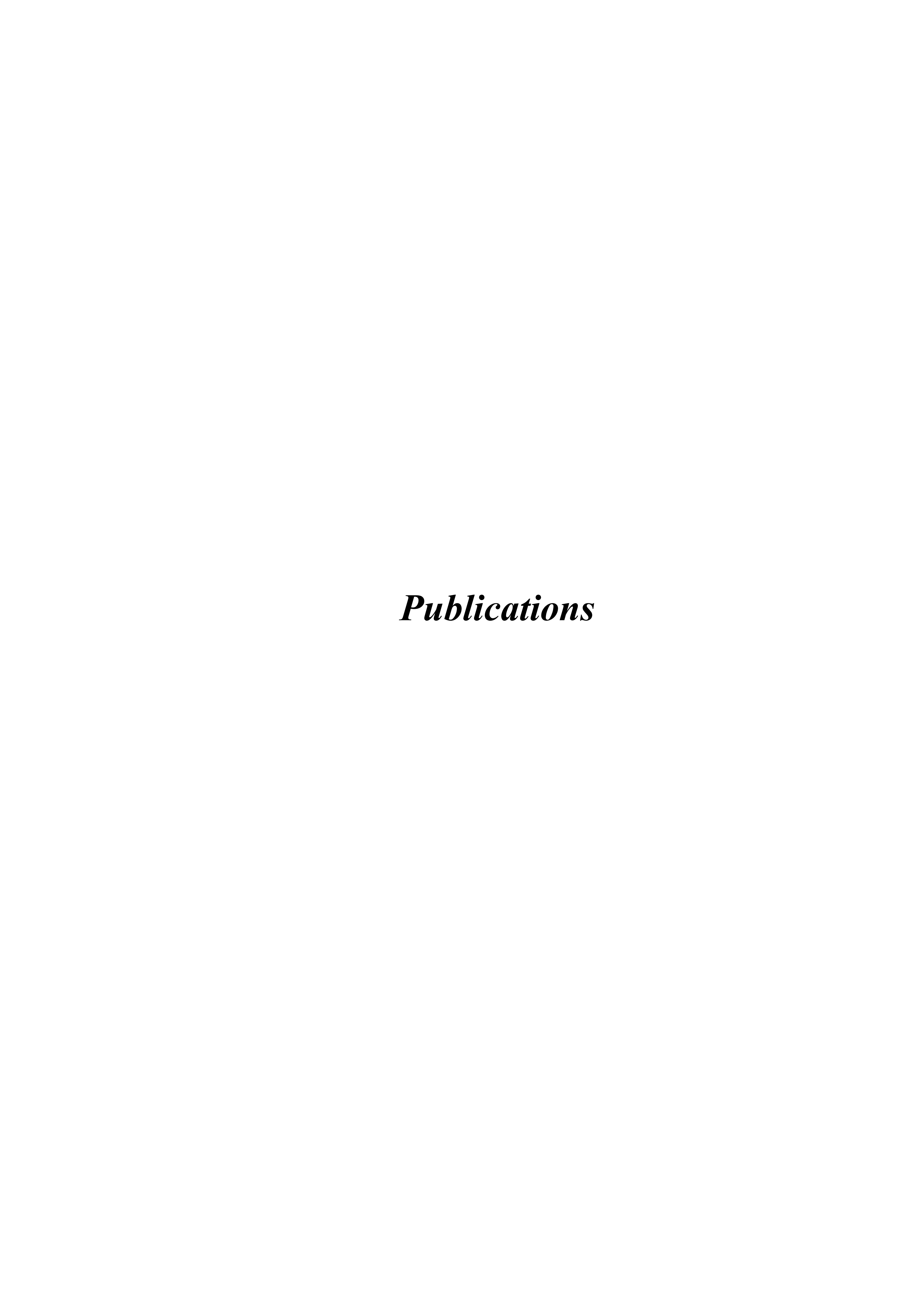
Furthermore, you are requested to submit the project no. to the AEC once the project is sanctioned. Moreover, you are requested to note further that the expenditure regarding maintenance of the animals has to be made from your project only. Special attention should be provided to prohibit to unlawful cruelty to the animals.

Thanking You
Yours sincerely,

30/07/2018

(Dr. Biswajit Mukherjee)

Chairman
Institutional Animal Ethics Committee
Jadavpur University
Kolkata-700032



Publications

Journal of Nanobiotechnology

RESEARCH

Open Access

Phosphorothioated amino-AS1411 aptamer functionalized stealth nanoliposome accelerates bio-therapeutic threshold of apigenin in neoplastic rat liver: a mechanistic approach

Moumita Dhara[1], Ashique Al Hoque[1,2], Ramkrishna Sen[1], Debasmita Dutta[3,4], Biswajit Mukherjee[1*], Brahamacharry Paul[1] and Soumik Laha[5]

Abstract

Hepatocellular carcinoma (HCC) is a leading cause of death globally. Even though the progressive invention of some very potent therapeutics has been seen, the success is limited due to the chemotherapeutic resistance and recurrence in HCC. Advanced targeted treatment options like immunotherapy, molecular therapy or surface-engineered nanotherapeutics could offer the benefits here owing to drug resistance over tumor heterogenicity. We have developed tumor-sensing phosphorothioate and amino-modified aptamer (AS1411)-conjugated stealth nanoliposomes, encapsulating with apigenin for precise and significant biodistribution of apigenin into the target tumor to exploit maximum bio-therapeutic assistances. The stable aptamer functionalized PEGylated nanoliposomes (Apt-NLCs) had an average vesicle size of 100–150 nm, a smooth surface, and an intact lamellarity, as ensured by DLS, FESEM, AFM, and Cryo-TEM. This study has specified in vitro process of optimum drug (apigenin) extrusion into the cancer cells by nucleolin receptor-mediated cellular internalization when delivered through modified AS1411 functionalized PEGylated nanoliposomes and ensured irreversible DNA damage in HCC. Significant improvement in cancer cell apoptosis in animal models, due to reduced clearance and higher intratumor drug accumulation along with almost nominal toxic effect in liver, strongly supports the therapeutic potential of aptamer-conjugated PEGylated nanoliposomes compared to the nonconjugated formulations in HCC. The study has established a robust superiority of modified AS1411 functionalized PEGylated nanoliposomes as an alternative drug delivery approach with momentous reduction of HCC tumor incidences.

Keywords Aptamer, Apigenin, Stealth nanoliposomes, Intratumor drug accumulation, Apoptosis, Pharmacokinetics

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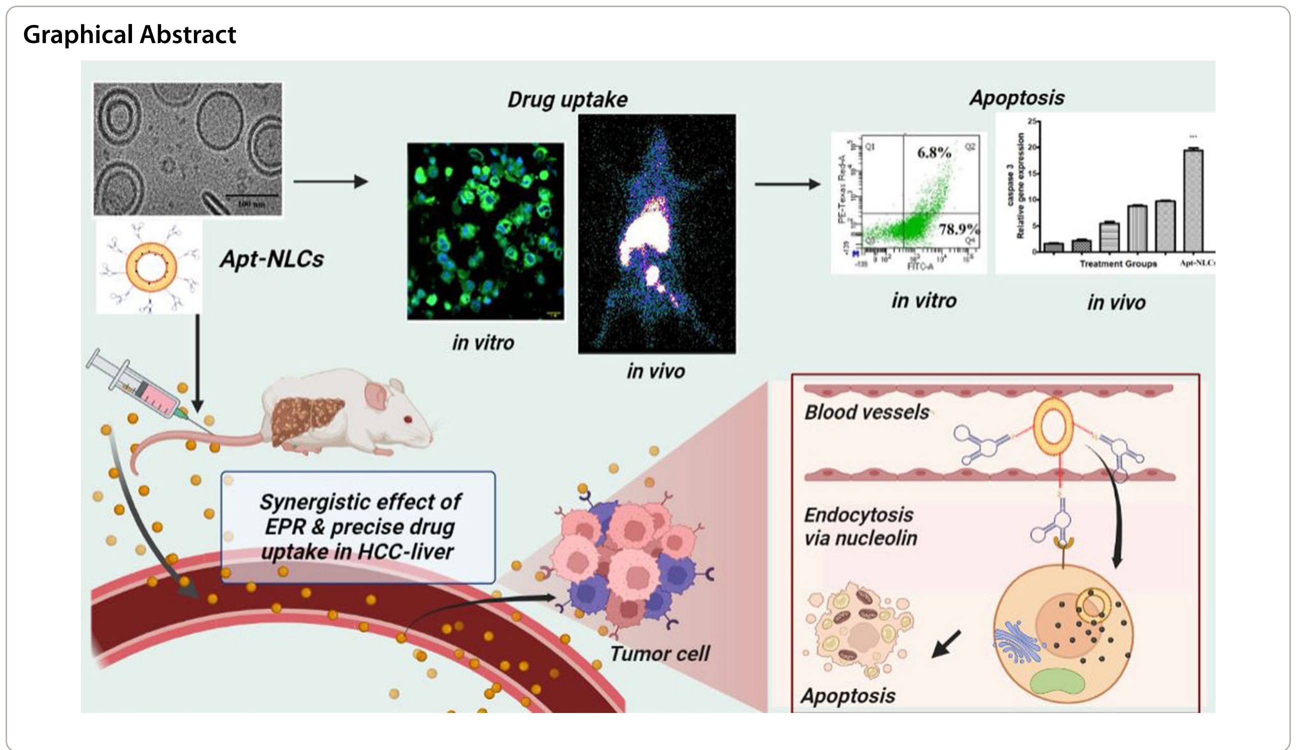
Graphical Abstract



Introduction

Despite some landmark development in chemotherapy, molecular or immunotherapy, treating or curing hepatocellular carcinoma (HCC) is still a global health issue, and the shocking fact is future estimated incidences of hepatic malignancy that could cross one million by 2025 [1]. The development of more and more potential, alternative, and cost-effective therapeutics is on-demand to combat this dynamic challenge. Naturally occurring flavonoids, such as apigenin, quercetin, and many having enormous potential as anticancer agents, have already been tried to formulate by encapsulating in nanoparticles owing to their poor solubility and bioavailability [2]. But in clinics, therapeutic challenges such as lack of specificity toward the target organ and desired curing response along with bare minimum side effects to the normal or healthy cells are yet to be adequately addressed [3]. Optimizing precise therapeutic accumulation to the target site with ligand-functionalized nanoformulations could minimize drug resistance and improve therapeutic tolerances [4]. Thus, it can accelerate their scope for preclinical to clinical translation [5]. Apigenin has been well demonstrated both in vitro and in vivo, promoting apoptosis, inducing cell cycle arrest, suppressing the cancer cell invasion along with its autophagy and immunogenic activities [6]. Apigenin-encapsulated nanoparticles were already applied effectively for delaying the progression of different types of cancer, including HCC [7]. However, along with refining solubility and bio-availability, precise delivery through ligand-functionalized lipid nanocarriers could improve the apoptotic potential of apigenin in a great extent [8]. Aptamer fabricated nano liposomal drug delivery is already in prime focus relating to advanced targeted drug delivery for cancer [9]. Further, apigenin-loaded nano liposomes modified with ligand-targeted drug delivery system have not been explored yet, for HCC. Formulation optimizations, the process of incremental biotherapeutic accumulation in tumor cells, and related apoptosis still need scientific clarity. We hypothesize that the therapeutic threshold of apigenin might be uniquely upgraded by precise, predictable, and effective drug accumulation to the target, cancerous hepatocytes, applying stable, aptamer functionalized PEGylated nanoliposomes.

AS1411 is a non-immunogenic, thermostable 26-mer guanine-rich DNA aptamer with quadruplex structural advantages, which provides exceptional affinity toward the nucleolin proteins overexpressed on the surface of HCC cells [10]. Customized phosphorothioate backbone modification in amino-modified DNA aptamer (AS1411) offers superior pharmacological stability to the aptamer and is used for the effective target-specific nano-drug delivery system [11]. DSPE-PEG is intended to provide a longer half-life in vivo with reduced clearance due to its stealthy coating over the nano liposomal surface [12].

Here, we have developed apigenin-encapsulated, PEGylated nanoliposomes functionalized with phosphorothioated amino-modified-AS1411 aptamer. We explored the process of incremental drug internalization vs apoptosis through dual functionalized aptamer conjugated PEG-nanoliposomes over pegylated (non-aptamer conjugated) or normal nanoliposomes for apigenin both in vivo and in vitro models. Finally, we concluded the therapeutic benefits and tried to establish the additional biological evidence mostly relevant to the preclinical or clinical research and outcome.

Experimental section

Materials

Apigenin was obtained from Sigma–Aldrich Lab. (MO, USA). Carboxymethyl-PEG2000-1,2-Distearoyl-sn-glycero-3-phosphoethanolamine or (DSPE-PEG2000-COOH) was purchased from Laysan Bio Inc. (AL, USA). 1-Ethyl-3-(3-dimethylamino propyl) carbodiimide, chloride (EDC, HCl), *N*-hydroxy succinimide (NHS), cholesterol, dimethyl sulfoxide, butylated hydroxytoluene (BHT), ethanol, chloroform, sodium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphate were procured from E Merck Specialties Ltd. (Mumbai, India). Fluorescein isothiocyanate (FITC) and soya-l-α-lecithin (SPC) were acquired from Hi-Media Lab. Pvt. Ltd. (Mumbai, India).

The 26-mer- AS1411 DNA aptamer, having sequence 5′-GT GGT GGT GGT TGT GGT GGT GGT GG-3′ (molecular weight: 8674.3 g/mole) was custom-synthesized as phosphorothioate backbone and 3′-amino-modified form by GCC Biotech (Kolkata, India).

Cell lines

All the in vitro cell-based studies were conducted on human liver cancer cell lines, Hep-G2/Huh-7 cell lines purchased from NCCS (National Centre for Cell Science, Pune, India). The cell lines were sub-cultured in DMEM media, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution, and maintained at 5% CO_2 containing humidified air at 37 °C.

Hep G2 is a well-differentiated immortal hepatocellular carcinoma cell line obtained from a Caucasian male aged 15 years [13]. The popularity of the use of Hep G2 cells for in vitro cellular study is related to the fact that it is primary liver cancer (well represented by hepatocellular carcinoma, HCC). Since Hep G2 cells have a high degree of morphological and functional differentiation in vitro, they are considered a suitable model for studying the intracellular trafficking and dynamics of various chemicals, including drugs, formulations, proteins, and ligand molecules in vitro [14]. Several studies reported Hep G2 as in vitro model for detecting cytoprotective, cytotoxic, genotoxic, and antigenotoxic agents in hepatocarcinogenesis and drug-targeting investigations [15, 16]. Thus, we selected Hep G2 cells for the in vitro investigation.

Huh 7 is also a well-differentiated hepatocellular carcinoma cell line, isolated from the tumor originated from liver hepatocytes, from a 57-year-old Japanese male.

Sprague Dawley (SD) rats for hepatocellular carcinoma (HCC) model

Sprague Dawley (SD) rats (120–150 g) were procured from NIN (National Institution of Nutrition), Hyderabad, India, and animal studies, conducted here were approved by the Institutional Animal Ethical Committee (AEC), Jadavpur University on submission of proper experimental protocol plan following guidelines of the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Pre-formulation studies (FTIR-Fourier-transform infrared spectrometry)

Fourier -transform infrared spectrometric (FTIR) analysis was applied to examine if there was any molecular interaction between the drug and the excipients used during the formulation development [17]. FTIR spectroscopy was carried out with the drug (pure apigenin, Api), drug excipients (SLY, CHC, DSPE-PEG, BHT) individually along with their physical mixture, formulated plain nanoliposome (NLCs) with or without the drug, PEG-NLCs and finally, aptamer conjugated PEGylated nanoliposomes, Apt-NLCs, within the wave number array of 4000–400 cm^{-1} under inert atmospheric conditioning KBr palate by an FTIR spectrophotometer (Bruker-(Alpha), Ettlingen, Germany) and were analyzed.

Preparation of apigenin (Api)-loaded nanoliposomes (NLCs, PEG-NLCs) and aptamer functionalized PEGylated Api-loaded nanoliposomes (Apt-NLCs)

Apigenin-encapsulated plain nanoliposomes (NLCs) and PEGylated NLCs were prepared by the thin-film hydration method [18]. For subsequent preparation of the aptamer functionalized PEGylated nanoliposomes (Apt-NLCs), we have used the PEGylated NLCs (PEG-NLCs). The coupling of aptamer on the liposomal surface was accomplished by a covalent attachment of the amino group of the aptamer (NH_2-modified AS1411) with the carboxyl group of PEG-DSPE-COOH in the PEG-NLCS [19]. Surface grafting of amino-modified AS1411 aptamer on the prepared pegylated NLCs was conducted through the activation carboxyl group of PEG component (PEGylated-1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (PEG-DSPE-COOH)). The pegylated NLCs were suspended in a mixture of 150 mM *N*-hydroxy succinimide (NHS) and 300 mM ethyl (dimethyl aminopropyl)

carbodiimide (EDC) at room temperature for 1 h by maintaining all through a neutral of 1 M Tris HCI buffer [19, 20]. The resulting solution was added to 25 µl aliquot from 100 µM of 3′amine-modified AS1411 aptamer stock solution (the final concentration of AS1411 in experimental nanoliposomes Apt-NLCs was 5 µM) [21]. Here, the portion of the sample aptamer solution was previously arranged by applying a denaturation–renaturation process so that it would interact in a biochemical reaction readily. The solution mixture was kept in a shaker incubator overnight at normal room temperature to accomplish aptamer ligation on the surface of PEGylated NLCs. Finally, we removed the excess and unconjugated aptamer from aptamer-conjugated nanoliposomes (Apt-NLCs) by centrifuging and washing the mixture thrice with nuclease-free deionized water and the aptamer conjugated formulations were stored at 4 °C for further analysis [22].

The blank aptamer conjugated liposomes (blank-Apt-NLCs) were also prepared following a similar process but without encapsulating apigenin in the liposomes. Both NLCs and Apt-NLCs were labeled with FITC (fluorescein isothiocyanate) dye by incorporating 10 µl of FITC (0.4% w/v ethanolic solution) into the organic phase during thin layer formation of liposome preparation [22].

Determination of aptamer conjugation by agarose gel electrophoresis

To determine the successful ligation of aptamer AS1411 with the PEGylated liposomes (PEG-NLCs), aptamer conjugated nanoliposome (Apt-NLCs), free aptamer (AS1411), and a commercial 50 bp DNA ladder were sequentially placed in the wells of previously prepared 1% agarose gel plate. Through running electrophoresis for 20 min at 100 V, we observed the migration pattern for different samples as mentioned above [22, 23]. Here, 0.5 mg/ml ethidium bromide was used in the agarose gel to visualize bands in the gel electrophoresis, and gel loading dye was used for staining DNA during the electrophoresis process. Finally, free DNA aptamer, DNA ladder, and the DNA-conjugated nanoformulations in gel plate were visualized using a UV transilluminator as reported earlier [24].

To determine the conjugation of AS1411 aptamer on to PEG-NLCs quantitatively, first we repetitively washed out (more than three times) the Apt-PEG-NLCs solution with nuclease free water to remove all the unconjugated free aptamer available in Apt-PEG-NLCs. Finally, with proper dilution adding nuclease free water to it, we compared the amount of AS1411 aptamer in aptamer conjugated nanoliposomes, Apt-PEG-NLCs along with 1 µl of a free AS1411 (DNA aptamer) and a plain PEGylated nanoliposomes, PEG-NLCs (without aptamer) by applying nanodrop UV spectrophotometric (nano 300 micro spectrophotometer, IGene Labserve Pvt Ltd. India) method [25, 26].

Percentage of drug loading and drug entrapment efficiency

To determine drug-loading, the accurately weight of lyophilized liposome (2 mg), NLCs/PEG-NLCs or Apt-NLCs was dissolved in 2 ml of ethanol-acetonitrile-dimethyl sulfoxide solvent mixture at a ratio of 0.5:1:1(v/v) as the best suit solvent composition for testing drug loading of the experimental formulations. It was determined by the trial-and-error method. The resulting solution was vortex-mixed for 1–2 h and then centrifuged for 20 min at 10,000 rpm, clear supernatant (1 ml) was collected to measure the absorbance intensity by the UV/VIS-spectrometer at the corresponding λ_{max} of apigenin 340 nm, and the drug content in the test liposomes were analyzed from the corresponding standard calibration curve prepared early [7, 18]. Thus, percentage drug-loading and drug encapsulation efficacy were obtained using the formulae mentioned below.

$$\%\ \text{Drug loading} = \left(\text{Amount of drug in liposome/Amount of liposome used}\right) \times 100 \tag{1}$$

$$\%\ \text{Yield} = \left(\text{Weight of dry powdered liposome/Total weight of all the components used in the formulation}\right) \times 100 \tag{2}$$

Particle size distribution & ζ-potential measurements

Particle size distribution and ζ-potential of NLCs, PEG-NLCs, and Apt-NLCs were analyzed by Malvern Zetasizer Nano-ZS 90; (Malvern Instruments, UK) applying dynamic light scattering (DLS) technique with sample suspension of the experimental liposomes prepared by diluting them properly, vortex-mixing and sonication

process. The results represented were the average particle size considering the standard deviation of at least three different batches of the experimental liposomes.

Field emission scanning electron microscopy (FESEM)

Surface morphology of prepared test nanoliposomes (NLCs, PEG-NLCs, and Apt-NLCs) were observed under FESEM by forming a very thin layer of samples coated with platinum with a platinum coater at an accelerating voltage of 10 kV [27].

Atomic force microscopy (AFM)

The three-dimensional architecture of Apt-NLCs was observed under atomic force microscopy (AFM) by placing one drop of properly diluted and air-dried formulation suspension on a mica surface coverslip plate. Thus, shapes of the Apt-NLCs were observed through AFM by fixing a resonance frequency at 150–250 kHz under ACAFM mode [28, 29].

Cryo-transmission electron microscopy (Cryo-TEM)

The internal bilayer morphology, membrane stability and surface functionalization characteristics of the prepared experimental nanoliposome, Apt-NLCs, were determined by cryo-TEM. Here test liposomes were dispersed (100 µg/ml) in Milli Q water, and an aliquot (4 µl) of it was placed on a glow-discharged 300 mesh carbon-coated copper grid (TEM grid), and the air-dried samples were visualized under the cryo-TEM instrument (200 kV Talos-Arctica electron microscope, FEI/Thermo Scientific) [30].

In vitro drug release

In vitro drug release study was conducted by taking 2 ml of experimental liposomal suspensions (which were prepared by dispersing 2 mg of lyophilized NLCs, PEG-NLCs, and Apt-NLCs individually in 2 ml of buffer solution, PBS phosphate buffer with pH 7.4/acetate buffer, with pH-5) into a dialysis bag (Dialysis Membrane-110, Himedia, India). Then, the sample was dialyzed into 50 ml of respective drug release media, PBS/acetic buffer, containing 0.01% (w/v) β-cyclodextrin at room temperature (37 °C) by placing the dialysis system on a magnetic stirrer with very slow stirring at 100 rpm. During each study, 1 ml of drug release media was collected from 50 ml of solution at predetermined intervals up to 96 h, and was replaced by 1 ml of fresh media immediately [31]. Finally, all the samples were analyzed by UV–VIS spectroscopy at 340 nm wavelength against the respective medium without drug, and drug concentration was determined using a calibration curve prepared earlier. In vitro cumulative drug release data were represented through various release kinetic models such as zero order, first order, Higuchi model, Korsmeyer–Peppas and Hixson–Crowell models, and the highest R^2 (correlation coefficient) value was evaluated after plotting the drug released data to determine the best suit drug-release kinetic model [32].

Stability study

Stability assay of the intended experimental formulation, aptamer-conjugated PEGylated nanoliposomes (Apt-NLCs), was performed as per ICH guidelines [22], at the storage conditions (40 ± 2 °C and 75 ± 5% RH) and in the refrigerated form at (~ 4 °C) for 6 months. The samples were then subjected to examination for any changes in particle size or morphology using FESEM and variation in drug content in the prepared nanoliposome (Apt-NLCs) by conducting a drug loading assay.

MTT assay to evaluate in vitro antiproliferative activity of test liposomes

Two different liver cancer cell lines, Hep G2 and Huh-7, were procured from the National Centre for Cell Science, Pune, India. The cells were cultured in DMEM media, maintaining all necessary conditions [33]. The cell (taking 1×10^3 cells/well) viability expressed as IC_{50} doses for the free drug (apigenin), NLCs/PEG-NLCS, Apt -NLCs and blank-Apt-NLCs (Apt -NLCs without the drug) in both the cell lines were evaluated through MTT [(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide] assay using a wide range of individual equivalent drug concentration (1 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM) following the standard protocol [34].

Human peripheral blood mononuclear cell (PBMC) processing

This process is performed by isolating peripheral blood mononuclear cells (PBMC) from anticoagulated blood. Anticoagulated blood was added with an equal volume of Ficoll-Hypaque (Histopaque-1077) and centrifuged at 400*g* for 30 min to separate the cells from the whole blood. The PBMCs were collected from the interface of two liquids, washed with PBS (0.01 M, pH 7.4) twice, and resuspended in the RPMI 1640 medium [22]. The cytotoxic activity of different nanoliposomes suspended in PBS in the presence of a very minute amount of dimethyl sulfoxide, DMSO (final DMSO concentration < 0.1%), was evaluated for PBMC using an MTT assay.

Apoptosis assay

To verify the apoptotic potential of aptamer functionalized nanoliposome, Apt-NLCs, in comparison with the nonconjugated ones (NLCs/Peg-NLCs) in Hep G2 cells, we executed a flow cytometric (BD LSRFortessa™, BD Biosciences) study using FITC-Annexin V staining assay kit (BD Biosciences). The apoptotic activity of the experimental nanoliposomes containing an equivalent amount of active drug (considering the IC_{50} values from MTT) was determined upon 48 h of prior treatment and following the protocols [29].

Determination of cell-targeting potential of the aptamer (AS1411) assessing in vitro cellular uptake of the experimental nanoliposomes

In vitro uptake of FITC-labeled PEG-NLCs and FITC-labeled Apt-NLCs by HepG2 cells treated with the formulations containing the equivalent amount of drug was evaluated quantitatively at the three successive time points (1 h, 2 h, 4 h) by flow cytometric method. To confirm the receptor-mediated cellular uptake of an aptamer (AS1411) functionalized PEGylated nano-liposome (Apt-NLCs) over the other non-functionalized pegylated nanoliposome (PEG-NLCs), a competitive assay was performed. In this competitive assay, Hep G2 cells were preincubated with an excess amount (0.2 µg/well) of free aptamer (AS1411) for 2 h to block available coupling receptors (majorly nucleolin) [34, 35]. After that, it was treated with Apt-NLCs maintaining all other conditions the same. A quantitative comparison of in vitro cellular uptake between FITC-labeled formulations PEG-NLCs and Apt-NLCs in Hep G2 cells and FITC-labeled Apt-NLCs against aptamer-pretreated Hep G2 cells were estimated to verify receptor-mediated cellular uptake of aptamer-conjugated experimental nanoliposomes, Apt-NLCs. FACS Diva software (BD Biosciences) was used to analyze the data [35]. We have also captured confocal microscopic (Olympus Fluo View FV10i, Olympus) images of Hep G2 cells treated with FITC-labeled PEGylated-NLCs and -Apt-NLCs at 1 h and 4 h intervals to visualize the cellular uptake qualitatively.

Study of cell cycle arrest and apoptosis-related proteins

FACS (BD Biosciences, USA) analyzer was also used to analyze different phases of cell cycle during cell propagation, referring to apoptotic behavior of test nanoliposomes (an equivalent IC_{50} dose of apigenin containing in the respective amount of NLCs, PEG-NLCs and Apt-NLCs was used to treat 1.5×10^5 Hep G2 cells per well in six-well plates for 24 h). After washing in 50 µl RNase-free water and treating with Anexin-V, propidium iodide (PI) solution, the cells were subjected to analysis [36].

Similarly, apoptosis-related signaling proteins (p53, cleaved caspases, Bcl-2) were also estimated using respective protein markers by flow cytometric assay. These proteins have a major influence on the genetic regulation process (DNA synthesis, damage, or repair) during apoptosis. The experiments were conducted in Hep G2 cells, treated with Apt-NLCs, PEG-NLCs, and NLCs, taking an equivalent dose of apigenin for 24 h following the published protocol and guidelines [36].

Development of DENA-induced hepatocellular carcinoma in Sprague Dawley (SD) rats

The Institutional Animal Ethical Committee Jadavpur University approved all the animal studies. Development of hepatocellular carcinoma induced by chemical carcinogens such as diethyl nitrosamine and 2-acetylaminofluorine (AAF) has been well-known in animal models for the last several decades [37, 38]. In the present study, we used Sprague Dawley rats. Carcinogen-induced initiation-promotion model was used. Hepatocellular carcinoma (HCC) was developed in Sprague Dawley male rats using chemical carcinogens, applying initially a single intraperitoneal dose of 200 mg/kg of diethyl-nitrosamine (DENA), followed by a bi-weekly oral administration of 0.5% of w/w 2-acetylaminofluorene (2-AAF) for 16 weeks [38, 39]. Experimental groupings (A–G) were done as normal control rats (Group A), carcinogen control rats (Group B), carcinogen-treated rats received free apigenin (Group C), carcinogen-treated received NLCs (Group D), carcinogen-treated rats received PEG-NLCs (Group E), carcinogen-treated rats received Apt-NLCs (Group F), and normal rats received Apt-NLCs (Group G). All the carcinogen-treated groups were injected (through the i.v. route) 20 mg/kg body weight of free apigenin or an equivalent dose of the experimental formulations once a week for 8 weeks after the 16th week of carcinogen-treated animals [7, 22].

Pharmacokinetic study

The plasma and liver pharmacokinetic profiles for free drug, NLCs, PEG-NLCs, and Apt-NLCs were evaluated in HCC-induced Sprague Dawley (SD) rats (body weight, 125–150 g). Plasma and liver samples were collected from the experimental animals after injecting a single i.v. bolus dosage of 2 mg/kg of body weight of apigenin or corresponding amount of NLCs, PEG-NLCs, and Apt-NLCs containing equivalent apigenin, at 2, 4, 8, 12, 24, 48, 72, 96, and 120 h. The final analytical samples were prepared from the biological samples

through the liquid–liquid extraction process (using TBME, t-butyl methyl ether as a volatile solvent for drug extraction from plasma and liver homogenate) and analyzed by LC–MS/MS method applying naringenin as an internal standard [22]. The experiments were repeated in triplicate.

Assessing biodistribution of NLCs, PEG-NLCs, and Apt-NLCs by Gamma scintigraphy study

To investigate the accumulation efficiency of liposomes, NLCs, PEG-NLCs and Apt-NLCs, toward the target organ (liver), the formulations were radiolabeled by technetium-99 m (^{99m}Tc) following the stannous chloride reduction method with a radiolabeling accuracy almost above 90% [33]. Radiolabeled formulations (equivalent drug dose) were injected through the cannulation process to carcinogen-treated rats to ascertain the pattern of bio-distribution of different test nanoliposomes (NLCs/PEG-NLCs/Apt-NLCs) at 4 h and 8 h after their administrations. The results were articulated as % administered dose distributed per gram (%ID/g) of tissues or organs. Gama-scintigraph images of the animals were captured at 4 h and 8 h post-injection through GE Infinia γ Camera facilitated along with Xeleris Work Station, USA [29, 33].

In vivo hepatocyte uptake of fluorescent-labeled formulations by confocal microscopic study

To elucidate the accumulation pattern of different test nanoliposomes in the neoplastic hepatocytes, we have injected FITC-labeled NLCs, PEG-NLCs, and Apt-NLCs into the carcinogen-induced HCC animals through the tail veins. Taking a time interval of 4 h and 8 h post-injection, tumors and tumor-adjacent tissues were collected by sacrificing the animals and stored at 10% formaldehyde [22]. The tumors and tumor-adjacent tissue sections were fixed on slides and evaluated under confocal microscopy.

Assessing antitumor efficacy of the experimental nanoliposomes using a prepared animal model

To ascertain the antitumor potencial of the test nanoliposomes, we divided the animals into seven groups, Gr.A, normal (control), Gr. B, HCC positive control animals, HCC control, Gr.C, HCC induced animals treated with the free drug, Gr.D, HCC induced animals treated with NLCs, Gr.E, HCC induced animals treated with PEG-NLCs, Gr. F, HCC-induced animals treated with Apt-NLCs, Gr G, normal animals treated with Apt-NLCs. In the case of Gr C, D, E, F, and G, we followed a treatment schedule of once-weekly i.v. dose of 20 mg/kg body weight of free apigenin or equivalent amount formulations for twelve weeks. There were seven experimental animals in each group. At the end of the treatment, we collected the whole liver, and liver sections from the sacrificed experimental animals were assessed for the degree of antitumor potency of Apt-NLCs compared to NLCs/PEG-NLCs or free drug.

Gross and histopathological examination for liver morphology

We performed a gross macroscopic examination of the whole liver to identify the tumor incidences. Further, microscopic observation of tumor tissue sections by histopathological staining (with hematoxylin and eosin, periodic acid-Schiff) was conducted to evaluate the microscopic changes in the liver histopathology and to identify hepatic altered focal lesions [7].

Identifying apoptotic gene expression level through the qRT-PCR analysis

Total RNA was extracted from frozen (stored at −70 °C) liver tissue sample (from Gr B, C, D, E & F animals) with Trizol reagent and was evaluated through nanodrop (QI Aexpert) after following the manufacturer's protocol. Then, with the required amount of sample RNA, cDNA was prepared and using this template, RT-PCR studies were performed with the aid Bio-Rad SYBR green PCR master mix along with corresponding primers as per manufacturer's instructions [40, 41]. The specific synthetic primers (for p^{53}, F:5′-ATGTTTTGCCAA CTGGCCAAG-3′, R:5′-TGAGCAGCGCTCATGGTG -3′); (for caspase 3, F: 5′-GTGGAACTGACGATGATA TGGC-3′, R: 5′-CGCAAAGTGACTGGATGAACC-3′); (for Bcl-2, F:5′-TGTGGATGACTGACTACCTGA ACC-3′, R:5′-CAGCCAGGAGAAATCAAACAGAGG -3′) and (for β-actin, F:5′-AAGATCCTGACCGAGCGT GG-3′, R:5′-CAGCACTGTGTTGGCATAGAGG-3′) were purchased from Saha Gene, Hyderabad, India and maintaining the thermo-cycling conditions as per the manufacturer's protocol. The qRT-PCR studies were performed on Rotorgene (Qiagen) instrument. The fluorescence activity in qRT-PCR demonstrated the relative gene expression level for corresponding target genes and it was determined by applying 2-ΔΔCt calculative process, where β-actin was taken as the housekeeping gene [42].

Hepatic function test

Blood samples were collected from all the groups of experimental animals (Gr A-G), and after processing as per the reported method [33], the level of AST (aspartate

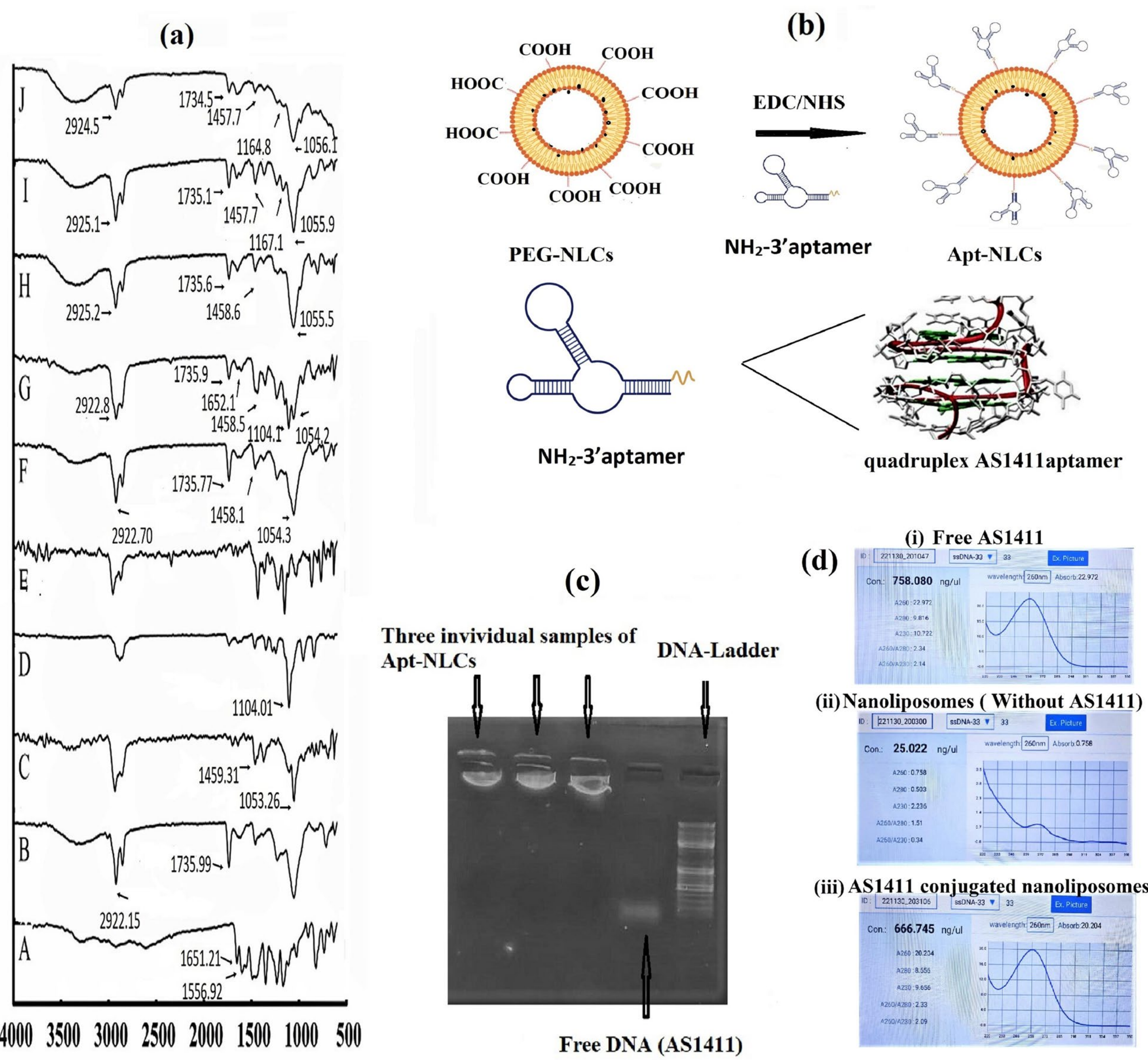


Fig. 1 Drug-excipients interaction and aptamer conjugation on stealth nanoliposome. **a** FTIR spectra of apigenin (A), soya lecithin (B), cholesterol (C), DSPE-PEG-2000-COOH (D), BHT (E), Blank formulation (F), the physical mixture of all constituents (G), NLCs (H), PEG-NLCs (I), and Apt-NLCs (J); **b** Pictorial representation of surface functionalization of PEG-NLCs with modified NH2-AS1411; **c** Determination of aptamer conjugation by agarose gel. The first three lanes from the left had (Apt-NLCs) showing no sample run, the 4th lane showed free aptamer (AS1411) showing movement, and the 5th lane had DNA ladder; **d** i, ii, iii represented spectrometric reading for free AS1411, plain nanoliposomes AS1411 conjugated nanoliposomes by nanodrop UV spectrophotometer, (experiment was conducted on triplicate set)

aminotransferase), ALT (alanine transaminase), and ALP (alkaline phosphatase) were determined using commercially available bioassay kits (Coral Clinical Systems, Goa, India) following manufacturer protocols [33].

Statistical analysis

The data were statistically analyzed using ORIGIN 8.0 software, one-way ANOVA followed by post hoc Dunnett's test using Graph Pad Prism Software, considered 5.0. $p < 0.05$ was as a minimum level of significance.

Results and discussion

Physical characterization

Pre-formulation and preparation of NLCs/PEG-NLCs/Apt-NLCs

Thin film hydration method was followed for the preparation of experimental nanoliposomes. FTIR spectra for apigenin (Api), each of the excipients (SLY, CHC, DSPE-PEG, BHT), their physical mixture with drug, along with test nanoliposomes NLCs, Apt-NLCs with Apigenin (Api) (Fig. 1a) showed the presence of their

characteristic functional groups, for Api (C=C stretching at 1556.92 $cm^{-1}$and C=O stretching at 1651.21 cm^{-1}); for SLY (C=O stretching at 1735.99 cm^{-1} and C–H stretching at 2922.15 cm^{-1}); for CHC (C–H bending at 1459.31 $cm^{-1}$and C–O stretching at 1053.26 cm^{-1}); and for DSPE-PEG (2000)-COOH (C–N medium intensity stretching at 1104.01 cm^{-1}) in their physical mixture and in the formulations as in original components, suggesting that nanoliposomes were formulated successfully without any chemical interaction among the ingredients. However, minor shifting of bands (H–O stretching) suggests the formation of weak Van der Waal interaction or weak hydrogen bonds due to physical interaction during formulation development. The characteristic peak of apigenin was absent in the NLCs without the active drug (called here Blank formulation). It was also missing in the prepared test nanoformulations (both in NLCs and Apt-NLCs), indicating complete drug encapsulation. Thus, we confirmed the chemical compatibility of apigenin and other excipients using an FTIR study and successful drug encapsulation in the developed nanoliposomes (NLCs, PEG-NLCs, and Apt-NLCs).

Determination of aptamer conjugation

The successful conjugation of phosphorothioated amino-modified AS1411(DNA-aptamer) with PEG-NLCs was determined using agarose gel-electrophoresis by observing prominent fluorescence bands under UV-transilluminator. Figure 1c depicted a gel-electrophoresis assay, and the migration of parallel luminating bands for 50 bp commercial DNA ladder was noticed clearly. Free DNA (AS1411) was also migrated near 25 bp position of 50 bp commercial DNA ladder, while little or no migration for aptamer-conjugated nanoliposomes (Apt-NLCs) was observed. The three stable luminating bands near the starting wells for three individual samples of Apt-NLCs from three different batches indicated successful DNA conjugation with the nanoformulations. Aptamer-conjugated DNA could not migrate through the agarose gel plate with the same speed of free aptamer as it was covalently attached to the bulky PEGylated nanoliposomes.

Through nanodrop UV spectrophotometer, we observed that 1 µl of free AS1411 contained 758.04 ± 55 ng/µl of ssDNA at 260 nm and compared this with the equivalent amount of AS1411 from the diluted solution of Apt-NLCs (we had added 20 µl of 100 µM AS1411 aptamer to 20 mg of PEG-NLCs to get 5 µM AS1411 in sample) depicted in Fig. 1d. The ssDNA concentration reading in Apt-NLCs was 666.01 ± 03 ng/µl, while non-conjugated plain nanoliposomes (without aptamer conjugation) did not showed any significant absorbance at 260 nm range in nanodrop UV spectrophotometer. This result indicated that more than 80% of AS1411 aptamer used for conjugation was coupled with PEG-NLCs, so 1 mg of PEG-NLCs had capacity to bind approximately 0.20 µM of AS1411 of ss DNA.

The modification ratio of aptamer conjugation to nanoparticle surface may not affect in vivo or in vitro performances of Apt-NLCs. Since single or more aptamers bound to nucleolin receptors would have a similar fate of receptor-mediated cellular internalization. However, the aptamer's binding probability to the nucleolin receptors would obviously be more or faster if the number of aptamers conjugated on the NLC surface is more.

Physicochemical characterization for test nanoliposomes

The mean particle size, PDI, ζ-potential, drug loading, and encapsulation efficacy were optimized for NLCs, PEG-NLCs, and Apt-NLCs. NLC had an average diameter size of 30 nm (Additional file 1: Table S1), which increased to 140 nm (Additional file 1: Table S1) and 150 nm, respectively, upon PEGylation (PEG-NLCs) and PEGylation followed by aptamer conjugation (Apt-NLCs). (Fig. 2). The average surface charge (ζ-potential) was found to be 1.16 mV, − 55.9 mV, and − 22.4 mV for NLCs, PEG-NLCs, Apt-NLCs, respectively (Additional file 1: Table S1 and manuscript Fig. 2). The presence of DSPE PEG-2000-COOH in the liposome has provided a negative charge, which has been decreased for using amino-terminated aptamer conjugation. PDI values (Additional file 1: Table S1) for the respective formulations were 0.251, 0.256, and 0.316, referring to the formulations as uniformly distributed and could possess minimal aggregation in deionized water [22]. Respective drug loading for NLCs, PEG-NLCs, and Apt-NLCs (4.59 ± 0.02%, 4.38 ± 0.04%, and 4.33 ± 0.05%) along with drug entrapment efficiencies in case of all the optimized experimental nanoliposomes (NLCs, PEG-NLCs, and Apt-NLCs) were estimated in Additional file 1: Table 1. Satisfactory drug entrapment efficacy (> 85%) in the experimental nanoliposomes was determined depicted in Additional file 1: Table S1.

Indicated physiochemical characterizations were suitable for both in vitro and in vivo drug applications, particularly for hepatocellular drug delivery [43]. Mean zeta potential values (− 22 mV) in the case of Apt-NLCs referred to their stability when dispersed in aqueous suspension for drug administration [44].

The in-vitro drug release profile of NLCs, PEG-NLCs, and Apt-NLCs with follow-up kinetics

In vitro cumulative percentages of drug release (Fig. 2) for NLCs, PEG-NLCs and Apt-NLCs were plotted against time in PBS (7.4) containing (0.1% W/V) β-cyclodextrin. Here, we observed all the formulations initially upheld almost similar rapid drug release patterns up to 12 h.

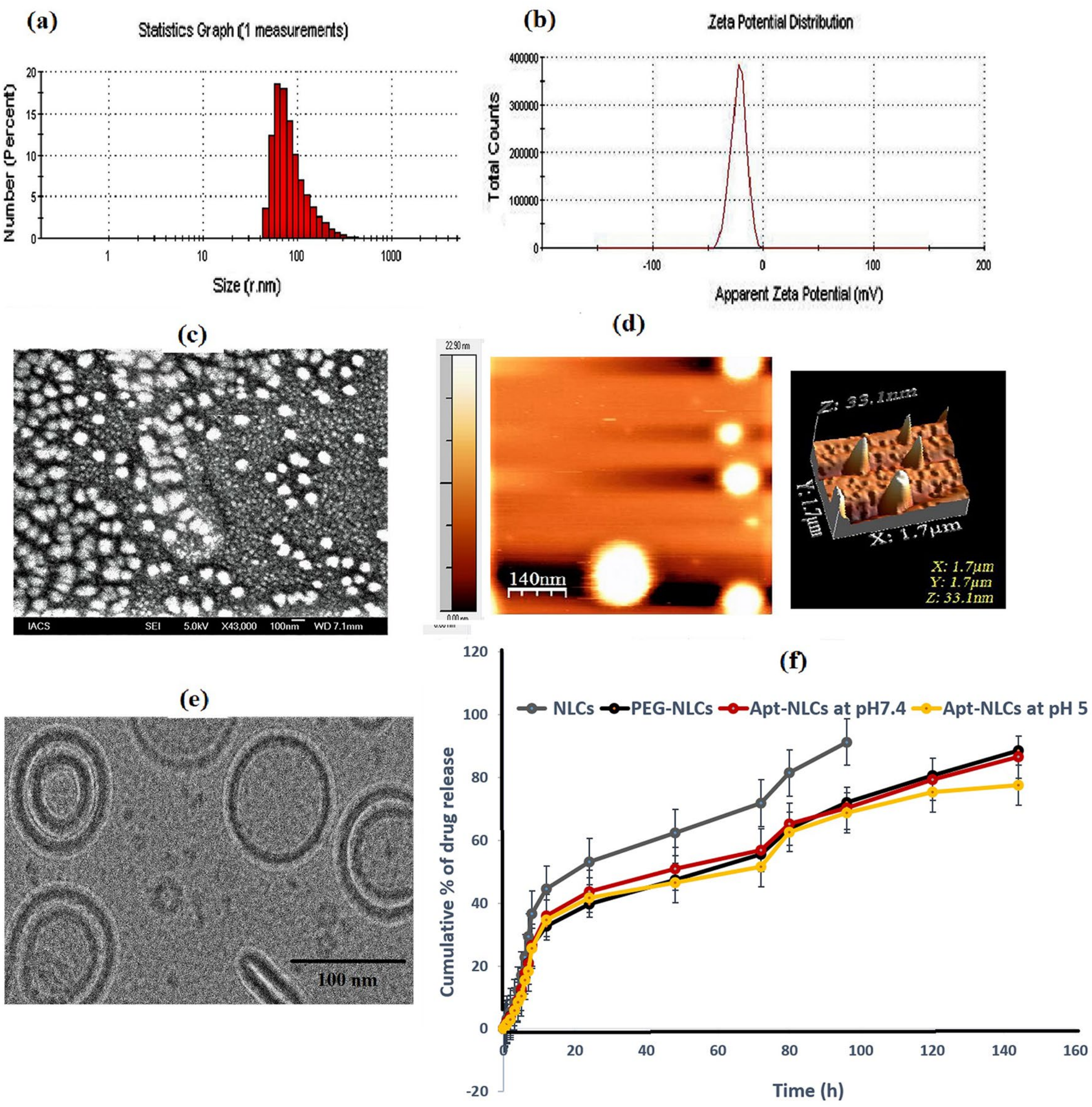


Fig. 2 Characterization of aptamer conjugated nanoliposomes Apt-NLCs. **a** average particle size distribution, and **b** Zeta potential, **c** surface morphology applying FESEM images at 43 000 ×. **d** Data by atomic force microscopy. **e** Cryo-TEM image depicts internal morphology, **f** Cumulative % drug release against time for Apt-NLCs as compared NLCs/PEG-NLCs. Data show mean ± standard deviation applying three different experimental values

Later phase PEG-NLCs and Apt-NLCs exhibited a prolonged drug release profile compared to NLCs, most likely because of the PEG coating present outside the test liposomes. NLCs took 92 h to display 90% of cumulative drug release in β-cyclodextrin-PBS medium, whereas PEG-NLCs and Apt-NLCs took 144 h for almost 90% drug release. Further, drug release at acetate buffer media (pH 5) also showed a similar pattern for PEG-NLCs and Apt-NLCs depicted in Additional file 1: Fig. 2.

The drug release data were tested on different kinetic models. The regression coefficient (R^2) values for each Zero-order Kinetics, First-order kinetics, Korsmeyer–Peppas kinetics, Hixson–Crowell, and Higuchi kinetics were elaborated in (Additional file 1: Table S2). The data suggest that drug release from the prepared formulations best fit with the Higuchi kinetic model ($R^2 = 0.9811$) and specified release component (n) value in derived from Korsmeyer–Peppas kinetic equation suggested the range

Table 1 Plasma and liver pharmacokinetic parameters in the HCC rats treated with NLCs/PEG-NLCs/Apt-NLCs and free apigenin as iv bolus form through tail vain

		Api	NLCs	PEG-NLCs	Apt-NLCs
In vivo plasma pharmacokinetic data	Cmax (ng/ml)	15.5 ± 0.16	17.2 ± 1.13	18.1 ± 0.55	19.2 ± 0.51
	AUC last (ng/h/ml)	125.8 ± 52	496.4 ± 62.9	826.4 ± 53[#]	986.2 ± 47[#]
	AUMC	1608 ± 1419	15,290 ± 3089	30,457 ± 2350	37,988 ± 1994
	MRT (h)	12.81 ± 1.58	30.83 ± 1.09	36.94 ± 0.41[$]	38.91 ± 0.21[$]
	AUC 0-∞ (ng h/ml)	130.61 ± 53.4	539.64 ± 79.34	1072.47 ± 171.21*	1446.21 ± 182.65*
	$t_{1/2}$ (h)	5.25 ± 0.25	13.51 ± 0.2	39.02 ± 0.2	43.02 ± 0.25
	Clearance (L/h/Kg)	15.9 ± 0.55	4.03 ± 0.63	2.42 ± 0.66	2.03 ± 0.52
In vivo liver pharmacokinetic data	Cmax (ng/ml)	160 ± 7.87	165.7 ± 7.37	178.9 ± 8.14	223.4 ± 6.64
	Tmax (h)	1 ± 0.15	3 ± 0.2	6 ± 0.22	6 ± 0.26
	AUC last (ng.h/ml)	1828 ± 194	4336 ± 474	9410 ± 553	14,658 ± 592
	AUMC	25,535 ± 3589	119,549 ± 17,352	330,328 ± 2126*	582,753 ± 21,258*
	MRT (h)	13.2 ± 3.8	27.55 ± 6.1	35.48 ± 5.9	42 ± 4.6
	AUC 0-∞ (ng.h/ml)	1898 ± 197	6440 ± 542	8961 ± 705	12,955 ± 892
	T1/2 (h)	5.65 ± 0.52	16 ± 1.2	34 ± 2	50 ± 1.45
	Clearance (L/h/Kg)	1.01 ± 0.01	0.461 ± 0.033	0.213 ± 0.006^	0.136 ± 0.005^

Data from the three independent experiments denote mean ± standard deviation (n = 3)

[#, $] Indicated significant (p < 0.05) improvement in AUC and MRT values in plasma for Apt-NLCs/PEG-NLCs treated animals in comparison to NLCs. (*) Indicated significant (p < 0.05) improvement in AUC in liver values in Apt-NLCs/PEG-NLCs treated animals in comparison to NLCs. ^ indicated significant (p < 0.05) reduction in hepatic clearance in Apt-NLCs/PEG-NLCs treated animals in comparison to NLCs

(0.81 to 0.86), which indicated non-Fickian type of diffusion and drug release patterns were maintained by all the prepared test nanoliposomes in the used media.

An extended drug release profile showed that it took almost 7 days for 90% of drug release in 1% β-cyclodextrin-PBS media (pH 7) from PEG containing nanoliposomes (with or without aptamer conjugation, Apt-NLCs/PEG-NLCs). This pattern was due to the contributary stealthy property of DSPE-PEG-2000 in the test nanoliposomes, which predicted prolonged circulation time. An almost similar pattern of drug release by Apt-NLCs at lower pH (pH 5) also assumed their accumulation toward the target tumor [45].

Surface and internal morphology of NLCs/PEG-NLCs and Apt-NLCs

FESEM, AFM, and Cryo-TEM evaluated the surface and internal morphology of the prepared nanovesicles. FESEM observation for NLCs /PEG-NLCs (Additional file 1: depicted Fig. S1) and Apt-NLCs (Fig. 2) suggested that the vesicles were nanosized (20–120 nm) with a smooth surface and homogenously well-distributed. Further, the physical architecture of the particles in a three-dimensional mode in AFM revealed that the average mean height for Apt-NLCs was 21.20 ± 0.05 nm (Fig. 2). The thick and intact uni- and bi-lamellar PEG-coated-oligo functionalized surface morphology for Apt-NLCs having a particle size within 100 nm range was clearly visible upon Cryo-TEM observation (Fig. 2). These types of morphological characteristics ensured their stability, cellular accumulation, and sustainability during in vitro and in vivo drug application [19].

Stability testing

FESEM studies for Apt-NLCs were conducted after 6 months of storage. When refrigerated at − 4 °C, the samples did not portray any major distinguishable changes as compared with freshly prepared Apt-NLCs, while storing at 40 ± 2 °C and 75 ± 5% RH, the morphology differed (Additional file 1: Fig. S2 and Table S3). Further, through estimating drug loading and zeta potentials, it revealed that the Apt-NLCs remained equally potent at refrigerated conditions on 6-month storage (depicted in Additional file 1: Table S3). Still, differences were present when stored at 40 ± 2 °C and 75 ± 5% RH setting. Hence, the formulations were absolutely stable over the period of 6 months in refrigerated conditions.

In vitro cellular studies

In vitro antiproliferative activity

We investigated in vitro antiproliferative profile of free drug, NLCs, PEG-NLCs, Apt-NLCs, and blank test nanoliposomes against two different types of liver cancer cell lines (Hep G2 and Huh-7 cell lines) over a range of concentrations (1–100 μM) following 48 h incubation

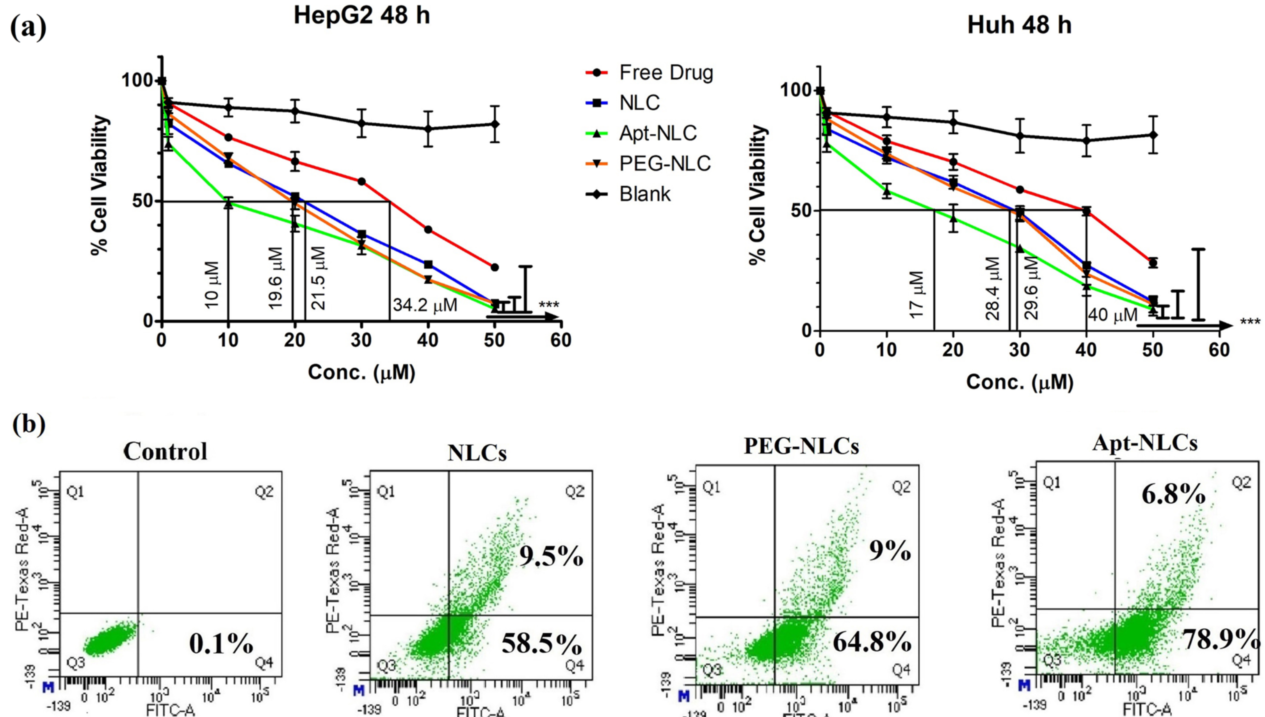


Fig. 3 In vitro cytotoxicity and apoptosis analysis. **a** Viability assay in HepG2 and Huh-7 cells after treating them with free drug (apigenin), different experimental formulations (NLCs, PEG-NLCs, and Apt-NLCs), and AS1411 functionalized blank liposomes for 48 h with drug concentration range (10-100 μM) respectively. **b** FACS analysis of cellular apoptosis applying Annexin V-FITC staining in HepG2 cells treated with NLCs, PEG-NLCs, and Apt-NLCs with their equivalent IC50 doses of the drug at 48 h

period (Fig. 3). We found a similar trend of cytotoxic profile in order of IC_{50} values, as Apt-NLCs < PEG-NLCs < NLCs < free drug < blank in both the cell lines (Fig. 3a). The results reflected the highest cytotoxic potential of aptamer conjugated nanoliposomes (Apt-NLCs) toward liver cancer cells compared to free drug or other nonconjugated nanoliposomes. A negligible cytotoxic activity produced by aptamer-conjugated blank nanoliposomes revealed that neither the aptamer nor the carrier nanoliposome had any interference on the cytotoxicity of Apt-NLCs. The lowest IC_{50} value, 10.1 μM, was observed for Apt-NLCs, against HepG2, while the corresponding IC_{50} value of Apt-NLCs toward Huh-7 was 17.1 μM. So, we have proceeded with Hep G2 cell lines for further in vitro studies to evaluate the apoptotic potential of aptamer-conjugated nanoliposomes over nonconjugated nanoliposomes. Additionally, insignificant cytotoxicity toward normal Human peripheral blood mononuclear cells, PBMC (> 50 μM) proved that Apt-NLCs were suitable to use as a delivery system through the blood to target HCC (Depicted in Additional file 1: Table S4).

Apoptosis

We have quantified the apoptotic potential of the experimental nanoliposomes by applying Annexin V-FITC staining using the flow cytometric method. Hep G2 cells were exposed to the nanoliposomes (NLCs, PEG-NLCs, and Apt-NLCs) with their equivalent IC_{50} drug doses for 48 h. The treated group with aptamer functionalized

(See figure on next page.)

Fig. 4 Cellular uptake studies. Flow cytometric data of cellular uptake. **a** PEG-NLCs, **b** Apt-NLCs, **c** Apt-NLCs in the presence of free AS1411 (receptor blocking condition) in Hep G2 cells at 1h, 2h, and 4h **d** Histogram representation of FACS mean fluorescence values obtained through above-mentioned uptake studies. Data represents mean ± SD, (n=3), bar (-) indicates groups between which the comparisons were made. ns: statistically insignificant, *** refer statistical significance at a level of P<0.05. **e** and **f** Confocal microscopic images of cell uptake of PEG-NLCs and Apt-NLCs in Hep G2 at 1h and 4h (Green color shows for FITC-labelled experimental nanoliposomes, PEG-NLCs/ Apt-NLCs, and blue color indicates nucleus stained by DAPI

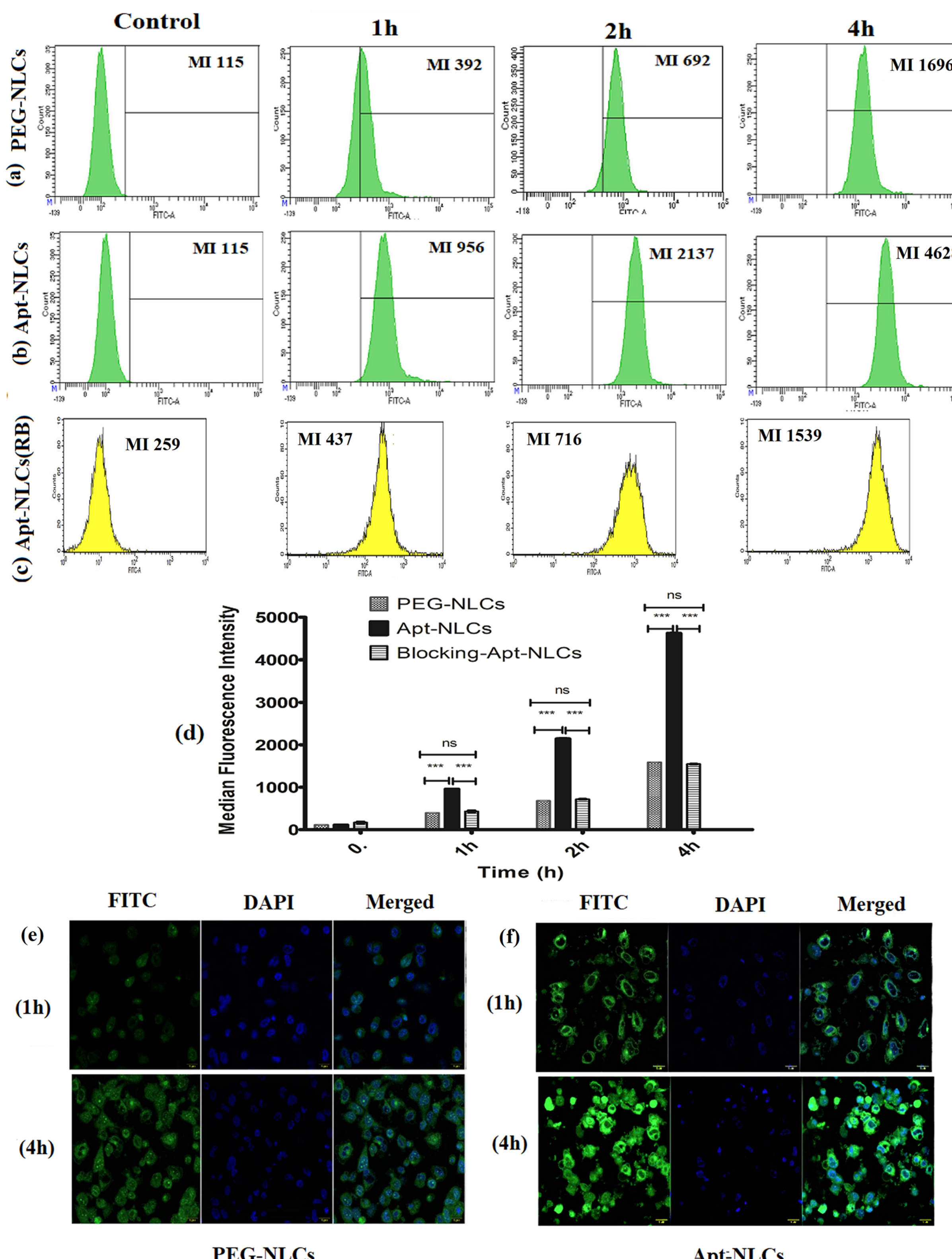


Fig. 4 (See legend on previous page.)

liposomes (Apt-NLCs) showed impressive improvement in the percentage of apoptosis (78.9% in the early and 6.8% in the late phases, a total 85.6%) compared to non-conjugated groups (NLCs/PEG-NLCs) (Fig. 3). NLCs and PEG-NLCs portrayed a total of 68% and 74% of apoptosis, respectively.

This indicated the antitumor potential of apigenin increased even at a low concentration (IC_{50} value in Hep G2) when delivered through an aptamer functionalized nanoformulations.

Receptor-mediated cellular uptake

We estimated uptake of FITC-labelled nanoliposomes, PEG-NLCs and Apt-NLCs (with their equivalent IC_{50} doses of the drug), in Hep G2 cells quantitatively through the flow cytometric method at different time intervals. Parallelly, we run a set with FITC-Apt-NLCs treated HepG2 cells pretreated with an excess amount of free aptamer (AS1411) denoted as a receptor blocking study. There was no considerable difference in mean fluorescence intensity in the case of normal (without the pre-treatment) Hep G2 cells treated with PEG-NLCs and in the receptor-blocked Hep G2 cells (using aptamer pre-trearment) treated with Apt-NLCs. In contrast, there was a significant improvement in uptake values in the case of normal Hep G2 cells treated with Apt-NLC in a time-dependent manner (Fig. 4a–d). These flow cytometric analyses strongly supported our hypothesis that aptamer (AS1411) functionalized nanoliposomes (Apt-NLCs) augmented cellular uptake in comparison with the nonconjugated nanoliposome (PEG-NLCs), most likely by the nucleolin receptor-facilitated endocytosis as in the cytotoxicity and apoptosis study, we observed notable superior antiproliferative potencial of aptamer-conjugated nanoliposomes (Apt-NLCs) over the non-conjugated nanoformulations. FITC-Apt-NLCs showed much superior cellular uptake in Hep G2 cells at 1 h and 4 h in comparison with PEG-NLCs during confocal microscopy (Fig. 4e and f).

In this competitive cellular uptake study, we tried to understand the probable receptor-mediated endocytosis approach for cell uptake. We observed significant improvement of in vitro uptake of Apt-NLCs (aptamer conjugated PEGylated liposomes) in Hep G2 cells as compared to uptake of Apt-NLCs in free-aptamer pretreated HepG2 cells. Also, bio-ligand AS1411 already reported as a biosensor aptamer had shown a tremendously high affinity toward the carcinogenic biomarker nucleolin receptor of HCC cells [46]. Here, AS1411 aptamer possibly facilitated nucleolin-dependent superior cellular uptake of Apt-NLCs to Hep G2 cells.

Cell cycle arrest and comparative estimation of apoptosis-related proteins

Arresting cell cycle by modulating the phases of cell propagation was postulated as the experimental nanoliposomes (NLCs, PEG-NLCs, Apt-NLCs) caused DNA damage in Hep G2 cells during cellular apoptosis. After flow cytometric analysis, we found that there was a distinguishable increase in the G2/M (73.1%) phase with some changes in the S phase in the cells treated with Apt-NLCs compared to NLCs (51.5%) and PEG-NLCs (55.1%) treatments (Fig. 5). This clearly suggests the enhanced antitumor potential of apigenin while delivered through receptor-targeted drug delivery of nano-system, Apt-NLCs. The damage or repair in DNA during apoptotic processes (either extrinsic or intrinsic) is closely monitored with the different apoptotic-related proteins. Flow cytometric estimation of tumor suppressor proteins, such as p53, caspase, and anti-apoptotic proteins, such as Bcl-2 was conducted. The study depicted the highest mean values for p53 and caspase-3 expressions and the lowest Bcl-2 values with Apt-NLCs compared to other nonconjugated formulations (NLCs/Peg-NLCs) in Hep G2 cells (Fig. 5). Arresting cell cycle at G2/M phase through upregulation in p53 and caspase activities and downregulation of Bcl-2 activity strongly indicates the cellular apoptotic potential of apigenin.

Apigenin allowed apoptosis in neoplastic cells following several apoptotic signaling pathways (extrinsic and Intrinsic), where tumor suppressor protein p53 and anti-apoptotic proteins Bcl-2 (Bcl-XL, Bcl-W, etc.) are very much associated with initiating the apoptotic process [5].

In contrast, caspase activation (majorly caspase-3, caspase-8, and caspase-9) and breakdown or damage in DNA irreversibly play a central role in promoting apoptosis in the tumor cells [47]. Here, the noticeable advanced apoptotic event of arresting p53-mediated cell cycle propagation by apigenin delivered through Apt-NLCs was observed as compared with non-functionalized plain nanoliposomes. This suggests that the modified drug delivery system (Apt-NLCs) boosted the apoptotic potential of apigenin through significant in vitro drug accumulation in the immunomodulatory Hep G2 cells by regulating corresponding signaling pathways irreversibly, thus, strongly checking apoptotic precedence of Apt-NLCs over the nonconjugated nanoliposomes.

In vivo studies

Plasma and liver pharmacokinetic study

Amounts of apigenin in plasma and liver at predetermined time intervals were estimated after injecting free-apigenin, NLCs, PEG-NLCs, and Apt-NLCs (2 mg

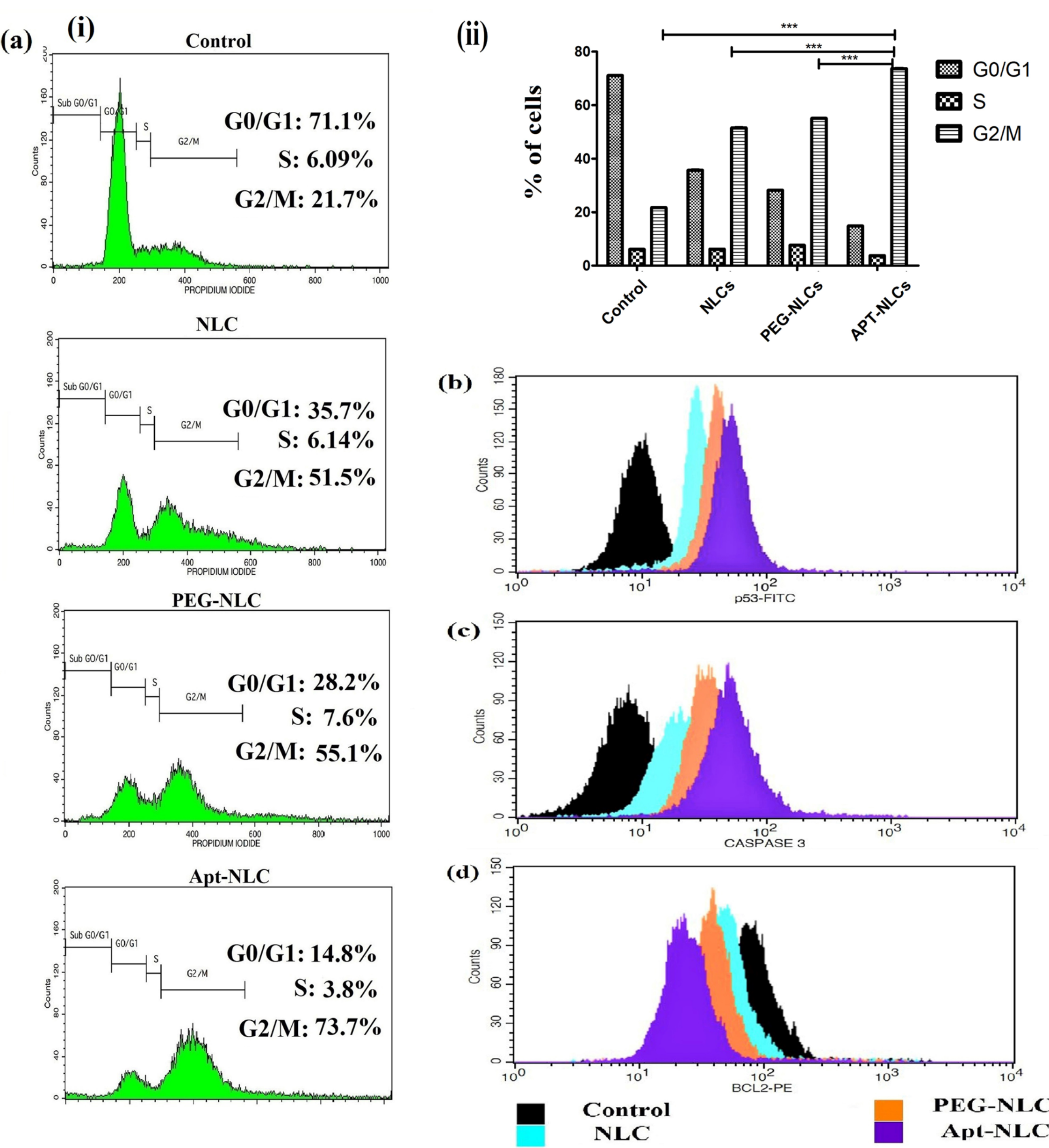


Fig. 5 Flowcytometric representation of cell cycle analysis and apoptotic protein expressions studies in Hep G2 cells. **a** i & ii Cell cycle analysis of different experimental nanoliposomes NLCs, PEG-NLCs and Apt-NLCs (highest improvement in G2/M phase in aptamer conjugated nanoliposomes, Apt-NLCs in comparison to non-conjugated nanoliposomes, NLCs/PEG-NLCs. Data represents mean ± SD, (n=3), *** refer statistical significance at a level of P<0.05. **b**, **c** and **d** p53, Casapae-3, and Bcl-2 protein quantifications upon NLCs, PEG-NLCs, and Apt-NLCs treatments, respectively, using FACS

apigenin or equivalent dose of formulations /kg body weight) through the tail vein, and the corresponding bio-samples were collected and analyzed using LC–MS/MS method [17]. After plotting the data (representing) graphically, the pharmacokinetic parameters were derived for the experimental nanoliposomes along with the free drug (Fig. 6a). All the nanoformulations maintained a steady drug plasma concentration up to 96 h in contrast with a negligible amount of free-apigenin in plasma at 48 h. However, there were significant improvements in plasma $t_{1/2}$ (~3.5-fold), AUC (~twofold), and MRT (~1.2-fold) values in PEG-NLCs and Apt-NLCs treated animals compared to NLCs treated animals were observed. Plasma drug clearance was reduced by up to 50% in the case of both PEG-NLCs and Apt-NLCs compared to NLCs. Further, hepatic apigenin accumulation was highest in the

case of Apt-NLCs, and it was increased by 1.2-fold and threefold compared to PEG-NLCs and NLCs, respectively. Elevated hepatic MRT value and reduced hepatic clearance (Table 1) in the case of Apt-NLCs compared to PEG-NLCs and NLCs strongly suggest a greater hepatic accumulation of apigenin when administered as an encapsulated form through a target-specific aptamer mediated-PEG containing drug delivery system.

In vivo, animal models play a vital role in understanding the drug-delivery features and their efficacy in the physiological systems [48]. Here, in vivo pharmacokinetic evaluation of the nanoliposomes (NLCs/PEG-NLCs/Apt-NLCs) in HCC-induced experimental animals (SD, rats) revealed Apt-NLCs carried the highest Plasma half-life ($t_{1/2}$) and AUC. Apt-NLCs also exhibited maximum hepatic drug deposition over more extended periods with the highest MRT values as compared with the nonconjugated nanoliposomes NLCs/PEG-NLCs. They endorsed the lowest drug clearance in HCC-positive experimental rats. All of these superior pharmacokinetic parameters directed prolong and optimum drug bioavailability for apigenin in system as because PEGylated nanoliposomes might improve the circulation time and sustainability of tumor surrounding areas [49].

Drug biodistribution study by γ-scintigraphy

After injecting ^{99m}Tc-NLCs/^{99m}Tc-PEG-NLCs/^{99m}Tc-Apt-NLCs in the carcinogen-treated animals, radiolabeled nanoliposomes accumulation was visualized through γ-scintigraphy at 4 h and 8 h of post-injection with estimating bio-distribution of the different experimental nanoliposomes among the different organs of the body. In γ-scintigraphy images, the radio signals were visible in most of the peritoneal region in animals treated with the experimental nanoliposomes, NLCs, and PEG-NLCs, at 4 h, although exclusive and maximum hepatic signals were initiated in Apt-NLCs treated animals (60.69 ± 1.63% ID/g in liver tissue) at 8 h (Table 2, Fig. 6b). Further, at 8 h, a time-dependent drug distribution pattern was observed. Clear and predominant hepatic restricted signals were noticed in Apt-NLCs treated animals (49.51% ID/g of tissue) (Table 2), whereas, NLCs/PEG-NLCs offered indistinct drug accumulation (18.89 %ID/g and 33.078% ID/g of tissue, respectively) in the liver, along with considerable detectable signals in stomach, lung, kidney and to the other adjacent tissues of the body (Table 2). This could mention selective hepatic accumulations of aptamer functionalized nanoliposomes over nonconjugated formulations in vivo, most likely after following the receptor-mediated uptake of Apt-NLCs through the attachment of aptamer, AS1411 to the target oncogenic biosensor nucleolin receptor overexpressed on neoplastic hepatocytes.

All these data indicated the rationally designed aptamer (AS1411) functionalized PEGylated nanoliposomes offered significant drug retention (Apt-NLCs > PEG-NLCs/NLCs) selectively in the carcinogenic liver due to the uptake of aptamer (AS1411) conjugated nanoliposomes (Apt-NLCs) by the neoplastic hepatocytes. Most likely, it followed aptamer (AS1411) sensitive biomarker nucleolin receptor-mediated cellular uptake. Thus, the synergic effect of precise drug accumulation and their sustainability in neoplastic hepatic tissues reinforced the dominance of Apt-NLCs as the finest as well as promising drug delivery vehicles in vivo.

Intratumor deposition of FITC-labeled nanoliposomes

We investigated the neoplastic hepatic area after injecting FITC-labeled nanoliposomes into HCC-positive animals to compare the uptake of different test nanoliposomes within the neoplastic tissue and their surrounding liver tissues. We visualized a distinctive higher fluorescence in the neoplastic hepatic tissue of the Apt-NLCs injected animals at different time points compared to the surrounding liver tissues (Fig. 6e). At the same time, much less localized fluorescence was observed in tumors treated with the other nanoformulations than Apt-NLCs in HCC animals. It suggests the superiority and specificity of AS1411 conjugated drug delivery toward the HCC tumors.

(See figure on next page.)

Fig. 6 Plasma and liver pharmacokinetics of free drug and all the test formulations (NLCs, PEG-NLCs and Apt-NLCs), biodistribution by gamma-scintigraphy studies, and accumulation of FITC-labelled test nano formulations in cancerous and normal hepatic tissues. **a** Plasma concentration of apigenin vs. time, upon iv administration of NLCs, PEG-NLCs and Apt-NLCs in HCC induced rats, respectively (Data from the three independent experiments show mean ± standard deviation (n = 3); **b** liver concentration of apigenin vs. time curve, upon iv administration of NLCs, PEG-NLCs and Apt-NLCs in HCC induced rats, respectively (Data from the three independent experiments show mean ± standard deviation (n = 3). (***) significant (P< 0.05) improvement in plasma and liver drug concentration in PEG-NLCs and Apt-PEG-NLCs applied animal Gr in comparison to NLCs over the specific time point as indicated in figure; **c** γ Scintigraphy imaginings of HCC rats at 4h and 8h after injecting 99mTc-labeled – (NLCs, PEG-NLCs and Apt-NLCs) through venous cannulation process. **d** Confocal microscopic observation of tumor tissues sections of carcinogenetic rats upon the treatment of FITC-labeled – (NLCs, PEG-NLCs, and Apt-NLCs) at4h and 8 h of administration of iv injection. The figure showed green fluorescence of FITC labeled formulations within the cancerous tissue. The right column showed the photos of the same tissue sections without fluorescence. **e** a comparative study of fluorescence level in both tumor tissue and healthy tissue by FITC-Conjugated Apt-NLCs, also in tumor tissue by FITC-conjugated NLCs/PEG-NLCs

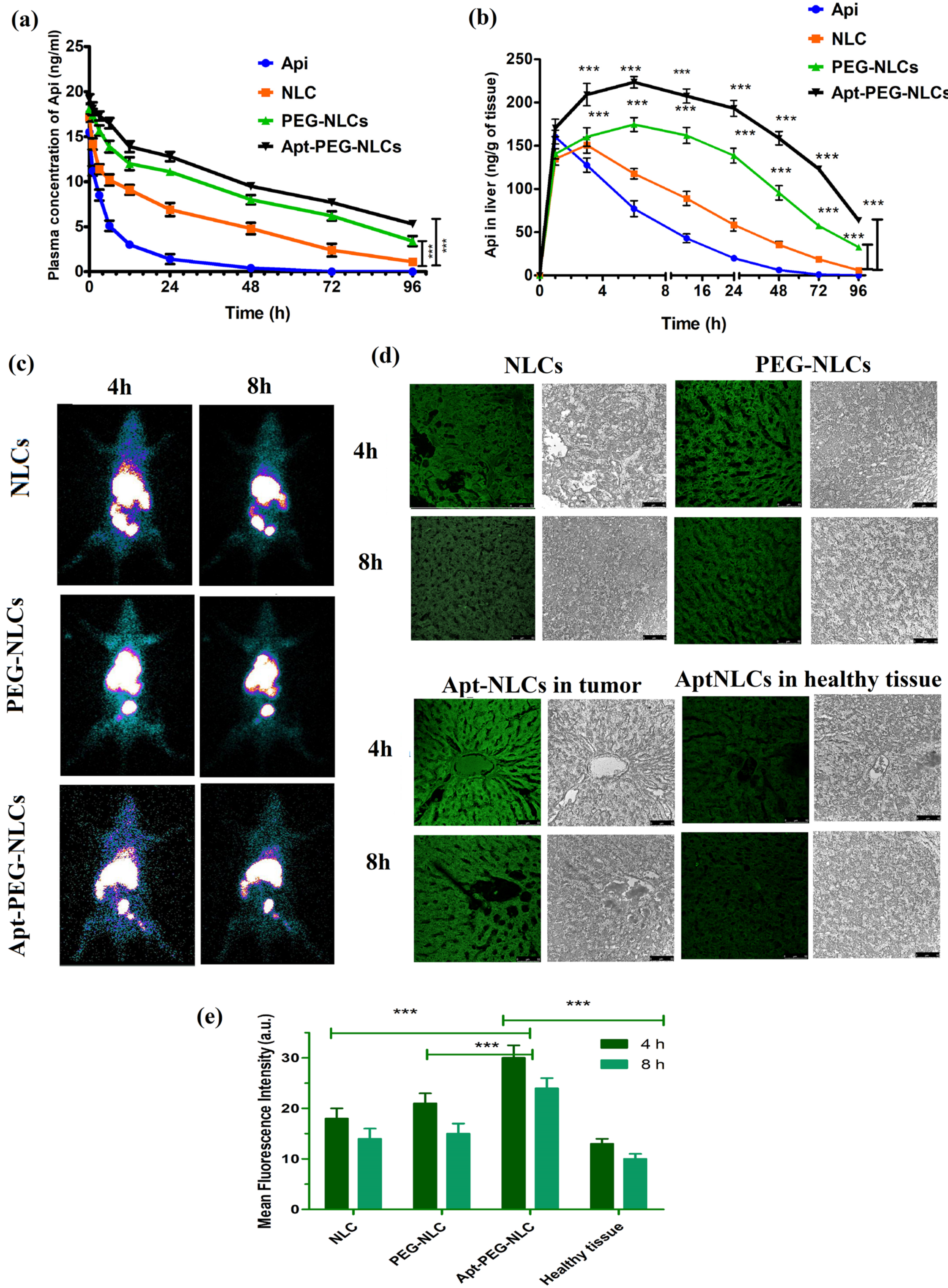


Fig. 6 (See legend on previous page.)

Table 2 Results of biodistribution of experimental formulations

Organ/Tissues	Biodistribution of ^{99m}Tc-labeled experimental nanoliposomes in HCC induced rat model					
	^{99m}Tc-NLCs		^{99m}Tc-PEG-NLCs		^{99m}Tc-Apt-NLCs	
	4 h	8 h	4 h	8 h	4 h	8 h
Heart	0.587 ± 0.043	0.347 ± 0.065	0.489 ± 0.047	0.309 ± 0.028	0.199 ± 0.026	0.155 ± 0.007
Blood	2.538 ± 0.067	1.484 ± 0.091	5.8973 ± 0.53	2.369 ± 0.366	4.392 ± 0.201	2.523 ± 0.115
Liver	37.234 ± 0.33	18.899 ± 1.25	47.686 ± 1.92[#]	33.078 ± 1.06	60.69 ± 1.63*	49.51 ± 0.478^
Lung	3.778 ± 0.209	2.554 ± 0.411	2.502 ± 0.295	2.678 ± 0.135	1.667 ± 0.349	2.319 ± 0.260
Spleen	0.627 ± 0.033	0.545 ± 0.036	0.833 ± 0.047	0.633 ± 0.106	0.724 ± 0.052	0.446 ± 0.042
Muscle	0.163 ± 0.013	0.142 ± 0.013	0.1893 ± 0.01	0.172 ± 0.022	0.118 ± 0.024	0.156 ± 0.011
Intestine	13.902 ± 0.29	8.988 ± 0.932	17.974 ± 0.61	14.27 ± 0.249	5.828 ± 0.074	3.651 ± 0.048
Stomach	0.487 ± 0.018	0.342 ± 0.028	0.653 ± 0.039	0.355 ± 0.012	0.387 ± 0.016	0.308 ± 0.032
Kidney	10.033 ± 0.71	4.461 ± 0.465	4.475 ± 0.276	3.254 ± 0.127	3.631 ± 0.406	2.591 ± 0.268
Urine	15.616 ± 0.14	14.706 ± 0.24	10.702 ± 0.82	9.755 ± 0.769	6.929 ± 0.851	5.654 ± 0.650

Data are expressed in % mean of injected dose (ID) per gram of organ/tissue ± SD (n = 3)

(#) Indicated significant ($p < 0.05$) amount of drug accumulation in liver in PEG-NLCs treated Gr over NLCs, while (*) indicated significant ($p < 0.05$) amount of drug accumulation in liver in Apt-PEG-NLCs treated Gr over PEG-NLCs Gr. at 4 h. (^) indicated significant ($p < 0.05$) amount of drug accumulation in liver in Apt-PEG-NLCs treated Gr over PEG-NLCs at 8 h

Macroscopic and microscopic examinations of the liver of rats treated with experimental nanoliposomes

One or multiple tumorigenic hyperplastic nodules (HN) with different tumor volumes were developed in experimental animals receiving carcinogens and various drug formulations. Post-treatment, we observed Gr B animals (HCC control) showed enormous tumorigenic growth (Fig. 7). There was no gross improvement of tumor suppression noticed upon free apigenin treatment in Gr C animals. In the case of nonconjugated nanoliposome (NLCs/PEG-NLCs) treated groups (Gr D & E), an almost 50–60% reduction in average tumor volume was detected in comparison to the control group. Liver weight against total body weight of animals varied upon the experimental treatments (Fig. 7c). However, the value was close to normal in the case of carcinogen-treated rats treated with Apt-NLCs. Nevertheless, a significant diminishing effect in tumor incidences (> 90%) in the case of Gr F animals was noticed compared to other treated groups. The carcinogen-treated animals (Gr F) that received Apt-NLCs comprised some tiny tumor lesions with an average size of < 50 mm^3, confirming the superior therapeutic potential of Apt-NLCs as a commanding anticancer formulation.

We have done an extensive microscopic examination of hepatic tissues collected from all the treatment group animals at the end of the treatment protocol. In histopathological images (Fig. 7), we observed characteristic HAF lesions with lobular or spongy inflammatory ducts in the case of Gr B, carcinogen control animals. However, upon anticancer treatment with Api/NLCs/PEG-NLCs/Apt-NLCs, various degrees of improvement in reforming typical hepatocellular architecture were observed. The appearance of scattered apoptotic artifacts in the case of group D & E animals was observed. In the case of Group F animals, Apt-NLCs promoted restructuring the cellular panache almost toward normal hepatocytes. The highest degree of reduction in hepatic altered focal lesions (HAF) occurrence (Fig. 7) was observed in the case of Apt-NLCs (Gr F), along with a significant reduction of tumor volume as well as HAF area (Table 3) compared to NLCs/PEG-NLCs (Gr D & Gr E) treated experimental animals, which also supported the optimum curative potential of aptamer-conjugated nanoliposomes rather than other normally developed nonconjugated nanoformulations (NLCs/PEG-NLCs).

Assaying apoptosis-related signaling proteins in the experimental rats

Histograms (Fig. 8) representation from the data generated through RT-PCR assay with liver tissue samples obtained from different experimental groups of rats expressed the maximal upregulation of p53, caspase-3 activation, and the maximum downregulation in Bcl-2 in carcinogen-induced rats treated with Apt-NLCs (functionalized nanoliposomes), suggesting the highest degree of apoptosis caused by Apt-NLCs in comparison to nonconjugated nanoliposomes (NLCs/PEG-NLCs).

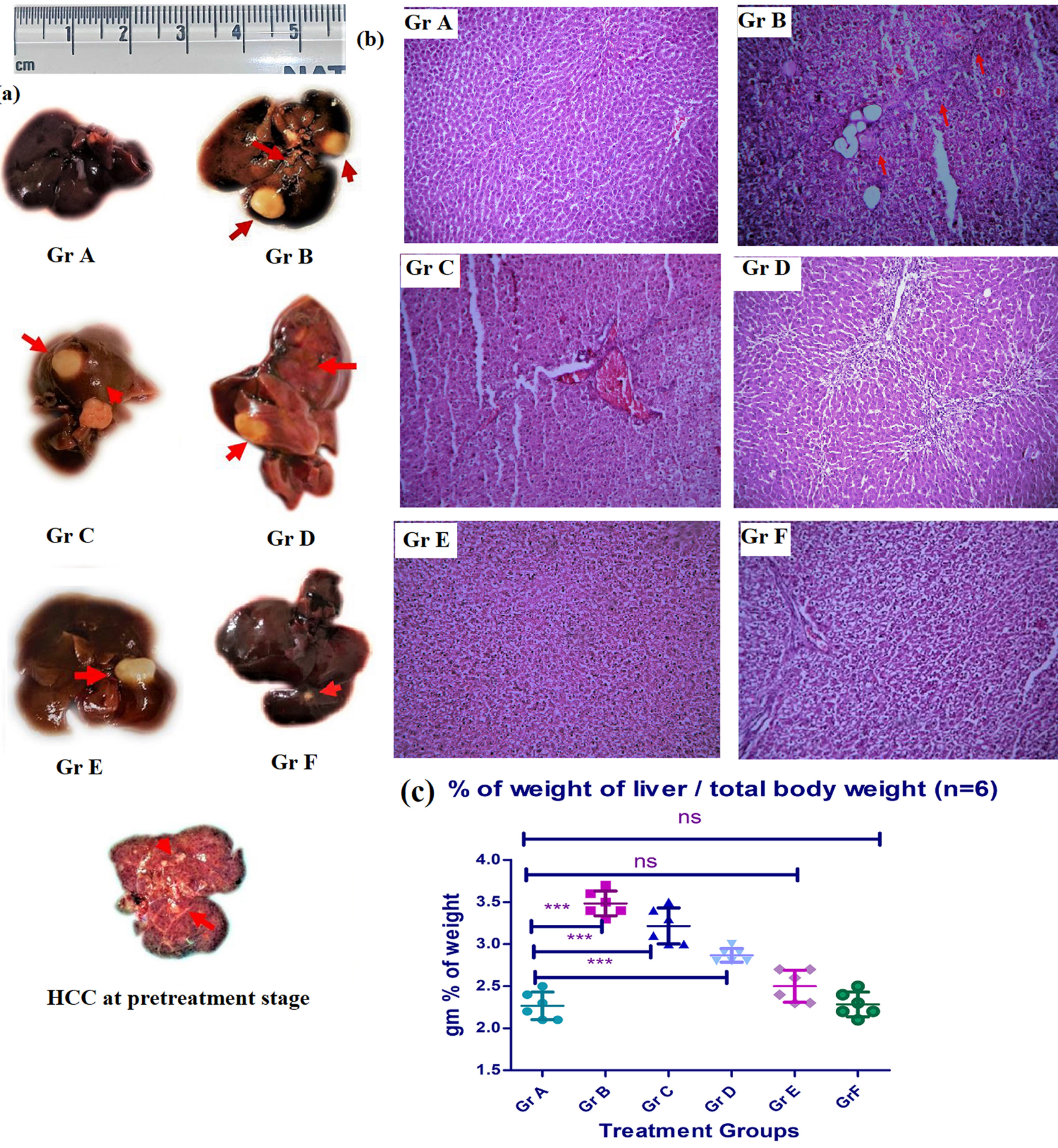


Fig. 7 Microscopic and macroscopic hepatic analysis of the experimental rats. **a** Macroscopic (in 100X magnification) liver images for the different experimental animal groups, Gr A to Gr F. **b** H & E histopathological microscopic images of liver tissue sections for various animal groups. **c** Represented % of weight of liver vs total body weight in different treatment group. (***) represent significant ($p < 0.05$) changes among group as indicated in figure and ns denoted as non-significant changes

Assaying hepatic functionality in different groups of experimental animals

Serum AST, ALT, and ALP levels generally indicate normal or pathological conditions of the liver [27]. Here, serum AST, ALT, and ALT were increased in carcinogen-treated (carcinogen control) rats compared to the normal rats. Otherwise, the levels were decreased upon treatment of NLCs/PEG-NLCs and Apt-NLCs. (Additional file 1: Table S5). The data showed the highest level of improvement in Gr F animals (Apt-NLCs treated carcinogenic animals). Moreover, no significant changes in normal serum AST, ALT, and ALP values in Gr G animals (normal animals treated with Apt-NLCs).

Higher bioavailability and intratumor drug delivery by aptamer conjugated nanoliposomes, Apt-NLCs, in experimental animals produced the greater anticancer potential of apigenin than other plain nanoliposomes (NLCs/Apt-NLCs). Likewise, predominant and significant

Table 3 Quantitative data referring tumor development and total HAF (hepatic altered foci) area among different group of experimental animals

Treatment Groups	Number of the rat developed tumour post treatment	Average tumour volume (n = 6) (mm)^3	Number of HAF area per cm^2 under microscopic observation on treatment
Gr A	0/6	–	–
Gr B	6/6	1502.89 ± 9.98$^{\#}$	86.74 ± 5.90$^{\#}$
Gr C	6/6	1261.5 8 ± 8.09$^{\#\$}$	67.89 ± 8.09$^{\#\wedge}$
Gr D	5/6	821.08 ± 4.67$^{\#\$}$	56.89 ± 6.06$^{\#\wedge ns}$
Gr E	3/6	786.73 ± 5.56$^{\#\$ns}$	49.97 ± 0.58$^{\#\wedge **}$
Gr F	2/6	35.89 ± 7.56$^{\#\$***}$	12.63 ± 0.43$^{\#\wedge ***}$
Gr G	0/6	–	–

$^{\#}$ Data represented mean ± SD (where, n = 6 in each group of animals)

$^{\$,\wedge}$ indicated significant ($p < 0.05$) reduction of tumor volume and HAF observed in (Gr C-Gr F) in comparison to positive control group Gr- B. Again, compared among the test nanoliposome (Gr D, Gr E, and Gr F), in Gr F (aptamer conjugated nanoliposome treated group) significant ($p < 0.05$) reduction of both tumor volume and HAF were observed mentioned in figure (***); while in Gr E (PEG-NLCs treated group showed non-significant (ns) amount of tumor volume reduction, (**) significant amount of HAF reduction and Gr D (NLCs treated animal group) showed non-significant (ns) amount of HAF reduction

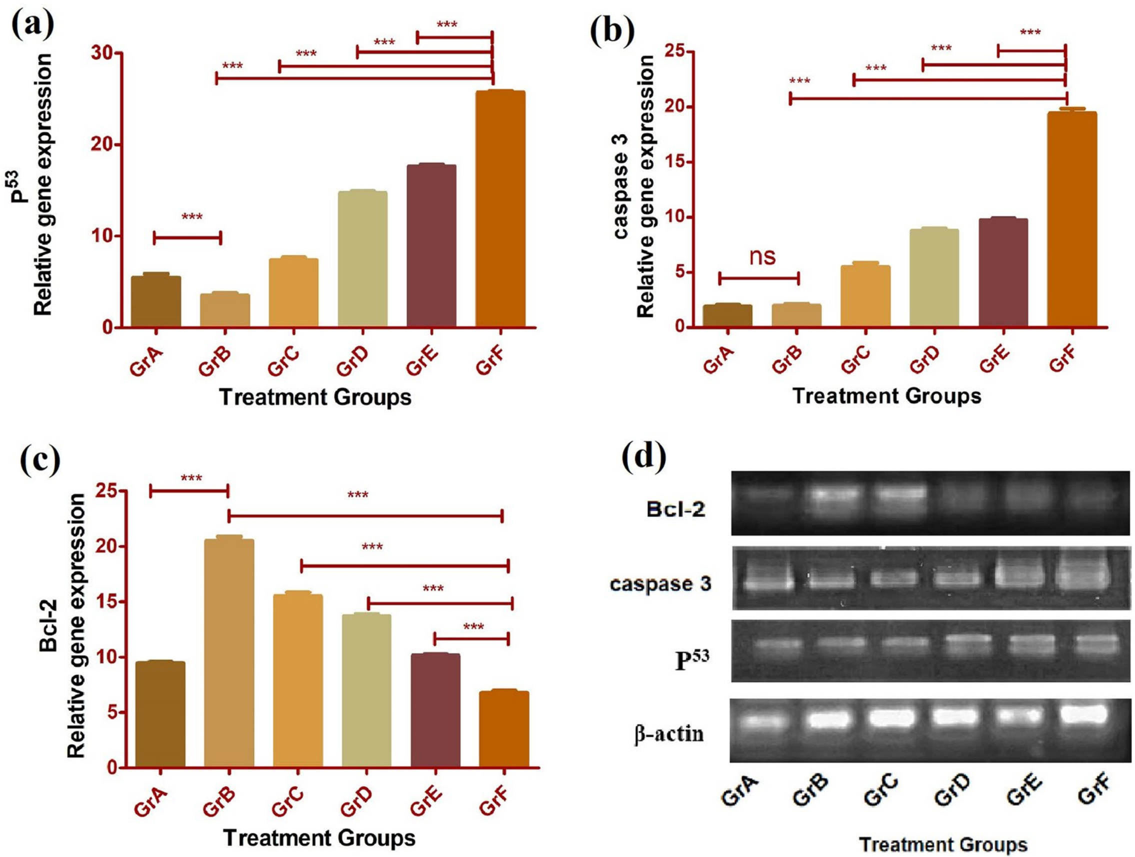


Fig. 8 Comparative apoptotic gene expression analysis through RT-PCR with liver tissue samples from different experiential groups of animals (A-F). **a** Representing increases level of p53, **b** caspase-3, and **c** decreased level of Bcl-2 expression. Data indicated ± SD (n=3), (***) expressed significant ($p < 0.05$) upregulation or downregulation in gene, in conjugated nanoliposomes Apt-NLCs treated animals (GR F), samples in comparison to non-conjugated NLCs/ PEG-NLCs nanoliposomes (Gr D/F), free apigenin (Gr C) and carcinogen positive animals (Gr B). ns represented no significant changes among Gr A and Gr B for caspase expression assay. **d** Representing β- actin, p53, caspase -3, Bcl-2 gene expression of different experiential groups of animals in agarose gel electrophoresis study

therapeutic progress was observed in HCC-developed rats treated with Apt-NLCs by controlling tumor incidences in the liver and restoring HAF toward normal in H&E-stained liver tissue sections over the nonconjugated nanoformulations and free apigenin. Further, in vivo apoptosis-related protein expression estimation among the different groups of experimental rats using qRT-PCR revealed the highest activities in caspase-3, caspase-9, p53, and the lowest activity in Bcl-2 in Apt-NLCs treated animals (Fig. 8). There were not many changes observed in liver functionality (ALT, AST, ALP) in Apt-NLCs treated in normal animals. The findings, thus, support the remarkable opportunity of aptamer functionalized PEG-containing nanoliposomes (Apt-NLCs) as a potent drug delivery system that could deliver chemotherapeutics, importantly active bio-flavonoids (here apigenin) to the target neoplastic hepatic region.

Conclusion

Achievement of any chemotherapeutic or any bioactive compounds in cancer therapy depends on their effective and rational drug delivery approach. Bioflavonoids, as a lipophilic anticancer agent, have to overcome robust pharmacokinetic as well as molecular drug delivery challenges in HCC. In our present study, we have developed aptamer functionalized nanoliposomes and highlighted first time the mechanistic approach of drug uptake, accumulation, and modulation in apoptotic signaling pathways for apigenin in neoplastic hepatic cells, which was triggered by reasonably designed aptamer functionalized PEG-containing nanoliposomes. Phosphorothioated amino-modified AS1411 aptamer-conjugated apigenin-loaded PEG-NLCs successfully induced apoptosis in Hep G2 liver cancer cells and arrested cell-cycle mostly at G2/M phase by upregulation in p53 and caspase activities and downregulation of Bcl-2 activity. Accumulating the aptamer conjugated formulation (Apt-NLCs) was distinctively more in the liver than in the other tissues. Further, Apt-NLCs accumulation in tumors in the liver was predominantly greater than in the surrounding non-cancerous hepatic tissue, suggesting successful site-specific drug distribution. Apt-NLC reduced tumor incidences and neoplastic hepatic altered lesions, suggesting its potential anticancer effect in vivo. Apt-NLCs impressively targeted the neoplastic hepatic region in rats. Further, we can explore the functional efficacy of aptamer functionalized nanoliposomes in additional preclinical animal models for gathering more relevant scientific pieces of evidence. Thus, the process for translation from preclinical to clinical drug development will be narrow.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12951-022-01764-4.

Additional file 1. Table S1: Details of physicochemical characterization of NLCs, PEG-NLCs, Apt-NLCs: Table for particle size, drug loading and zeta potential of plain nanoliposomes (NLCs), PEGylated Nanoliposomes (PEG-NLCs) and Aptamer functionalized PEGylated nanoliposomes (Apt-NLCs); 2. Fig S1: Particle size and surface characterization of plain nanoliposomes (NLCs) and PEGylated Nanoliposomes (PEG-NLCs). (a), (c) average particle size distribution for NLCs and PEG-NLCs respectively, (b), (d) surface morphology applying FESEM images for NLCs and PEG-NLCs respectively, 3. Table S2: In vitro drug release kinetics: The kinetic equations of drug release data tested for NLCs/PEG-NLCs/Apt-NLCs on various kinetic models with corresponding R2 (Regression coefficient) values were studied; 4. Fig. S2: Stability studies: (a) FESEM image of Apt-NLCs on (-4 °C) storage, (b) FESEM image of Apt-NLCs on (40 ± 2°C and 75 ± 5% RH), Table-S3: Drug loading and zeta potential of Apt-NLCs stored at (-4 °C) and (40 ± 2°C and 75 ± 5% RH) for six months; 5. Table S4: Cytotoxicity studies by MTT-assay: Table depicted respective IC50 (μM), Half maximum inhibitory concentration for apigenin, NLCs, PEG-NLCs, Apt-NLSs, Apt-BNLCs in HepG2 cells, Huh-7 cells and PBMC cells, 6. Table-S5: Assaying hepatic functionality in different groups of experimental animals # Data represented mean ± SD (where, n=6 in each group of animals). Table depicted respective AST/ALT/ALP values in all the experimental carcinogenetic animal groups treated with apigenin (Gr C), plain nanoliposomes (Gr D), PEGylated nanoliposomes (Gr E), aptamer conjugated PEGylated nanoliposomes (Gr F) along with normal animals treated with normal saline (Gr A) and normal animal treated with aptamer conjugated nanoliposomes.

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Author contributions

MD and BM conceived and designed the experiments, performed data analysis, and wrote the manuscript. MD performed the experiments. AAH also participated in performing experiments and manuscript writing. DD and RS helped in performing some cell line studies and animal experiments. SL as a technical expert, helped to perform instrumental analysis, BP performed experiments, and helped in drawing curves and statistical data analysis. All authors read and approved the final manuscript.

Declarations

Ethics approval and consent to participate

Permission from the Animal Ethics Committee of Jadavpur University was received before commencing any animal experiments. All experiments have been conducted following the guidelines of the Animal Ethics Committee of Jadavpur University.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 7

Aptamers and Their Potential in Site-Specific Nanotherapy Against Hepatocellular Carcinoma

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Abstract: Globally, hepatocellular carcinoma (HCC) is one of the most devastating neoplasia and hasa remarkably high mortality rate. Furthermore, the long latent period associated with HCC lends the diagnosis at the intermediate or advanced stages where the chemotherapy is the solitary therapeutic intervention. The responsiveness of HCC towards conventional chemotherapeutic agents is notably poor due to multiple factors. Among them, multiple drug resistance, reduced drug concentration at the tumor site, quicker clearance, and non-specific distribution are the prime causes leading to remarkably high off-target toxicity and mortality. More importantly, the approval of several multikinase inhibitors (MKIs) by the United States Food and Drug Administration (FDA) for the treatment of HCC as targeted therapeutics has been found to be inadequate to make a notable impact on survival. Therefore, ligand-based targeted therapeutics capable of delivering the therapeutic modality specifically into neoplastic hepatocytes have been explored extensively by researchers worldwide. Among the plethora of HCC-targeting ligands, aptamer-based targeted therapeutics in HCC have gained significant momentum compared to others due to some signature characteristics of aptamer, namely non-immunogenicity, low cost, non-toxicity, thermostability, simpler manufacturing, and high suitability for chemical modification. Despite their enormous potential, aptamer-based targeted therapeutics are still in infancy and require smarter thinking and quick translation from e-clinical to clinical application. Thus, the fundamental focus of the book chapter is to highlight promising features of aptamers, their production, chemical modification, mechanism of action, and finally, detailed emphasis has been given on the overall scenario of aptamer-based targeted therapeutics in HCC.

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INTRODUCTION

The term 'cancer' can be defined simply as the uncontrolled growth in abnormal cells present in any part of our body. Several types of carcinogens and genetic alterations are considered the primary factors responsible for cancer development (Maitra *et al.,* 2013). The causative agents that can develop normal cells into abnormally behaving cancer cells may include exposure to different chemicals or toxic compounds, pathogenic factors, ionizing radiation, and inherited or acquired mutations in the human genome. Classification of cancer depends on its location in the body and the kind of originating tissue or fluid, although some cancers may be of mixed type. Carcinoma, sarcoma, lymphoma, leukemia, and myeloma are five broad categories of cancer (https://stanfordhealthcare.org/medical-conditions/cancer/cancer/cancertypes.html).

Hepatocellular carcinoma (HCC), commonly characterized as the initial development of malignancy in hepatocytes, is one of the most frequently diagnosed cancer globally but is extraordinarily heterogeneous in nature. Generally, the heterogeneity in HCC is described by rapid dysregulation of cellular signal transduction pathways and the highly unpredictable tumor microenvironment in the neoplastic hepatocytes. Again, these abnormal alterations in the cellular pathophysiological environment are developed through some genetic and epigenetic changes in hepatocytes, and they are very comprehensively associated with multiple metabolic syndromes such as diabetes, obesity or alcohol abuse, non-alcoholic steatohepatitis (NASH) with altered sex, aging, and various environmental factors, including hepatitis B and hepatitis C along with other viral infections and immunogenic disorders (Dagogo-Jack *et al*., 2018). Thus, the therapeutic strategies of HCC are extremely challenging, along with minimum off-target cytotoxicity. Depending on the type and extent of cancer spreading, chemotherapy is chosen to treat cancer. For many years in the past, chemotherapy and radiation were options to save or at least prolong the life span of cancer patients. Radiation delays cancer growth, and on the other hand, chemotherapy helps manage different symptoms caused by cancer. However, the medical community is now aware ofthe extent of damage the chemotherapy and radiation therapy cause during treatment and the consequent health hazards that follow in the future (https://www.ctoam.com/precision-oncology/why-we-exist/standardtreatment/treatment/chemotherapy/). The need for more sophisticated tools for the treatment of cancer will provide selective cell-specific accumulation of chemotherapeutics with reduced side effects to normal cells.

Although chemotherapy has importance in treatment for cancer patients, the toxic effects (*e.g*., hair loss, nausea, and vomiting, *etc*.) of this conventional cancer therapy that occur due to the inability of chemotherapy to differentiate between cancerous and healthy cells, tendency to develop resistance to the chemical agents, and the necessity of using other treatment forms along with it limit its use (http://www1.udel.edu/chem/C465/senior/fall98/Cancer2/methods.html). Surgery, chemotherapy, and irradiation are the mainstream therapeutic approaches for cancer (Padma, 2015; Xu *et al.,* 2001; Dutta *et al.,* 2015; Dutta *et al.,* 2016). The main strategy of targeted therapy is to deliver a drug at the cancer-promoting specific tissue environment or to specific cancerous cells to target a particular protein or genes. The success of the therapy solely depends on targeted drug action in specific cancer cells, thus reducing toxic side effects in normal healthy cells (Gerber *et al.,* 2008; Padma, 2015). By utilizing the "enhanced permeability and retention effects" (EPR) effect, passive targeting to the tumor can be attributed to various macromolecules (Dutta *et al.,* 2019; Kale *et al.,* 2020, Hazra *et al.,* 2021).

On the other hand, direct targeting to a particular target antigen can be achieved through specific monoclonal antibodies or by small molecule drugs that act by altering their signaling. Under indirect approaches, some proteins express specifically on the tumor cell surface and serve as special targeting devices. These can be actively targeted by conjugating the antigen-specific ligand molecules to the drug carrier (Wu *et al.,* 2006).

Different newer technologies have got rapid and huge expansion for the molecular level diagnostics and tumor targeting strategies which made it essential to develop more specific targeting ligands to target definite cell surface molecules expressed uniquely in tumor tissues or cells (Kim *et al.,* 2018). Extensive research is on the pipeline, involving the active targeting to cancer cells utilizing different ligands such as proteins, peptides, monoclonal antibodies, small molecules, and last but not least, the nucleotide sequence-based targeting molecule, aptamers (Ray *et al.,* 2021; Mondal *et al.,* 2019; Mukherjee *et al.,* 2020; Mukherjee *et al.,* 2021). They can recognize cancer cell-specific and unique receptors and bind with them (Sivashankari *et al.,* 2016). The use of aptamer has been widely investigated for its role in targeted therapy, the discovery of biomarkers, *in vivo* imaging, and *in vitro* diagnosis (Sun *et al.,* 2014; Dutta *et al.,* 2018). Aptamers are small, structured oligonucleotides, *i.e*., RNA or single-stranded DNA ligands that are selective and have a high affinity towards the target molecules.

In this chapter, our main objective is to explore aptamer-mediated targeted therapy, the diagnostic and theranostic use, its target receptor-specific designing

procedures, modifications, characterizations, mechanism of actions, and valuable applications for the treatment of HCC.

Therapeutic Approach for HCC: Active *vs.* Passive Targeting and Aptamer.

Apart from developing newer systemic chemotherapeutics and immunogenic drugs, the establishment of a novel drug delivery system through active or passive targeting has been in trend for the last two decades. The invention of nanotechnology has embarked on a novel platform to manage malignancy or cancers considerably better and intrigued scientists worldwide for its characteristic versatility. The foremost advantages are accumulating therapeutics at the tumor site through enhancing permeability and EPR effect with its unique nanosize and physio-chemical properties, thus overcoming systemic adverse effects (Adiseshaiah *et al*., 2016). This process, described as passive targeting, relies upon the availability of these tiny nanoparticles besides the leaky or damaged vasculatures of the impaired lymphatic system, one of the distended features of tumorous tissues (Kalyane *et al*., 2019). However, the dependability of EPR-based passive targeting has always been under doubt. In recent times, it was reported that the uptake of nanoparticles in tumor tissues was limited to 0.7% of the injected therapeutics (Wilhelm *et al*., 2016). Researchers are obligated to design a smart nanocarrier system that can proactively reach the target tumor and steadily improvise exclusive neoplastic cellular uptakes. Thus, active targeting has been instigated through conjugating of various tumor-specific ligands such as peptides, aptamers, antibodies, small molecules, *etc*., with the nanocarriers to recognize and attach with their complementary receptor partners or surface membrane proteins generally overexpressed on target tumor cells (Bazak *et al.,* 2015). Many impediments such as by-passing systemic toxicity, blood-brain barrier, and overcoming multi-drug resistance in tumors seem to be sensibly resolved with active targeting in curing cancer.

Some bio-conjugates such as asialofetuin or their synthetically modified analogues could be applied as conjugating ligands with nanocarriers to reach fibrotic liver efficiently through targeting tumor-specific ASGPR (Asialoglycoprotein receptor is commonly found overexpressed in mammalian liver cells). The site-specific biodistribution of an Alexa647-labeled polyvalent drug carrier to the neoplastic hepatocytes rather than normal parenchymal tissues in rodents was effectively explored depending on ASGPR-directed hepatocellular accumulation of the therapeutics, while prolonged retention time of the formulation was confirmed through GalNAc attached pegylated polymers (Sanhueza *et al*., 2017). PES (Polyehelenesebacate) -Gantrez®AN- 119 encapsulated Doxorubicin nanoparticles have been formulated for restoring safety and efficacy in hepatocellular carcinoma following this asialoglycoprotein-

mediated active targeting (Pranatharthiharan *et al.*, 2017). Another smart bioconjugate has established the potential of ligand-fitted biodegradable micelles (Gal-CLMs) in HCC. Gal-CLMS, a cross-linked copolymer attached with galactose (targeting ligand), efficiently delivers paclitaxel to the tumor-bearing tissues of some nude mice and maintains controlled drug release patterns in a specified physiological pH *in vitro* (Zou *et al.*, 2014). Again, therapeutic superiority of doxorubicin encapsulated liposomes (CHOL-Al-AG) when conjugated with arabinogalactan (a modified galactose with high affinity towards liver tumor tissues) is demonstrated by Pathak *et al.,* compared to basic liposomes *in vitro* (Pathak *et al.*, 2015). Peptide sequence RGD performs as a labeling moiety for collagen VI and is popularly used to target integrin (overexpressed in fibrotic hepatocyte) (Bartneck *et al.*, 2014). Integrin- αv β3 targeted nanoliposomal paclitaxel (PTX) was developed by Chen *et al.* to improve solubility and bioavailability of paclitaxel (PTX) for effective drug delivery in HCC (Chen *et al.*, 2015). Both *in vitro* and *in vivo* studies confirmed that encapsulated and conjugated drugs to DSPE-PEG with RGD-motif (RGD-L--PTX) had successfully improved the drug accumulation and enhanced the antitumor activity than the unconjugated or free form of PTX. Another effective amalgamation of doxorubicin and sorafenib with iRGD is fabricated, entrapping these in smart lipid-based hybrid NPs for their efficacious delivery to the target neoplastic hepatocyte both *in vivo* and *in vitro* for boosting anticancer activity (Zhang *et al.,* 2016). Wang *et al*., 2016 demonstrated that CD44 antibody-triggered nanoliposomal anticancer drug delivery for molecular imaging and therapy in HCC. In a comprehensive review, Ravichandran and Rengan described various therapeutic uses of aptamers as active drugs targeting moiety through attaching with nanocarriers or acting as labeled biosensors (Ravichandran and Rengan, 2020). Dual aptamer (A15 and CL4) factionalized pegylated nanoparticles were successfully developed to target both CD133 and EGFR of HCC to potentiate the cytotoxicity of salinomycin towards HCC stem cells (Jiang *et al.*, 2015). In another comparative study, Chakraborty *et al.* described the affinity of different types of aptamers towards different liver cancer cell lines (Chakraborty *et al*., 2020).

Aptamer as a Targeting Molecule and its Modifications for *In Vivo* Stability

Single-stranded nucleic acid oligonucleotides combine with their complementary strand and can fold into 3D scaffold conformation to interact with specific target protein receptors overexpressed on the surface of the diseased organ. Small single-stranded DNA or RNA sequences within the size range of 15 to 50 base pairs designed to target several types of receptor molecules are called chemical antibodies or aptamers (Pang *et al*., 2018, Tabarzad and Jafari, 2016). The term ‘Aptamer’ originates from the Latin word, ‘Aptus’, which denotes ‘to fit’, and the

Greek word 'Meros', which means 'part'. The aptamer is synthesized by systematic evolution of ligands by exponential enrichment (SELEX) method generating a random single-stranded oligonucleotide library for a particular protein receptor family (Tuerk and Gold, 1990). The receptor affinity and selectivity are regulated by conditional modifications such as ionic strength, pH, and temperature of SELEX technology. The chemically synthesized target-specific oligonucleotides are developed according to the 3D conformational shape of the receptor of interest. There are several types of SELEX methodology named Capillary Electrophoresis SELEX (CE-SELEX), Crossover-SELEX, Conditional-SELEX, Tissue slide-based SELEX, Subtractive-SELEX, High-Throughput Sequencing SELEX (HTS-SELEX), Conditional-SELEX, Microfluidic SELEX (M-SELEX), apart from the conventional technology for the modifications of aptamers (Cerchia and de Franciscis, 2010; Hung *et al*., 2014; Zou *et al*., 2018). Many advantageous applications are available for aptamer as a targeting moiety over other targeting ligands, primarily antibodies. Aptamers can form into different defined secondary structures such as loop, stem, bugle, hairpin, G-quadruplex, pseudoknot, which are followed by the development of unique form 3D structures that can bind specific receptor molecules by hydrogen bonding, hydrophobic and electrostatic interactions, and van der Waals forces interactions (Camorani *et al*., 2017). The aptamer and receptor-binding interaction are similar to the antigen-antibody interaction. However, the small size of the aptamer is favorable for deep tissue penetration, especially the desmoplastic stromal barrier developed in different types of tumors with a wide range of targeting possibilities and long shelf-live. The aptamer can be easily conjugated chemically with a variety of nano formulations for targeting purposes to overcome off-target cytotoxicity to the normal tissues. The short production time and low cost have made it a preferable choice of targeting ligand. Apart from targeting specificity, an aptamer can be considered as a therapeutic molecule capable of hindering protein-protein interactions by receptor-ligand binding; therefore, it can function as an antagonist. Thus, some aptamers have agonist-like activities for extracellular receptor domains followed by inhibition of receptor-mediated activation of the downstream signaling pathway. Furthermore, there are various other aptamer applications in the biomedical field, including biosensors, diagnostic, aptasensors, and imaging systems.

Despite its wide range of biological applications, the long-time *in vivo* stability of aptamer is a matter of concern due to the presence of nuclease in the bloodstream. Chemical modifications in the oligonucleotide sequence of aptamers can introduce the nuclease stability in blood circulation sequences. Among several types of aptamer modifications, the most common chemical modifications are the phosphate backbone modification such as oxygen replacement with sulfur in ribose unit or inside the phosphodiester linkage, locked nucleic acids, and

circular, multivalent, and dimerization of aptamers, end-capping at the terminal end. The 3′ to 5′ exonuclease attack can be blocked by capping the 3′-end of the aptamer. Another strategy to protect the aptamer from exonuclease activity is the capping of the 3′ end with inverted deoxy-thymidine modification. Cholesterol can be conjugated at the oligonucleotide end by a spacer, resulting in many fold increase in plasma half-life. A major drawback of the small-sized aptamer is the high clearance rate that the conjugation of large PEG molecules can control at the 5′ end of aptamer sequences. The 2′ position of the sugar modifications can improve the half-life of the aptamer sequence. The 2′ -Fluoro (2′ -F), 2′ -amino (2′ -NH2) and 2′ -O-methyl (2′ -OMe)2′ -substitute modifications are the most common 2′ position substitution on ribose unit (Camorani *et al*., 2017; Esposito *et al*., 2011; Chen *et al*., 2019, Mallikaratchy, 2017). The phosphorothioate backbone modification is another aptamer stability procedure that can ensure the long-term stability of the aptamer in plasma. The phosphorothioate backbone modified aptamer conjugated nanoparticle was reported to target EpCAM receptor overexpressed on the surface of colorectal carcinoma (Dutta *et al*., 2018).

Aptamer and HCC

The steep rise in HCC cases along with poor prognosis acts as a driving force that directs the researchers around the globe to develop strategies that lead to substantially superior and well-organized management of HCC, resulting in distinctively superior outcomes in the present scenario (Zhang *et al.,* 2016b; Li *et al.,* 2016; Mohamed *et al.,* 2017; Sun *et al.,* 2014; Zhou and Rossi 2014**)**. The efficient management should address two key issues: (a) identification of highly efficient biomarkers which will help diagnose the diseases at the early stages; (b) development of substantially effective and safer targeted therapeutics based on the biomarker proteins or receptors having remarkably improved penetration and retention in HCC tissues and significantly lowered off-target toxicity (Zhang *et al.,* 2019). The multiple promising features of aptamers enable them to successfully utilize both the aspects of effective management of HCC (Zhang *et al.,*2016b; Li *et al.,*2016; Mohamed *et al.,*2017; Fu and Xiang 2020; Zhou and Rossi 2014; Ladju *et al.,*2018**)**. For example, TLS11a, one of the most-studied aptamers, was utilized to develop an electrochemical biosensor (aptasensor) which enables straightforward, selective, highly sensitive, and label-free diagnosis of neoplastic hepatocytes (Shangguan *et al.,* 2008; Kashefi-Kheyrabadi *et al.,* 2014**)**. Further, they revealed that the developed aptasensor had the ability to detect the liver cancer cells within a very low concentration (2 cells/ml) and at a wide linear dynamic range. A microcantilever biosensor based on TLS11a that worked on a similar principle as that of aptasensor for the analysis of neoplastic hepatocytes was developed. However, its responsiveness was found to be less as compared to aptasensor (Chen *et al.,*2016**)**. TLS11a was conjugated with gold

nanoparticles and horse reddish peroxidase enzyme (HRP), leading to the development of cytosensor, which allows simple and sensitive (30cells/ml) detection of neoplastic hepatocytes (Jo and Ban 2016). In a sequential advancement in the development of diagnostic tools, a combination of TLS11a, indium tin oxide electrode assay, and multifunctional nanoprobes had been used, which showed a much-improved detection limit (Sun *et al.,* 2017). Xu *et al.* (2015) synthesized a series of aptamers capable of distinguishing HCC cells from normal liver cells and had K_D (dissociation constant) values within the range of 64-349 nM, indicating their high affinity. Further, treatment with trypsin substantially decreased the binding of aptamer with HCC cells, signifying that target proteins for aptamer binding were present at the surface of neoplastic hepatocytes. From the outcomes of the study, they concluded that the aptamers developed by them could be successfully explored to develop a tool for early detection, targeted therapeutics, and imaging agents. Santra *et al.* (2010) highlighted the successful implication of fluorescent silica nanoparticles in the imaging of malignant tissue owing to their photostability, brightness, and high emission. Hu *et al.* (2017) labeled the HCC cells with biotinylated TLS11a aptamer (Bio-TLS11a) followed by co-incubation with fluorescent silica nanoparticles functionalized with streptavidin (Sa-FSNPs). The interaction between streptavidin and biotin enabled *in vitro* assessment of HepG2 cells. Further, they claimed that Sa-FSNPs had the ability to specifically detect HepG2 cells with satisfactory sensitivity and were devoid of any notable off-target toxicity. Wang *et al.* (2016) developed an aptamer-based innovative strategy that enabled the detection of circulating tumor cells (CTCs) in HCC. The surface of nanofilm made up of a hydroxyapatite/chitosan (HA/CTS) was functionalized with carbohydrate sialic acid Lewis X (SLex), leading to the formation of the CTC-^{BioT}Chip platform, which exhibited efficient capture and identification of HCC CTCs. HCC can develop upon exposure to a small number of aflatoxin B_1(AFB_1). Joo *et al.* (2017) developed graphene oxide (GO) and fluorescein imide functionalized AFB_1-specific aptamer-based rapid and efficient detection method for AFB_1. The binding of the aptamer to AFB_1 resulted in a conformational change, which enabled the aptamer to interact with GO leading to a decrease or quenching of fluorescence. The published reports in the literature (Watany *et al.,* 2017; Mohammed *et al.,* 2016; Fezza *et al.,* 2019) pointed out the substantial potential of Dickkopf-1 (DKK1), secretory protein (an inhibitor of Wnt signaling transduction pathway) in early detection of HCC. Zhou *et al.* (2019) developed two DKK1-targeting aptamers, such as slow-acting and fast-acting. Experimental evidence showed that slow-acting aptamer was suitable for aptamer-based enzyme-linked immunosorbent assay (ELISA), whereas fast-acting variety was found to be useful in flow cytometry and spot-blot. The latest advancement in the arena of HCC detection based on TLS11a aptamer has involved its conjugation

with a fluorescent probe that can differentiate between malignant and normal hepatocytes. Findings of *in vitro* study in liver cancer cells and analysis of frozen HCC tissue section revealed that aptamer-functionalized fluorescent probe emitted fluorescent signal upon binding to targeted cancer cells.

Aptamers and *In Vivo* Imaging in HCC

In vivo imaging plays a pivotal role in the clinical diagnosis of tumors, and it utilizes different techniques such as magnetic resonance imaging (MRI), ultrasound molecular imaging, computed tomography (CT), and fluorescence molecular tomography (Zhang *et al.,* 2019). For remarkable improvement in the efficiency of different *in vivo* imaging techniques, the applications of various aptamer-based contrast agents, probes, and fluorescent groups have been explored. The findings have shown a highly promising outcome. For example, magnetic resonance imaging (MRI) has gained a substantial preference for *in vivo* imaging in HCC to detect tumors because of its exceptional spatial resolution for soft tissues. However, short circulation half-life and a deficiency in tumor-targeting specificity of commercially available MRI-contrast agent, gadolinium-DTPA (diethylenetriamine penta-acetic acid) (Gd^{3+}-DTPA), leads to the feeble outcome of MRI-based imaging in HCC (Bayat *et al.,* 2019; Bouvier-Muller and Duconge 2018; Zhang *et al.,* 2019**)**. To overcome drawbacks along with remarkable improvement of imaging efficiency, Yan *et al.* (2018) synthesized an aptamer by SELEX capable of targeting Endoglin (CD105), type I membrane glycoprotein overexpressed in neoplastic hepatic cells and in the neovasculature of the endothelial cells. The abundant expression of CD105 has been observed in the tumor periphery. The aptamer showed high affinity (K_D 98pmol/L) for the target protein. They successfully developed an HCC-targeting nanoprobe upon conjugating aptamer and imaging reporter, and the prepared nanoprobe allowed successful visualization of orthotopic HCC xenografts with diameters as small as 1-4 mm. Upon successfully completing the study, they concluded that the multimodal nanoprobe has the potential of non-invasive imaging of tiny HCC by pre-operative MRI leading to successful tumor removal *via* intraoperative near-infrared (NIR) fluorescence imaging. Zhao *et al.* (2018) developed ultrasmall superparamagnetic iron oxide (USPIO)-based magnetic resonance probe which was functionalized with AP613-1, capable of targeting glypican-3 (GPC3), a heparan sulfate proteoglycan highly expressed in the plasma membrane of most HCC cells, but not in normal liver tissue or benign hepatocellular nodules, with high affinity and specificity. They synthesized oleic acid-coated USPIO nanoparticles whose surfaces were tailored with amino polyethylene glycol. AP613-1 functionalized USPIO nanoprobes were prepared by the reaction of a 5′-carboxyl group of aptamer with an amino group of USPIO. Findings of *in vitro* investigations in malignant hepatocytes and *in vivo* studies in GPC3-expressing

HCCs in xenograft mice revealed high specificity of the aptamer-functionalized magnetic probe for GPC3 cells leading to targeted imaging effect exclusively in malignant hepatic tissues.

Aptamers and Targeted Delivery in HCC

The significantly high mortality rate in HCC is due to the multiple potential drawbacks of currently available chemotherapeutics in HCC. Among them, certain potential drawbacks that result in greatly compromised therapeutic outcomes in HCC include faster clearance, low drug concentration in HCC cells (only 5-10% of dose accumulated in neoplastic hepatocytes as compared to dose accumulated in the normal liver), resulting in sub-optimal effect and multi-drug resistance (MDR) and non-specific biodistribution, and therefore, severe toxicity in healthy tissues (Zhang *et al.,* 2016). Thus, the rapid development and availability of targeted therapeutics capable of specifically delivering therapeutic payloads into neoplastic hepatocytes and the massive reduction in toxicity in healthy hepatocytes are badly needed at the clinical level for remarkable improvement in therapeutic outcomes. The plenty of evidence in the literature has revealed significant superiority of aptamer over the other targeting ligands in the development of targeted therapeutics in HCC (Sun and Zu 2014; Zhang *et al.,* 2016; Li *et al.,* 2016; Mohammed *et al.,* 2017; Elnaggar *et al.,* 2019; Chen *et al.,* 2019; Li *et al.,* 2016; Ladju *et al.,* 2017**)**. Despite the fact that significant research on targeted chemotherapeutics involving conjugation of aptamer with antitumor drugs, oligonucleotides, or nanocarriers has been undertaken (Shangguan *et al.,* 2008; Trinh *et al.,* 2015; Dong *et al.,* 2017; Weigum *et al.,* 2016), successful targeted therapeutics in HCC is still in infancy. Chakraborty *et al.* (2020) explored the potential of phosphorothioate backbone-modified TLS 9a (designated as L5) selective delivery of therapeutics in HCC cells for the induction of target-specific apoptosis in them. Further, they compared the targeting potential L5 with previously reported HCC-targeting aptamers (TLS-11a, AS-1411, APS 613-1) and non-aptamer ligands (galactosamine/transferrin) by conjugating them with polymeric nanoparticles which were prepared from US Food and Drug Administration (USFDA)-approved polymer, poly(lactic-co-glycolic) acid (PLGA) and evaluated through a series of well-defined *in vitro* and *in vivo* investigations. Findings of the study revealed that L5-functionalized drug-nanocarrier (PTX-NPL5) showed maximum potency to induce apoptosis preferentially in neoplastic hepatocytes among the different types of investigated formulations used in this study. Further, it showed no appreciable toxicity against healthy hepatocytes both *in vitro* and *in vivo*, unveiling a new avenue in the arena of targeted therapeutic against HCC. They highlighted that chain-length and affinity (denoted by K_D value) are equally crucial in selecting aptamers to develop aptamer-functionalized targeted therapeutics by comparing the characteristics of

L5 and TLS 11a (designated as L2). Computational modeling showed L5 specifically targeted surface biomarker proteins, TAG-72 and HSP70, which are the surface biomarkers specifically present on the surface of neoplastic hepatocytes. The examples of aptamer-mediated targeted delivery systems capable of directing therapeutic payloads preferentially in neoplastic hepatocytes have been summarized in Table **1**.

Table 1. Summary of aptamer-functionalized nanocarriers for targeted delivery of therapeutics.

Name of Aptamer	Characteristics of Aptamer	Target	Brief Outcome	References
A15 to target CD133 cells and CL4 to target to target EGFR	A15 and CL4 both were DNA aptamers and their sequences are given below A15- (5′-NH2 -CCCUCCUACAUAGGG-3′) CL4- (5′-NH2-GCCUUAGUAACGUGCUUUGAUGUCGAUUCGACAGGAGGC-3′)	CD133 cells and EGFR receptor	Salinomycin-loaded PLGA nanoparticles (SN) were prepared by multiple emulsion solvent evaporation techniques. Aptamers were conjugated on the surface of SN by using the N-(--Dimethylamino propyl)-N'-ethyl carbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS). A15 functionalized, CL4-functionalized, A-15, and CL4-functionalized nanoparticles were designated as CSN, ESN and CESN, respectively. The cytotoxicity study revealed that the cytotoxic potential of CESN in HCC cells and CD133+ HCC cells (MDR cancer cells) was remarkably superior as compared to CSN and ESN nanoparticles. Further, *in vivo* studies in HCC tumor bearing xenograft mice supported the findings of *in vitro* study. Thus, they concluded that substantially superior antitumour activity of CESN might be due to the efficient targeting by dual aptamers leading to much improved preferential delivery of salinomycin to HCC cells.	Jiang *et al.*, 2015

Name of Aptamer	Characteristics of Aptamer	Target	Brief Outcome	References
EpDT3	RNA aptamer. The sequences of unmodified and modified EpDT3 are given below. (5'GCGACUGGUUACCCGGUCG-3') -unmodified 5′-FAM-GCGACUGGUUACCCGGUCG-$(CH2)_6$-NH_2-3′- modified aptamer	EGFR receptor	Despite the fact that tumor suppressor gene PTEN (Ad5-PTEN) has enormous potential as an antitumor agent in HCC, several factors such as auto-immunogenicity, non-specific toxicity to normal tissues, and inferior stability in the blood stream because of neutralizing antibody restrict its widespread application. The linker molecule PEG was used to conjugate EpCAM-targeting EpDT3 aptamer with Ad5-PTEN to form EpDT3-PEG-A-5-PTEN conjugate (EPAP) by adopting the chemical synthesis method. Results showed that EPAP was found to 16-times more stable as compared to naked Ad5-PTEN. It significantly elevated gene expression and cellular internalization of EPAP by HepG2 cells, inhibited cell proliferation and migration, and decreased the toxicity in normal liver cells, L02. *In vivo* study in HepG2 xenograft in nude mice showed the substantial antitumor potential of EPAP. In short, EpDT3 had the potential to bind with overexpressed EpCAM receptors with high affinity and specificity, leading to preferential delivery of Ad5-PTEN in HCC cells resulting in remarkably superior therapeutic outcomes.	Liu *et al.*, 2018

Name of Aptamer	Characteristics of Aptamer	Target	Brief Outcome	References
TLS11a	DNA aptamer, 63 bp long, the sequence of aptamer was given below 5′-ACA GCA TCC CCA TGT GAA CAA TCG CAT TGT GAT TGT TAC GGT TTC CGC CTC ATG GAC GTG CTG TTTTTTTTTT-SH-3′	Hepatoma cells	Bi-specific T-cell engagers (BiTE) have evolved as an innovative therapeutic approach because of their excellent therapeutic potential in HCC. However, several drawbacks have hindered their successful clinical applications. An aptamer/antibody bispecific system (AAbs) was developed by conjugating TLS11a aptamer and anti-CD3 antibody through a crosslinker, sulfosuccinimidyl 4-(--maleimidomethyl) cyclohexane-1- carboxylate (sulfo-SMCC) leading to the formation of bi-specific system, TLS11a/CD3. Findings of the study showed that TLS11a/CD3 showed preferential binding with hepatoma cells (H22) and T-cells, leading to the T-cell mediated antigen-specific lysis of hepatoma cells. Further, it promoted significant inhibition of implanted H22 tumors and elongated the survival of mice. In conclusion, simultaneous targeting of H22 and T-cell directed the T-cells to kill the tumor cells effectively, leading to substantial improvement in antitumor activities both *In vitro* and *in vivo*.	Hu *et al.,* 2018
ST21	Peptide aptamer (SFSIIHTPILPL-TAT-SFSIIHTPILPL	HCC cells	A cationic peptide, disulfide cross-linked stearylated poly arginine peptide modified with histidine (H_3R_5) was modified with peptide aptamer S21 which was composed of SP94 and cell-penetrating peptide TAT. SP94 directed the specific binding to HCC cells, whereas the job of the cell-penetrating peptide was to facilitate the movement across the plasma membrane. miRNA-195 (miR195) and fasudil were simultaneously loaded into peptide carriers by condensation through electrostatic interaction and ammonium sulfate-induced transmembrane electrochemical gradient, respectively. Findings revealed that the therapeutic potential of aptamer-functionalized peptide nanoparticles was significantly superior as compared to unmodified counterparts. Thus, aptamer-functionalized peptides possessed the ability of preferential delivery of therapeutic payloads simultaneously into HCC cells.	Liu *et al.,* 2016

Name of Aptamer	Characteristics of Aptamer	Target	Brief Outcome	References
TLS1c	DNA aptamer, 5'-ACAGGAGTGATGGTTGTT ATCTGGCCTCAGAGGTTCTCGGGTGTGGTCACTCCT G-3′	BNL 1ME A.7R.1 (MEAR) cells	TLS1c-functionalized cabazitaxel (Cab)-loaded liposome was prepared. Findings revealed that TLS1c resulted in preferential delivery of aptamer-functionalized liposome into neoplastic MEAR cells, leading to remarkably enhanced therapeutic potential and significant reduction in off-target toxicity	Cheng *et al.,* 2019
AS-1411	DNA aptamer, 5'-d(GGTGGTGGTGGTTGTGGTGGTGGTGG)-3′	Nucleolin receptor	Thiol-modified AS-1411 was tailored at the surface of biocompatible and redox-responsive mesoporous silica nanoparticles containing cytochrome c as sealing agent and DOX as therapeutic payload to achieve tumor-targeted triplex therapy. Cytochrome c was immobilized mesoporous silica nanoparticles (MSNs) *via* disulfide linkage resulting in redox-responsive intracellular delivery. Extensive investigations revealed that AS-1411 directed target specific delivery in HCC cells and reluctant, glutathione resulted in release of DOX into cellular microenvironment leading to apoptosis of malignant hepatocytes. Thus, they highlighted that the triplex therapy consisting of DOX, cytochrome c, and AS-1411 possessed a remarkable potential for successfully combating HCC as evidenced from the change of tumor size, TUNEL staining, and HE staining assays.	Zhang *et al.,* 2014

Name of Aptamer	Characteristics of Aptamer	Target	Brief Outcome	References
GT-75	A DNA aptamer 5′-TGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGT TTGTTTGTTTGTTTGTTTGTTTGT-3′	Elongation factor 1A (eEF1A), protein involved in the elongation step of protein synthesis	GT-75 aptamer containing GT repetition was tested in three types of HCC cells, such as HepG2, HuH7, and JHH6. The delivery of G-75 by liposome resulted in time and dose-dependent cytotoxicity. The apoptotic potential was found to be highest in JHH6 cells, however no notable effect on cell cycle was observed. They highlighted that G-75 showed its therapeutic potential upon interaction with eEF1A; as no distinct changes in mRNA or protein level were observed. Upon completion of the successful study, they concluded that liposome-mediated delivery was far superior in reducing the cell viability in panel of HCC cells compared to cholesterol-mediated delivery.	Scaggiante *et al.,* 2016
AP273	RNA aptamer, TCAGGTGCAGTTCTCGACTCGGTCTTGATGTGGGT	α-fetoprotein (AFP)	High expression of AFP has been observed in HCC whereas minimal expression is seen in adult healthy liver tissue. It is one of the key and widely used serum biomarker for the diagnosis of HCC. SELEX technology was employed to synthesize and characterize an RNA aptamer that showed significantly high affinity (K_D ~33nM). Results showed that aptamer exhibited dose-dependent significant inhibition on proliferation of HCC cells by substantially downregulated AFP-dependent oncogenes. Thus, they concluded that AFP had the potential to be acted as an efficient therapeutic and diagnostic agent to achieve significantly improved therapeutic outcome in AFP-related HCC. Later, the study by Dong *et al.,* further supported the outcome of previous study as they pointed out the ability of AP273 to specifically target the HCC cells.	Dong *et al.,* 2015

Aptamer-Functionalized Theranostic in HCC

A theranostic system comprising diagnostic agent and therapeutic modalities may have enormous potential in HCC therapy due to the simultaneous ability of detection or diagnosis and therapy, which could help overcome the problem associated with the diagnosis of HCC and therapy. Further, their functionalization

with aptamer boosts the combined outcome of theranostic because of highly preferential and superior delivery to HCC tumors(Usmani *et al.,* 2018). Pilapong *et al.* (2014) developed a smart magnetic–aptamer probe by functionalizing carboxymethyl cellulose modified MNPs (CMC-MNPs) with EpCAM aptamer to target overexpressed EGFR receptor at the surface of malignant hepatocytes. The delivery capability of nontoxic cellulose derivatives to deliver therapeutic payload DOX, targeting potential of EpCAM aptamer, and imaging quality of magnetic nanoparticles have made them popular. Detailed findings of the study revealed that developed aptamer-based magnetic nanoprobe possessed the satisfactory potential of magnetic resonance (MR) imaging *in vitro*, enhanced target-specific delivery potential to deliver DOX into HCC cells, leading to significantly superior cancer diagnosis and treatment. Babei *et al.* (2017) developed 5-fluorouracil (5-FU)-loaded PEGylated mesoporous silica nanoparticles (NPs), which were hybridized with gold nanoparticles as gate keeper leading to the formation of (PEG-Au@Si-5-FU) for theranostic application in HCC. Finally, the developed theranostic system was functionalized with an EGFR-targeting aptamer to make it target-specific for neoplastic hepatocytes due to the ability of aptamer to bind with overexpressed EGFR receptors on the surface of HCC cells. Findings of the study revealed that cytotoxic potential and cellular internalization of aptamer-functionalized theranostic nanoparticles were significantly superior compared to the unmodified counterpart. Thus, they concluded that their research had provided proof-of-concept that reveals the potential application of hybrid nanoparticles as multimodal nanoparticles. Two well-suited HCC cells, HCCLM9 with superior metastatic potential and MHCC97-L with inferior metastatic potential, were explored to identify a potent aptamer capable of detecting the HCC metastasis. The whole SELEX technology was employed by using HCCLM9 as the target cell population and MHCC97-L as the subtractive cell population. Outcomes of the study revealed that out of six different aptamers, LY-1 showed substantially superior binding affinity and specificity for metastatic cell populations' *in vitro* study, a preclinical study in a metastatic animal model, and clinical HCC specimens. Further, LY-1 functionalized magnetic nanoparticles possessed an excellent ability to pick up the HCC cells from whole blood, indicating a promising role of LY-1 as a molecular probe to identify metastatic HCC cells.

Mechanisms of Aptamer Internalization

Aptamers have been developed to target overexpressed proteins or receptors, leading to targeted delivery of therapeutic payload into neoplastic cells and significant reduction of off-target toxicity. Multiple well-defined pathways, namely clathrin- and caveolae-mediated endocytosis, macropinocytosis, and phagocytosis are responsible for the internalization of the aptamer. However, a growing number of published reports has demonstrated that mechanisms of

internalization of aptamers are broadly of two types; clathrin-and caveolae-mediated endocytosis, also known as receptor-mediated endocytosis (general mechanism of aptamer internalization) and macropinocytosis (mechanism of internalization of nucleolin-targeting aptamer, AS-1411) (Yoon and Rossi 2018).

The mechanism of aptamer internalization by clathrin-mediated pathway was confirmed by a colocalization study using fluorescently-labeled transferrin. Clathrin-mediated endocytosis (CME) takes place *via* a series of steps which include: (a) selection of cargo and formation of clathrin-coated pits; (b) induction of cargo followed by membrane-invagination; (c) fractionalization of vesicles and uncoating and (d) sorting by endosomes. Caveolae-mediated endocytosis has the following characteristics (a) (CvME) is triggered upon binding of ligand to receptor followed by separation of caveolae vesicles from the plasma membrane by dynamin, (b) the lipid rafts of the caveolae are composed of mainly cholesterol and sphingolipids and (c) the regulator of a caveolin protein family (caveolin-1/2) includes kinases [Src family protein tyrosine kinases, PTKs] and phosphatase (alkaline phosphatase); and the proteins responsible for caveolae-mediated sorting include caveolin-2, cavin, actin, integrin, and Rab family proteins (Yoon and Rossi 2018; Bareford and Swan 2007; Oh and Park 2014**)**.

The 41-mer sgc8 aptamer has a high binding affinity as K_D value is in the picomolar range for human protein tyrosine kinase-7 (PTK7), which is one of the important biomarker proteins present at the surface of leukemic cells and solid tumors. The aptamer is internalized by receptor-mediated endocytosis (RME), which includes invagination of the plasma membrane to form new intracellular vesicles that enclose the complex of sgc8 and PTK7, resulting in the protection of the complex before itreaches the endosomes (Shangguan *et al.,* 2008a). The internalization of other aptamers, such as aptamers against PSMA-targeting, EGFR-targeting, *etc*., takes place through RME. Plenty of studies have highlighted that RME is considered a standard mechanism for the internalization of aptamers (Soundararajan *et al.,* 2009; Wang *et al.,* 2016; Yoon and Rossi, 2018).

Macropinocytosis, an actin-dependent mechanism, is the process of formation of waving sheet-like extensions of the plasma membrane, which ultimately leads to the generation of large (>200 nm) organelles called macropinosomes upon the closure of the extensions, and unlike other endocytic mechanisms, macro-pinocytosis is not directly activated by drug or their associated receptors. The polymerization of cytosolic actin is the key for the formation of protrusions in macropinocytosis and is driven by the coordinated action of small family Rho guanosine triphosphatases (GTPases) and phosphoinositide 4,5-bisphosphate (PI(4,5)P2). It is worth mentioning that very few protrusions lead to the formation

of macropinosomes, while the rest are pulled back to the plasma membrane. Nucleolin-targeting AS-1411, the sole member in the aptamer family, is internalized by the process of macropinocytosis. Overexpression of nucleolin receptor has been reported in various types of malignancy such as breast cancer, glioma, ovarian cancer, HCC, *etc*. It can shuttle between cytoplasm and nucleus. The half-lives of cytoplasmic and cell-surface nucleolin are 45 minutes, whereas nuclear nucleolin has a half-life of more than 8 h. Evidence in the literature pointed out that the translocation of nucleolin is a temperature-dependent process, and endoplasmic reticulum (ER)-trans-golgi network transport does not play any significant role in the translocation process (Kotula *et al.,* 2012).

The Aptamer-receptor complex RME is fused with endosomes, leading to their sorting for recycling back to the plasma membrane or delivery to late endosomes and lysosomes for degradation. The subcellular distribution of aptamers has been studiedusing confocal microscopy and transmission electron microscopy (TEM) to gather information regarding the intracellular fate of aptamers (Yoon and Rossi 2018**)**. For example, colocalization of cell-type-specific DNA aptamers with lysosomal-associated membrane protein 1 (LAMP-1) was authenticated by *in situ* fluorescence hybridization in renal proximal tubule RPTEC/TRT1 cells. The fluorescence of PrP^c–functionalized fluorescent QDs provided distinctive clues regarding the intracellular dynamics of aptamer through fluorescent imaging. The findings of the immunofluorescent assay revealed that PrP^c-QD complexes were colocalized in the lysosome, endoplasmic reticulum, and Golgi apparatus (Chen *et al.,* 2012; Cesur *et al.,* 2015). Tailoring of aptamers on the surface of the nanoparticles has been done to detect the subcellular localization by optical microscopy or TEM devoid of any fluorescence tag. For example, subcellular localization of nucleolin-targeting AS-1411 was analyzed by TEM upon conjugating them at the surface of nanoparticles in C6 brain glial tumor cells and brain endothelial cells, bEnd.3. It was observed that AS-1411 aptamer-nanoparticle complex was primarily colocalized in endosomes of bEnd3 cells and mitochondria. In contrast, the complex was colocalized in the lysosome and Golgi apparatus of C6 cells, indicating varied subcellular localization of aptamer depending on the cell types. The DNA aptamer (5ECdsAP1) targeting transcription factor activating protein 1 (AP-1) was functionalized at the surface of iron oxide or gold nanoparticles, and the TEM study showed localization of aptamer in ER only. Thus, based on the published reports, it has been observed that aptamers are first internalized into endocytic vehicles followed by their redistribution into other subcellular compartments depending on the physiology of the host cells (Liu *et al.,* 2014; Gao *et al.,* 2014).

Factors Affecting *In Vivo* Therapeutic Potency of Aptamer

The development of aptamer having a high binding affinity for the target by *In vitro* SELELX is relatively simple. Translation of their potential for a therapeutic application requires an extensive investigation of their behavior *in vivo*. The following factors need to be adequately addressed to explore the potential of aptamers to a maximum extent for a better therapeutic outcome (Keefe *et al.,* 2010; Morita *et al.,* 2018**)**.

Resistant to Nuclease Enzyme

Aptamers made up of unmodified nucleotides have extremely short serum half-lives which range from several minutes to several tens of minutes due to the abundance of nuclease enzymes in the physiological fluid. The average time for the decay of aptamers in the blood depends on their structure. Increasing evidence in the literature suggests that chemical modification in nucleotide sugars or internucleotide phosphodiester linkages increases serum half-life.

Substitution of 2′-OH in ribose sugar of pyrimidine ring with 2′-fluro (2′-F), 2′-NH_2 (2′-NH_2) and 2′-O-methyl (2′-OMe) imparts resistance to nuclease degradation leading to an elevation of serum half-life. Incorporation of locked nucleic acids (LNAs), which are the analogs of ribonucleotide having a methylene linkage between 2′-O and 4′-C of the sugar ring, results in strengthening of resistance to nucleases and thermostability, leading to the formation of most stable pairs. In contrast to LNA (which is a structurally rigid modification, flexible structural modification), unlocked nucleic acid (UNA), which lacks the bond between C2′- C3′ of sugar, has also been explored to increase the resistance to nucleases. Several modifications in the phosphodiester backbone have also been explored for predominant improvement in resistance to nucleases. Replacement of phosphodiester linkage of aptamers with methyl phosphonate or phosphorothioate analog has been attempted widely. Incorporation of phosphorodithioate linkage (PS2) formed by substituting non-bridging oxygen atoms with sulphur atoms results in a dramatic increase in target-binding affinity. Further, the replacement of phosphodiester linkages with triazole linkage is highly advantageous. Triazole linkage can be established by automated phosphoramidite synthesis using modified dinucleoside blocks or by performing click reactions between azide- and alkyne-bearing nucleosides (Sun *et al.,* 2014; Morita *et al.,* 2018; Keefe *et al.,* 2010**)**.

A chiral transition of the D-form of natural DNA has resulted in the formation of L-DNA, the mirror image of the former, which exhibits significantly better resistance to nuclease as compared to D-form without any notable change in binding affinity. Application of chiral transition in aptamers has resulted in the

generation of L-enantiomeric aptamers, known as Spiegelmers, by chemical synthesis. Among the series of chemicals as described above, 2′ -fluoro, 2′---methyl-substitutions, 3′-end capping with inverted thymidine and phosphorothioate backbone modifications have been investigated much extensively to develop aptamers highly resistant to nuclease degradation (Keefe *et al.,* 2010; Zhou and Rossi 2014; Sun *et al.,* 2014; Sun and Zu 2015; Zhu and Chen 2018; Lakhin *et al.,* 2013**)**.

Renal Filtration

The molecular masses of most of the aptamers (15-50 nucleotides long) range between 5-15 kDa, which is significantly lower than the molecular mass cut-off for renal glomerulus as it ranges between 30-50 kDa. Therefore, aptamers, irrespective of sensitivity/resistance to nucleases, are susceptible to renal filtration. The most widely used strategy to prevent renal excretion of aptamers is to increase the molecular weight of aptamers by functionalizing them with high molecular weight entities such as polyethylene glycol (PEG), cholesterol, protein, multimerization, and nanomaterials. Cholesterol can be conjugated at the 5′-end of an aptamer to form cholesterol-aptamer conjugate (cholODN) (Keefe *et al.,* 2010; Lakhin *et al.,* 2013; Morita *et al.,* 2018). A study conducted by Lee *et al.* (2013) showed that 2′-F pyrimidine modified RNA aptamer containing 29 nucleotides inhibits the replication of Hepatitis C virus. The aptamer was conjugated with cholesterol to form a cholesterol-conjugated aptamer. Results showed that cholesterol conjugation resulted in a longer half-life and significantly reduced clearance rate, which was approximately nine times lower than that of the non-conjugated aptamer. Conjugation with PEG is a widely used strategy and probably more extensively investigated than cholesterol conjugation to augment the circulating half-life. For example, conjugation of 40 kDa (PEG) to 2′-F or 2′-O-methyl aptamers increased circulating half-life from 0.5 to 12 h when administered to Sprague-Dawley rats (Soutschek *et al.,* 2004). Due to its high molecular weight, the functional hindrance of aptamer binding to its target can be created upon conjugation to PEG. Thus, a case-by-case determination regarding the effect of different parameters such as size, type of PEG, and length of PEG linked to the aptamer on the binding interaction of aptamer to its target should be performed (Morita *et al.,* 2018)

Safety of Aptamers

Majorly, aptamers lack the toxicology class effects associated with antisense oligonucleotides as reported in the literature (Keefe *et al.,* 2010; Morita *et al.,* 2018; Lakhin *et al.,* 2013). Visible accumulation of mononuclear phagocytes as determined histologically is the principal finding in the toxicity study of

therapeutic aptamers. This finding does not come under adverse events. A good safety margin between a pharmacologically effective dose and a good safety margin between a toxicologically established no-adverse-effect level has been shown by therapeutic aptamers (Geary *et al.,* 2003; Keefe *et al.,* 2010; Morita *et al.,* 2018**)**. Thus, very restricted information is available regarding the toxicological aspect of aptamers. In some literature, it has been reported that polyanionic effects, unnatural tissue accumulation, extensive chemical modification or conjugation, and non-specific immune activation, especially after continuous or repeated administration of aptamer therapeutics, may be the principal factors that contribute to the potential toxicities associated with aptamers. High negative charges associated with aptamers are responsible for non-specific binding of them with blood proteins, probably leading to their internalization in non-target tissues resulting in unwanted side effects and reduction in therapeutic efficacy. Pieces of evidence in the literature have revealed that accumulation of aptamers primarily occurs in the kidney, liver, and spleen upon intravenous administration (Morita *et al*., 2018), and different techniques, namely immunohistochemistry, *in situ* hybridization, and histopathology, have been employed for qualitative determination of oligonucleotides in various tissues. Although chemical modification is badly needed to impart resistance to nuclease enzymes, sometimes chemically modified aptamer may give rise to toxic chemical effects or become immunogenic. For example, a 2-fold increase in plasma C3a level in mice was reported upon i.v. administration of E-selectin thioaptamer at a dose of 128 µg as compared to that of mice treated with normal saline.

Interestingly, the elevated level was too low to initiate the symptomatic hypersensitive reaction (Kang *et al*., 2015). LNA-modified nucleic acids resulted in severe hepatotoxicity. Thus, it is recommended that chemical modifications be used judiciously, depending on the desired therapeutic effects by using aptamers. Apart from the chemical modification, formulations of therapeutic aptamers, especially PEGylation, can give rise to the adverse response. Pre-existing antibodies to the PEG group are responsible for a severe allergic reaction, as evidenced from a phase-III study of PEGylated RNA aptamer against clotting factor IXa (Morita *et al*., 2018).

SELEX Technology for the Identification and Selection of Aptamers

Systemic evolution of ligand by exponential enrichment (SELEX) is the process that is widely used for the generation of aptamers. Briefly, the SELEX process is composed of five steps (Sun *et al.,* 2014; Morita *et al.,* 2018**)** as follows: (i) at first, the target of interest is mixed with random oligonucleotide library; (ii) separation of bound sequences from unbound sequences; (iii) generation of the

enriched library by the amplification of target-bound sequences; (iv) an initiation of next round of enrichment by blending the target of interest with newly amplified library; and lastly, several repetitions of a round of selection followed by sequencing of enriched aptamer sequences. Several modifications of the conventional SELEX process have been attempted to improve the outcomes of this procedure. The objectives of these modifications are as follows: The exploration of different techniques such as bead-based aptamer selection (Yang *et al.,* 2002; Yang *et al.,* 2003), slow off-rate modified aptamer (SOMAmer) (Kraemer 2011, Huang *et al.,* 2021), magnetic-assisted rapid aptamer selection (MARAS) (Lai and Hong 2014**)**, monoclonal surface display SELEX (MSD-SELEX) (Zhu *et al.,* 2014); inclusion of additional properties in selected aptamers such as X-Aptamers **(**He *et al.,* 2012) and Cell-SELEX; selection of aptamers which can recognize target at their native conformational state under physiological conditions such as Cell-SELEX, hybrid-SELEX, *in vivo*-SELEX, tissue-based SELEX, *etc*. (Wilner *et al.,* 2012; Ren *et al.,* 2020**)**. Two classical methods, namely traditional purified membrane protein-based SELEX and live cell-based SELEX, have been implemented to develop nucleic acid aptamers, which are used as targeted delivery agents.

Purified Membrane Protein-Based SELEX

This technique develops aptamers against cell-surface biomarker proteins and consists of different steps (Mallikaratchy *et al.,* 2006; Gopinath, 2007; Martin *et al.,* 2013; Zhou and Rossi, 2014). Initially, a random ssDNA library consists of chemically synthesized ssDNA oligonucleotides having 10^{14} - 10^{15} random sequences flanked by conserved primer binding sites, generally, 5′-sense primer sequence-(random primer sequence), antisense primer sequence-3′ composed of 18 to 22 bases in primer sequence and 20-40 nucleic acids in random sequence. The 5′-sense primer sequence is labeled with a fluorochrome reporter to monitor aptamer selection while affinity molecule such as biotin is tagged at 3′-antisense primer to separate single-stranded oligonucleotides, which are generated at each amplification round. The initial pool of DNA aptamers can be selected from a random ssDNA library. In the case of the generation of RNA aptamers, two additional steps are required. These are: (i) addition of T7 RNA polymerase promoter to the 5′-sense primer after the generation of ssDNA library; (ii) Transcription is initiated in 5′-3′ direction using DNA as a template. The second step of SELX includes denaturation and rapid cooling of oligonucleotides to initiate the formation of 3D structures, followed by mixing with the required target for specific binding enrichment. The third step of SELEX involves eliminating unbound nucleotide sequences by using membranes, columns, magnetic beads, and capillary electrophoresis. Later, conventional PCR (DNA aptamers) or RT-PCR (RNA aptamers) is performed to amplify the enriched

sequences for the generation of new sequences, which will be used in the next steps of SELEX. Finally, 4-20 rounds of amplification and enrichment are performed for the selection of aptamers. The correct determination of the number of amplification and selection steps depends on the nature of the target, whether a purified protein or a living cell is used on the evolution of aptamer sequence library, which is established by gel electrophoresis, flow cytometry (for target binding), classical cloning and sequencing method and high throughput Next-Generation Sequencing (NGS). The purified recombinant proteins or peptides which express in prokaryotic or eukaryotic cells can serve as a target for the selection of aptamers by SELEX. Certain proteins, especially highly glycosylated proteins, require post-translational modifications, which can only happen under the physiological condition to become active. In that case, newly synthesized aptamers by the SELEX method may not have the ability of selective recognition and interaction with the target proteins, highlighting their demerit for successful biomedical application. To overcome the limitation, SELEX technology based on whole living cells known as Cell-based SELEX has been explored to develop aptamers capable of identifying the target proteins under native conditions.

Live Cell-Based SELEX

This technique provides significant promise for generating aptamers capable of recognizing particular membrane protein under native conditions, signifying the potential of live cell-based SELEX to overcome the drawbacks associated with purified membrane protein-based SELEX. Information such as native conformation, biological function, and identities of cell-surface proteins is not required. All the steps of SELEX described above are also performed in cell-based SELEX (Zhou and Rossi, 2014**)**. The principal objective of live cell-based SELEX is to differentiate between the target cell population (cells express the protein of interest) and the control cell population (cells do not express the protein of interest). Therefore, two selection processes, namely counter selection and positive selection, have been performed in cell-based SELEX (Hicke *et al.,* 2001; Zhou and Rossi, 2014). Incubation of nucleic acid library with the control cells (also known as negative cells) is known as counter selection resulting in the removal of non-specific binding agents. In the positive selection, the target cell population (known as positive cells) is incubated with the nucleic acid library to recover the target-bound sequences.

Some Prospective Use of Aptamers in HCC Therapy

We have already discussed different types of aptamers, their chemical properties, synthesis, isolation procedures, and their advantages during miscellaneous applications in HCC, as an imaging probe or biosensor for diagnosis and as

therapeutic agents or therapeutic targeting moiety to recognize respective biomarkers of malignant hepatocytes. Thus, they improve the therapeutic efficacy of presently available chemotherapeutics, whether in a single or a combination form. Here, in Table **2**, we have mentioned some of the very promising aptamers that have been successfully developed and applied against HCC for diagnostic and therapeutic purposes.

Table 2. Aptamers under scientific investigation with promising results for HCC.

Aptamer	Targeting Receptor/Proteins/Cells	Development	Application	Current status	References
AP613-1-DNA aptamer	Glypican-3 or GPC-3 overexpressed in HCC	Through C-SELEX evolution	Imaging Probe	Under scientific research	Zhao *et al.*, 2018
LNC-2,4 DNA aptamer	Lipocalin-2 in HCC	Through C-SELEX evolution	Diagnosis	Under scientific research	Lee *et al.*, 2015
Aptamer-07S	HepG2	Through C-SELEX evolution	Detection	Under scientific research	Yu *et al.*, 2020
C-2, DNA Aptamer	HepG2	Through C-SELEX evolution	Detection	Under scientific research	Ninomiya *et al.*, 2013
TLS11a, DNA Aptamer	HepG2	Through C-SELEX evolution/ signal Amplification/ Voltametric method	Detection/cell signaling sensors	Under scientific research	Shangguan *et al.*, 2008/ Sun *et al.*, 2015/ Sun *et al.*, 2017.
TLS11a-GC, DNA aptamer	LH86	Through C-SELEX evolution	Drug Delivery	Under scientific research	Meng *et al.*, 2012.
G12msi, a modified DNA aptamer	GPC-3 overexpressed in HCC	Through C-SELEX evolution	Therapeutic targeting	Under scientific research	Park *et al.,* 2020.
TLS11a, DNA Aptamer	HCC cell surface receptors	Through C-SELEX evolution and conjugated with polymeric nanoparticle	Drug delivery	Under scientific research	Weigum *et al.*, 2016
AS1411, DNA Aptamer	Nucleolin, Cancer cell surface receptors	AS1411-Dox adduct/ AS1411-conjugated gold nanoparticles	Drug delivery and therapy	Phase 2 clinical trials.	Trinh *et al.,* 2015 & Kabirian-Dehkordi *et al.*, 2019.
EPAP, RNA Aptamer	EpCAM, cancer stem cell biomarkers	Aptamer- Gene chemical ligation	Therapy	Under scientific research	Xiao *et al.*, 2017

Aptamer	Targeting Receptor/Proteins/Cells	Development	Application	Current status	References
OPN-R3, RNA aptamer	Osteopontin, unique cell growth factor in HCC	Through C-SELEX evolution	Therapy	Under scientific research	Bhattacharya *et al.*, 2012.
GT75, DNA aptamer	(eEF1A), Elongation of cellular growth factor 1A in HCC	Liposome-aptamer ligation	Drug delivery	Under scientific research	Scaggiante *et al.*, 2016.
TLS 9a, DNA aptamer	HCC	Aptamer guided polymeric nanoparticle	Targeted therapy	Under scientific research	Chakraborty *et al.*, 2020.
AFP, RNA Aptamer	AFP, Alpha-fetoprotein	Through C-SELEX evolution	Detection and treatment	Under scientific research	Lee *et al.*, 2012.
Ep-MNPs,DNA Aptamer	EpCAM	Magnetic nanoparticle-aptamer	Imaging and treatment	Under scientific research	Pilapong *et al.*, 2014.
AP273, DNA Aptamer	Alpha-fetoprotein	Through C-SELEX evolution	Imaging and treatment	Under scientific research	Dong *et al.*, 2015
SLeX-AP, DNA Aptamer	moving tumour cells	Biocompatible nanoparticles	managing personalized therapy	Under scientific research	Wang *et al.*, 2016.

CONCLUSION

The molecular environment of HCC can be considered to understand the targeting mechanism through aptamers. The aptamers are generated based on biomarkers specific to HCC. This chapter highlighted the active targeting potential of different possible aptamers for the target-mediated drug delivery in HCC. Streamline research about this particular area of aptamer-mediated targeting in HCC has a significant impact along with different existing patented works and formulations under clinical and preclinical testing in this field.

Compared to other targeting ligands such as antibodies, the aptamers show lesser toxicity due to their high specificity and low immunogenicity. Aptamers resemble antibodies in terms of recognition and target binding capability to the targeting molecule. They provide some advantages over antibodies: shorter generation time, higher range of modifiability, no variability between the batches, improved thermal stability, higher target potential, and low-cost manufacturing. Several useful applications and specifically minimizing off-target cytotoxicity of anticancer drugs make aptamers an extraordinary targeting moiety in the therapeutic and diagnostic tool for HCC treatment. Thus, aptamer-mediated targeted drug delivery can be translated from bench to bedside to combat HCC

successfully.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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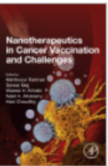
Nanotherapeutics in Cancer Vaccination and Challenges

2022, Pages 29-75



Chapter 3 - Recent developments in cancer vaccines: where are we?

Biswajit Mukherjee [1], Ashique Al Hoque [1], Apala Chakraborty [1], Samrat Chakraborty [1], Lopamudra Dutta [2], Debasmita Dutta [3], Soumyabrata Banerjee [4], Moumita Dhara [1], R. Manasa Deepa [5]

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Abstract

Cancer vaccines, also called therapeutic vaccines, improve the body's standard defense against cancer. Usually, the cancer vaccine is provided to the patient with already detected cancer. Immunotherapy is aimed more directly at cancer cells compared to conventional surgery, chemotherapy, or radiotherapy, offering patients the potential to provide improved reactions, enhance life-quality, or even cure the disease. Cancer vaccines might be designed to target tumor-associated antigens (TAAs), cancer germline antigens, virus-related antigens, or tumor-specific antigens (TSAs), which are also widely recognized as neoantigens. Sipuleucel-T (Provenge) and Talimogene laherparepvec (T-VEC) are the two FDA-approved vaccines currently being utilized for advanced prostate cancer and advanced melanoma skin cancer, respectively. Clinical trials are essential for learning more about cancer vaccines. Researchers are testing vaccines for several types of cancers, including bladder cancer, brain tumor, breast cancer, cervical cancer, colorectal cancer, lung cancer, and many more. This chapter discusses the literature and data on the value and use of cancer vaccines. Although cancer vaccines as a single therapy are not sufficient to declare a complete victory over this deadly disease until now, it must be a comprehensive approach and existing treatments on the concept of cancer

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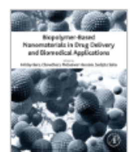
Biopolymer-Based Nanomaterials in Drug Delivery and Biomedical Applications

2021, Pages 3-28



Chapter 1 - Biopolymer-based materials in nanomedicine: Synthesis and characterization

Biswajit Mukherjee, Moumita Dhara, Debasmita Dutta, Apala Chakraborty, Shreyasi Chakraborty, Soma Sengupta, Laboni Mondal, Lopamudra Dutta, Kushal Pal

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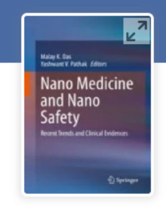
Abstract

Nowadays, the use of polymers is almost indispensable to the wide range of applications. Despite their unbeatable usefulness in the biomedical field, nonrenewable resourses, environmental pollution, and biocompatibility are the major limitations that restrict the predominant use of synthetic polymers. Thus, an expedition of the utilization of biopolymers is on a rise. Recently, the development of novel drug delivery systems or nanomedicine utilizing biopolymers enables us to prevent, treat, and diagnose early diseases with unparalleled efficiency and to ensure the accuracy even up to the molecular and genetic levels. This chapter focuses on the applications of biopolymers especially in the field of nanomedicine. Herein, we have briefly described the synthesis, characterization of the largely used and potential biopolymers, and their classifications. In addition, a special emphasis has been given on the uses of biopolymers in the drug delivery approaches from the research viewpoint to clinical applications. The chapter also describes the usefulness of aspiring biopolymers in the current nanomedicine development.



Nano Medicine and Nano Safety pp 175–211 | Cite as

Transdermal Nanomedicines for Reduction of Dose and Site-Specific Drug Delivery

Biswajit Mukherjee, Soma Sengupta, Soumyabrata Banerjee, Moumita Dhara, Ashique Al Hoque, Leena Kumari, Manisheeta Ray, Iman Ehsan & Alankar Mukherjee

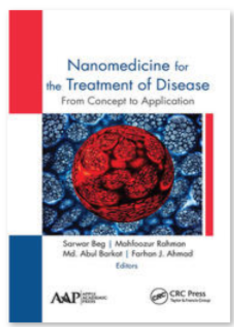
Chapter | First Online: 10 December 2020

Abstract

The emergence of new technologies provides unique opportunities to exploit novel approaches in drug delivery. Transdermal drug delivery systems (TDDS) are one of the imperative technologies of increasing interest with the benefits of sustained/controlled drug delivery leading to patient convenience and compliance. By definition, TDDS are topically administered medications, for example, patches or semisolids, which permeate the active ingredient through the intact skin for systemic effects in a sustained manner. Transdermal drug deliveries, therefore, are the noninvasive administration of active ingredients from the skin surface across its layers, to the systemic circulation. Nanomedicinal approaches through TDDS can be utilized for site-specific delivery of drugs which can lead to the reduction of dose, too. We have reported here TDDS providing nanomedicinal strategies to deliver drug(s) to the target tissues.

Keywords

Skin **Transdermal delivery** **Nanomedicine** **Dose** **Site-specific delivery**



Chapter

Nanomedicinal Genetic Manipulation: Promising Strategy to Treat Some Genetic Diseases

By Biswajit Mukherjee , Iman Ehsan , Debasmita Dutta , Moumita Dhara , Lopamudra Dutta , Soma Sengupta

Book Nanomedicine for the Treatment of Disease

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ABSTRACT

Genetic disease is characterized as an ailment due to a defect in an individual's genome, chromosomal abnormalities, and gene mutations. A successful therapeutic and diagnostic intervention on genetic diseases has been unrevealed predominately, and it demands technological development. Nanomedicine is a boon in various fields of medical applications from the development of diagnostic devices, contrast agents, in vivo imaging, analytical tools to drug delivery vehicles. Versatilities of nanomedicines owing to the dimension, biocompatibility, and manipulating capability to achieve successful delivery at the targeted site of action and their lower toxicity have made this field a technological revolution to combat different unmet needs in health. This is a possible alternative for combating chronic genetic disorders by targeting a particular gene(s) or tissue or cell, which creates a pathological state. Thus, nanomedicine could be a potential therapeutic tool to treat different genetic pathological conditions, such as diabetes, heart diseases, lysosomal storage disorders, Alzheimer's disease, cystic fibrosis, etc. In this chapter, we will discuss several strategies to manipulate or knock down the expression of the lethal gene(s) specifically responsible for some of such diseases. Here we mainly focus on diseases 104such as cancer and its fate while manipulating genetic alteration by the nanomedicinal strike.

Multifunctional Systems for Combined Delivery, Biosensing and Diagnostics

2017, Pages 261-280

Chapter 14 - Pulmonary Administration of Biodegradable Drug Nanocarriers for More Efficacious Treatment of Fungal Infections in Lungs: Insights Based on Recent Findings

Biswajit Mukherjee, Paramita Paul, Lopamudra Dutta, Samrat Chakraborty, Moumita Dhara, Laboni Mondal, Soma Sengupta

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Abstract

Lung fungal infection is a critical health problem, particularly in immunocompromised patients suffering from lung cancer, cystic fibrosis, HIV infection, etc. Due to rapid blood turnover in lungs, conventional drug delivery systems do not offer persistent blood level of drug in the lungs, causing inadequate drug bioavailability locally. Hence, a large amount of dose is generally required for a prolonged period, causing toxicity. The patients, therefore, cannot tolerate the treatment, and recurrence of fungal infection by the fungal spores may occur even after the conventional treatment requiring long-term medication. The pulmonary administration of drug nanocarriers thus may prove to be an underrated approach since this is a noninvasive method for local as well as systemic drug delivery, with various additional advantages, such as a large surface area with quick absorption, over other routes of administration, and local delivery of the medication for a prolonged time period. Devices, such as nebulizers, metered dose inhalers, and dry powder inhalers are used for delivering the drugs through the pulmonary route. Thus, the aim of this chapter is to summarize the recent findings related to the delivery of biodegradable nanocarriers for treating fungal lung infection through the pulmonary route.

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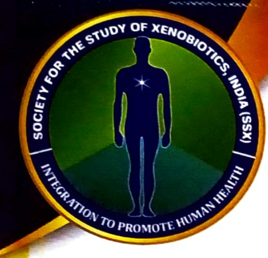

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