ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Formulation Development and Characterisation of Perfluorocarbon Nanobubbles for Sorafenib Delivery

Rangasamy Pasupathy, Pitchaimuthu Pandian, Subramanian Selvamuthukumar*

Department of Pharmacy, Annamalai University, Annamalai Nagar, Tamilnadu, India.

***Correspondence: Subramanian Selvamuthukumar**

Abstract

Nanobubbles (NBs) are novel drug delivery systems with combination of ultrasound contrast agents and drug carrier that could potentially act at specific target sites. Sorafenib is an anticancer drug for the treatment of hepatocellular carcinoma. In this study, sorafenib loaded nanobubbles were prepared by the thin film hydration method. Morphology was examined by Scanning electron microscopy and Atomic force microscope. Particle size and Zeta potential were determined by laser- scattering method. Ultrasound imaging was performed on a digital ultrasonic imaging system. The drug content in the sorafenib loaded NBs was determined by using HPLC method. The in vitro release profile of sorafenib loaded NBs was determined using a Franz diffusion cell method with absence and presence of Ultrasound exposure. The sorafenib NBs expected a spherical shape with a uniform particle size (170 nm), negative zeta potential (-52.47 mV) and polydispersity index (0.289). Ultrasound imaging confirmed the acoustical activity of perfluoropropane nanobubbles. Drug content was found to be 96 %. A slow and prolonged spontaneous drug release was observed in vitro, which was enhanced upon ultrasound stimulation. This result suggests that sorafenib loaded NBs can be considered a novel chemotherapeutic approach for treating liver cancer in combination with an ultrasound contrast agents.

Keyword: *Nanobubbles, Sorafenib, Perfluorocarbon, Ultrasound, Hepatocellular carcinoma*

Introduction

Sorafenib is a drug widely being used in the treatment of hepatocellular carcinoma that blocks several tyrosine protein kinases, such as VEGFR, PDGFR and Raf family kinases known as the crucial signalling pathway of serine/threonine kinases in oncogenesis [1]. It is the exclusively USFDA approved a drug for systemic treatment of advanced hepatoellular carcinoma (HCC) during liver transplantation or surgical resectioning as refractory treatment. Liver cancer is one of common cancer with a constant high rate of death due to cancer [2].

A required daily dose of 400-800 mg of sorafenib is required for optimum efficacy when it is given orally. The drug is practically insoluble in water resulted in poor oral bioavailability is extremely, at about 38-49%, and it is also hugely affected by the diet [3, 4]. The general side effects of sorafenib treatment include nausea, diarrhoea, fatigue, hand-foot syndrome, etc. [5]. Since the last few decades, research in different sorts of cancer is in boom that produced challenge for formulation chemist in altering the delivery systems of known drugs along with development of new chemical entity and its release including nanoparticle approach[5-11], liposomes[12, 13], nanomatrix[14] and microbubbles[15] etc. Nanobubbles (NBs) represent a novel system of encapsulation with therapeutic and diagnostic applications as both. Nanobubbles are round, globular particles with a shell and a gas-filled core structure that enables them to perceptibly dynamic properties.

The shell mostly composed of polymers, lipids, proteins, surfactant and polyelectrolyte multilayer, whereas core base can be charged up with different gases, such

as perfluorocarbon, carbon dioxide, sulfur hexafluoride and air [16]. The bubbles loaded with therapeutic drug and coupled with ultrasonication could potentially act at specific target sites. The microbubbles are blamed for instability and large size (about 1-6 μm) which makes them unfavorable for intravascular therapy. The microbubbles are formulated by using liquid perfluorocarbons and oils and hence described also as Perfluorocarbon nanobubbles (PNs). PNs are smaller in size, with a mean diameter below 200nm [17]. The nanobubble is formulated by using gas with poor water-soluble nature (viz. Perfluoropropane, Perfluorocyclobutane, and Sulfur hexafluoride) encapsulated with a shell consisting either polymer, protein, or lipid [18, 21].

Among these gases, the Perfluoropropane (C3F8) gas has known for useful ultrasound (US) imaging properties due to its perfect reflection and thus could be used as US contrast agents [22] during delivery of PNs at the particular targeted site. The procedure of ultrasonic imaging is known for its real-time and non-invasiveness, most often applied cause of cost-effective and safe for patients and clinical staff over repeated use as no ionizing radiation are utilized [23].

The earlier research also has shown the advantages of US-mediated imaging in lipid-based microbubbles and gene delivery. The nanobubbles based drug delivery consists of echogenicity while the lipid molecules in shell are bound together by weak type of physical forces, in the absence of chain entanglement, in compliance with area expansion and compression during US insonification [24].

Till the date, no studies were reported regarding the application of NBs for drug delivery to liver cancer. The present study aimed to develop nanobubbles formulation in the nanometer size range for use as an innovative drug delivery system for sorafenib.

During the formulation process, prime importance was given in reduction of the daily dose of sorafenib and effective delivery of the drug to the target site. The projected formulation consisting of nanobubbles with soybean phosphatidylcholine (SPC), cholesterol and oil used for bubble stabilization and addition of poorly soluble gas (perfluoropropane) to the core.

The resulting NBs formulation was characterized by morphological studies, particle size, size distribution and zeta potential, US imaging, and in-vitro behavior.

Materials and Methods

Materials

A gift sample of Sorafenib drug was received from Hetero labs limited. (Jadcherla, India), Coconut oil was procured from Sigma–Aldrich Chemical and Cholesterol was purchased from nice chemicals, Perfluoropropane (Micro C3F8) were purchased from Micromed. Fat-free soybean phospholipids with 70% phosphatidylcholine (SPC) (Lipoid S75) were gifted from Lipoid (GmbH, Germany). The other chemicals and reagents used during the work were of analytical grade.

Preparation of Sorafenib Nanobubbles

The thin film hydration method was followed during the formulation of Sorafenib loaded NBs. Briefly, 0.875g of Soybean phosphatidylcholine, 0.250g of cholesterol, and the drug were firstly dissolved in 5 ml of chloroform: ethanol (2:1) and placed in a round-bottom flask. Chloroform and ethanol were eliminated by vaporization via rotary evaporator at 40ºC, and the traces of solvent were eliminated by continuing the lipid film evaporation under a vacuum overnight.

About 50 ml deionized water was added to hydrate the lipid film by using probe-type sonicator (Make; Sonics-Vibra cell) for 10 min. Further, 2.5% of coconut oil was mixed into the system. It was blended by using high-shear homogenization (make: Remi) for 5 min. The resulted dispersion was cooled to 20ºC, and 3.75% of perfluoropropane was added into the dispersion, and the overall system was sonicated for 10 min to generate nanobubbles suspension. The blank NBs were similarly prepared without the addition of sorafenib.

Morphology Characterization

Determination of Particle Size and Zeta Potential

The zeta potential and particle size (z-average) of sorafenib loaded NBs were investigated by a laser-scattering method (Nano ZS® 90, Malvern). The sorafenib loaded and blank NBs were diluted 100-fold

with deionized water to measure the size and surface charge of NBs.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to visualize the structure of the NBs, and the images were recorded.

The samples were prepared by placing a drop of freshly prepared bubble solution on dust-free foil, and it was kept in a desiccator. After the sample was gold sputter-coated for 5 min, the images were obtained using a scanning electron microscope (JEOL JSM-5610LV, 30 kV).

Atomic Force Spectroscopy (AFM)

The surface topology of sorafenib loaded nanobubbles was examined using Atomic Force spectroscopy (AGILENT AFM-5500) The AFM was operated using silicon nitride tips in the non-contact mode.

Ultrasound Imaging

A latex tube with 10 ml of 1 × PBS was located in water and further exposed to ultrasound. The recordings of US images were performed on a digital ultrasonic diagnostic imaging system (Mindray, China) with a convex transducer at 5 MHz in visualization mode. The procedure was repeated by injecting sorafenib loaded NBs (200 µl) into same latex tube, and the ultrasound images were recorded.

Quantitative Determination of Sorafenib

The quantitative determination of Sorafenib by High-performance liquid chromatography (HPLC) was performed as proposed by Fucile C et al. [25] with slight modification. The HPLC system consisted of a quaternary gradient pump, a PDA detector, a standard auto sampler, a column oven, and a chromatography workstation (Shimadzu Technologies) following this HPLC method: A reverse phase Shim-Pack GIST C18 column was used (150 mm × 4.6 mm, 5 µm).

The composition of the mobile phase was 70 % acetonitrile and 30 % ammonium acetate (20 mM). The samples (50 µL) were injected into the HPLC column with mobile phase as a diluent, with a flow rate of 1.0 ml/min, and a temperature of the column at 40ºC. The drug peaks were detected at 260 nm, and the sorafenib concentration was calculated using an external standard calibration curve. The linear calibration curves were obtained with the concentration range of 2, 4, 6, 8, 10, and 12µg/ml and the regression coefficient of 0.999. The drug content in the sorafenib loaded NBs (3 ml) and DMSO (25 ml) were added to a standard flask, and then the solution was sonicated (bath sonicator) for 15 minutes. To detect the drug content in sorafenib loaded NBs, the solution was further diluted with mobile phase and injected into the HPLC system. The drug content % of Sorafenib loaded NBs was determined and further compared with the standard drug.

In Vitro Release of Sorafenib

The release of sorafenib from the perfluoropropane nanobubbles was determined using a Franz diffusion cell consisting of donor and receiving chamber separated by a cellulose membrane (cut-off, 12-14 kDa) with absence and presence of Ultrasound exposure. A 1.5 ml volume of sorafenib loaded nanobubbles was placed in the donor chamber. The receiving chamber was contained 25 ml of phosphate buffer saline (PBS) pH 7.4 with 1 % (w/v) Tween 80 as release medium. The temperature was kept at 37º C. The samples at regular intervals were withdrawn and completely replaced with the same volume of fresh buffer to maintain sink conditions from receiving chamber. The concentration of sorafenib in the withdrawn samples was determined by HPLC.

Results and Discussion

The nanobubbles filled with perfluoropropane was developed by introduction an inert gas and a novel ultrasound contrast agent. This perfluoropropane is filled in the inner core of nanobubbles. The SPC and Coconut oil both were respectively used during this formulation work as the interfacial membrane and oil phase in nanobubbles.

Thus these nanobubbles were formulated to load the interior with perfluoropropane, surrounded by coconut oil. Coconut oil, which was used as the oil phase, is superior in catalyzing the solubility of flavonoid in comparison to other oils [26, 27]. As cholesterol is an amphiphile in nature hence regulate the lipid structures and thus enhances the stability of formulations [28].

The density of Perfluoropropane is different than that of air, and since poorly soluble in water, it has a property to enhance the echogenicity of the US contrast agent as well as stability [29].Soybean phosphatidylcholine and cholesterol are the emulsifiers used during the work, were located at the oil/water interface surrounding sorafenib containing core as a phospholipid membrane. Visually, the nanobubbles were white and homogeneous, and though the exact solubility of sorafenib in internal phase could not be determined, it appeared that most of the input drug had been solubilized.

Characterization of Nanobubble Formulation

As far as the design of nanobubbles, the inner shell core surrounded comprised of SPC, Cholesterol, and coconut oil while shell core consists of perfluoropropane gas to ease the delivery of sorafenib. This novel NB formulation was designed to encapsulate sorafenib efficiently. Sorafenib-NBs were fabricated as innovative delivery systems to carry and release sorafenib at the target site. As nanobubbles enhance the drug uptake in the targeted region facilitated by therapeutic ultrasound, thus help in minimizing the daily administered dose and hence reduce systemic toxicity. The nanobubbles without sorafenib (blank) were used as control while characterization of sorafenib loaded nanobubbles.

The average diameters, zeta potentials, and polydispersity index of sorafenib loaded and unloaded NB formulations are reported in **Table 1.** The mean diameter of sorafenib loaded NB and blank NB both were presented less than 170 nm without significant difference whereas zeta potential measurements showed that the nanobubbles obtained were -47.57 and -52.47 mV for blank NBs and drug-loaded NBs samples respectively. Thus no statistical differences found in the zeta potential between both. The zeta potential plays a critical role in stabilization of nanobubbles as it generates repulsive force and arrest coalescence and inter bubble aggregation. The polydispersity index (PDI) of the blank NBs and Sorafenib NBs were 0.284 and 0.289. The low PDI values for the blank nanobubbles and sorafenib loaded NBs ensure uniform formed particles in the formulation. These results confirm the effective encapsulation of sorafenib inside the nanobubble without affecting its stability [12].

Table 1: Physico-chemical characteristics of Nanobubbles formulations

NB Formulations	Avg Diameter ± SD (nm)	Zeta potential ± SD (mV)	PDI ± SD
Blank Nanobubbles (Placebo)	165.3 ± 0.68	-47.57 ± 0.65	0.284 ± 0.003
Sorafenib loaded Nanobubbles	170.3 ± 0.95	-52.47 ± 0.93	0.289 ± 0.003

Results are shown as means ± SD (n=3)

Surface Morphological Properties of Sorafenib Nanobubbles

The surface morphology of sorafenib loaded NBs was measured using scanning electron microscopy (SEM). The SEM image of nanobubbles showed the spherical shape with a smooth surface **(Fig. 1).** The AFM investigations revealed the similar spherical shape of the bubble surrounded by a soft layer **(Fig.2).**

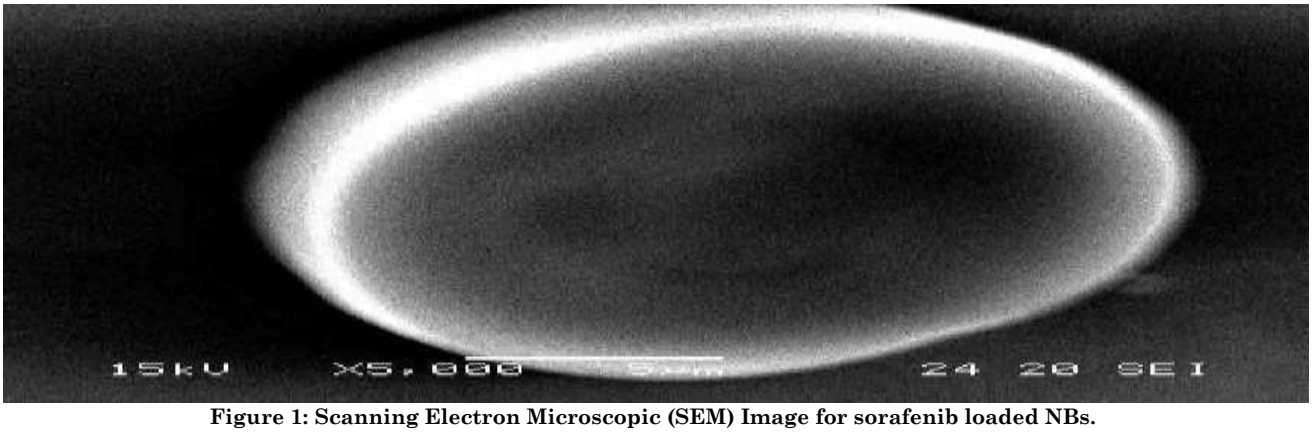


Figure 1: Scanning Electron Microscopic (SEM) Image for sorafenib loaded NBs.

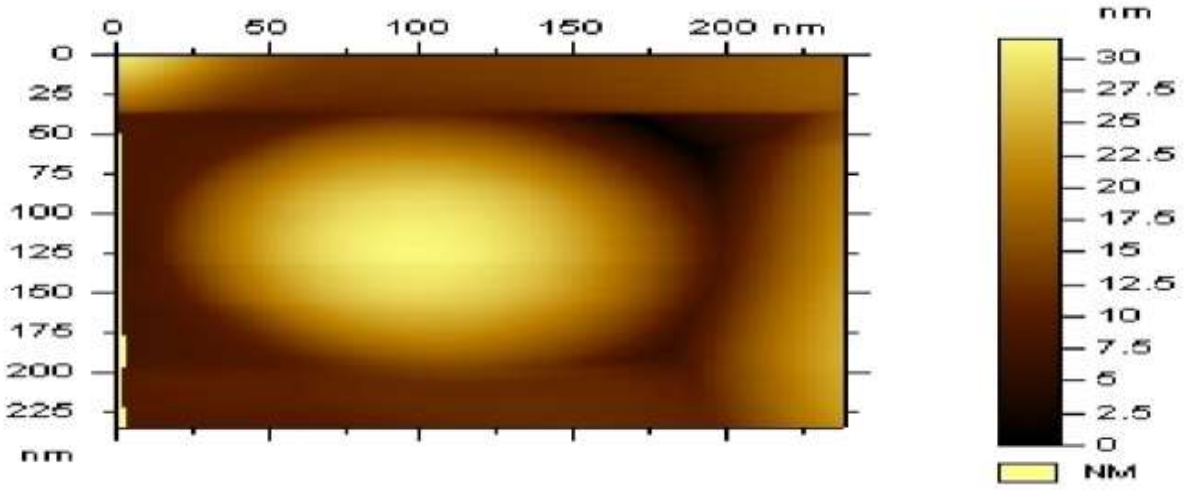
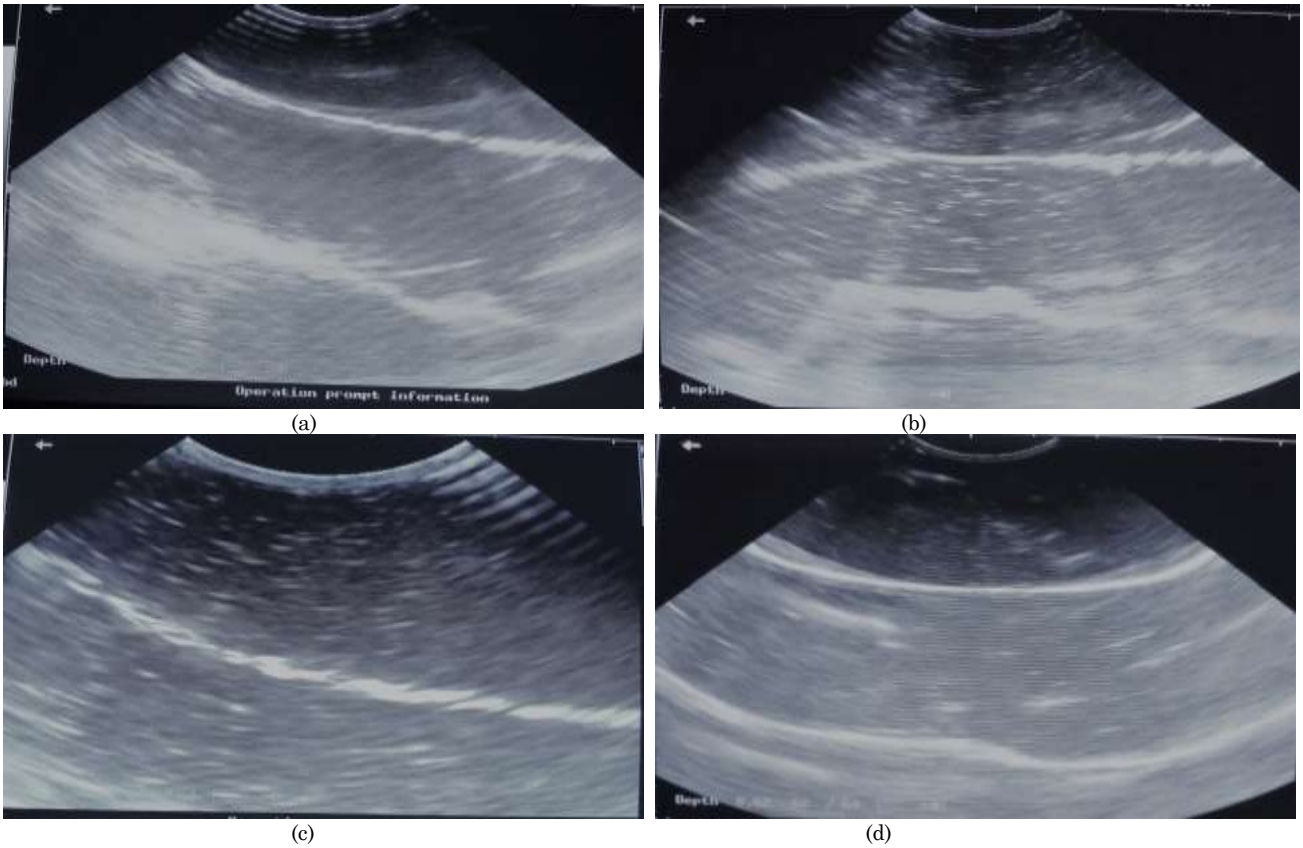


Figure 2: Atomic Force Spectroscopic (AFM) image for Sorafenib loaded NBs

Ultrasound Imaging

The active acoustic features of perfluoropropane based nanobubbles in this work were examined by ultrasound imaging where the dispersion of nanobubbles was visualized on a monitor. The low sonoporation frequency requires for administration, which offers better delivery presumably because the reduced size of bubbles enhances the internal pressure of entrapped gas in NBs core at a remarkable extent. It leads in better response toward sonoporation even at minimal US frequency [30]. Sorafenib loaded nanobubbles in the latex tube, shown in **Fig. 3**, which observed as an oscillated response towards acoustic pulses. No signals were generated while monitoring PBS by placing it in the latex tube **(Fig. 3 a).** As soon as the sample was added in the same latex tube, the color-coded spots were observed reflecting the echogenic movement signals of perfluoropropane nanobubbles. The acoustical activity of sorafenib loaded nanobubbles was thus confirmed by this observation **(Fig. 3 b & c),** the echo signals related to color-coded spots of particles almost got faded out later on the exposure of a 15-min US application **(Fig. 3 d).**



(a) (b)

(c) (d)

Figure 3: Ultrasonography of sorafenib loaded NBs. (A) PBS, (B and C) sorafenib loaded nanobubbles into same PBS, and (D) Representative images taken after ultrasound application (5 MHz) for 15 min

Drug Content Estimation

3 ml of sorafenib loaded NBs was mixed with 25 ml of DMSO, and then the solution was processed for sonication for 15 minutes using bath sonicator. The solution was further diluted and analyzed using HPLC. Drug content was found to be 96 % during estimation.

In Vitro Release of Sorafenib

In vitro release kinetics of nanobubbles containing sorafenib was investigated, and no initial burst effect was observed, ensuring the excellent and stable encapsulation of sorafenib within the nanobubble.

The release profile of the sorafenib loaded nanobubbles was determined in vitro at 37ºC in absence and presence of US energy. After application of ultrasound stimulation for 1 min at 5 MHz, the release of sorafenib was observed about 5 % while 2 % was detected in the absence of stimulus at the end of 1 h, shown in **Fig. 4.** After 24 h the sorafenib loaded NBs with the presence of ultrasound stimulus showed 49 % and 31 % of absence stimulus. The percentage of drug release after 48 h from the nanobubble formulation with the presence of ultrasound showed a 60 % and 41 % decrease in comparison with the absence of ultrasound stimulation, respectively.

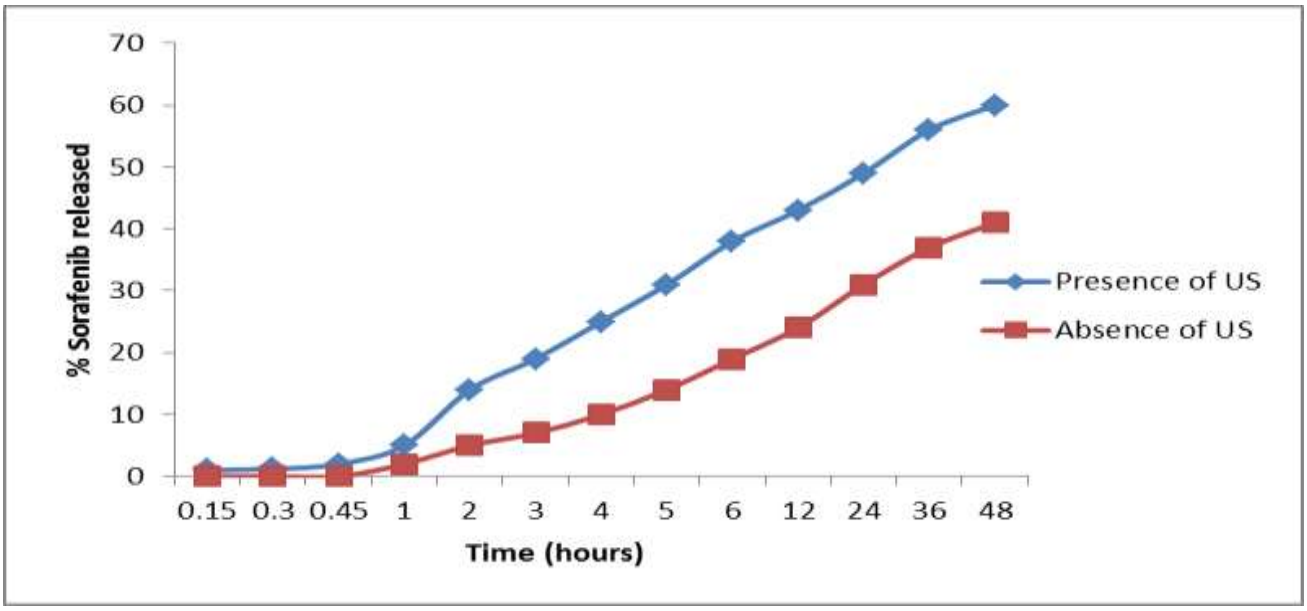


Figure 4: in vitro release profile of Sorafenib loaded NBs at 37◦C in presence and absence of US

Conclusions

The present formulation work throws light on the fabrication of novel therapeutic lipid-based NBs, filled by perfluoropropane gas and sorafenib as loaded drug molecule. The core and shell structure of NBs was not got affected by the incorporation of sorafenib. The addition of cholesterol and oil enhances the overall stability of the NBs. The enhancement of sorafenib release was notably achieved by inducing ultrasound pulses, to allow the bioactive entity to specifically targeted sites. A slow and prolonged spontaneous drug release was observed in vitro, which was enhanced upon ultrasound stimulation. The benefits of the prepared sorafenib loaded NBs assure the further investigations of this approach. In vivo studies are planned for evaluation of potentials of sorafenib loaded PNs as a system of parenteral drug delivery.

Acknowledgments

The authors are most grateful and appreciate towards Lipoid (GmbH, Germany) for gift sample of Lipoid S 75 sample. Authors also acknowledge Department of Pharmacy, Annamalai University, Chidambaram, Tamilnadu for providing necessary facility to success this project.

References

1. Potenza N, N Mosca, S Zappavigna, F Castiello, M Panella, C Ferri, et al (2017) MicroRNA- 125a- 5p is a downstream effector of sorafenib in its antiproliferative activity toward human hepatocellular carcinoma cells. Journal of cellular physiology, 232(7): 1907-1913.

2. Hsu M-H, S-M Hsu, Y-C Kuo, C-Y Liu, C-Y Hsieh, Y-C Twu, et al(2017) Treatment with low-dose sorafenib in combination with a novel benzimidazole derivative bearing a pyrolidine side chain provides synergistic anti-proliferative effects against human liver cancer. RSC Advances, 7(26): 16253-16263.

3. Liu C, Z Chen, Y Chen, J Lu, Y Li, S Wang, et al (2016) Improving oral bioavailability of sorafenib by optimizing the "Spring" and "Parachute" based on molecular interaction mechanisms. Molecular pharmaceutics, 13(2): 599-608.

4. Ranieri G, G Gadaleta-Caldarola, V Goffredo, R Patruno, A Mangia, A Rizzo, et al (2012) Sorafenib (BAY 43-9006) in hepatocellular carcinoma patients: from discovery to clinical development. Current medicinal chemistry, 19(7): 938-944.

5. Thapa RK, JY Choi, BK Poudel, TT Hiep, S Pathak, B Gupta, et al (2015) Multilayer-coated liquid crystalline nanoparticles for effective sorafenib delivery to hepatocellular carcinoma. ACS applied materials & interfaces, 7(36): 20360-20368.

6. Feczkó T, G Merza, G Babos, B Varga, E Gyetvai, L Trif, et al (2019) Preparation of cubic-shaped sorafenib-loaded nanocomposite using well-defined poly (vinyl alcohol alt-propenylene) copolymer. International journal of pharmaceutics.

7. Babos G, E Biró, M Meiczinger, T Feczkó (2018) Dual drug delivery of sorafenib and doxorubicin from PLGA and PEG-PLGA polymeric nanoparticles. Polymers, 10(8): 895.

8. Mato E, G Puras, O Bell, M Agirre, R Hernández, M Igartua, et al (2015) Selective Antitumoral Effect of Sorafenib Loaded PLGA Nanoparticles Conjugated with Cetuximab on Undifferentiated/Anaplastic Thyroid Carcinoma Cells. J Nanomed Nanotechnol, 6(281): 2.

9. Su Y, K Wang, Y Li, W Song, Y Xin, W Zhao, et al (2018) Sorafenib-loaded polymeric micelles as passive targeting therapeutic agents for hepatocellular carcinoma therapy. Nanomedicine, 13(9): 1009-1023.

10. Depalo N, RM Iacobazzi, G Valente, I Arduino, S Villa, F Canepa, et al (2017) Sorafenib delivery nanoplatform based on superparamagnetic iron oxide nanoparticles magnetically targets hepatocellular carcinoma. Nano Research,10(7): 2431-2448.

11. Kim DH, M-D Kim, C-W Choi, C-W Chung, SH Ha, CH Kim, et al (2012) Antitumor activity of sorafenib-incorporated nanoparticles of dextran/poly (dl-lactide-co-glycolide) block copolymer. Nanoscale research letters, 7(1): 91.

12. Liu J, B Boonkaew, J Arora, SH Mandava, MM Maddox, S Chava, et al (2015) Comparison of Sorafenib- Loaded Poly (lactic/glycolic) Acid and DPPC Liposome Nanoparticles in the In Vitro Treatment of Renal Cell Carcinoma. Journal of pharmaceutical sciences, 104(3): 1187-1196.

13. Xiao Y, Y Liu, S Yang, B Zhang, T Wang, D Jiang, et al (2016) Sorafenib and gadolinium co-loaded liposomes for drug delivery and MRI-guided HCC treatment. Colloids and Surfaces B: Biointerfaces, 141: 83-92.

14. Guo Y, T Zhong, X-C Duan, S Zhang, X Yao, Y-F Yin, et al (2017) Improving anti-tumor activity of sorafenib tosylate by lipid-and polymer-coated nanomatrix. Drug delivery, 24(1): 270-277.

15. Sivapalan N, B Leung, D Goertz (2015) Combining Sorafenib with the antivascular action of microbubbles for the treatment of hepatocellular carcinoma. in 2015 IEEE International Ultrasonics Symposium (IUS). IEEE.

16. Cavalli R, M Soster, M Argenziano (2016) Nanobubbles: A promising efficienft tool for therapeutic delivery. Therapeutic delivery, 7(2): 117-138.

17. Hwang T-L, Y-K Lin, C-H Chi, T-H Huang, J-Y Fang (2009) Development and evaluation of perfluorocarbon nanobubbles for apomorphine delivery. Journal of pharmaceutical sciences, 98(10): 3735-3747.

18. Lindner JR, J Song, AR Jayaweera, J Sklenar, S Kaul (2002) Microvascular rheology of Definity microbubbles after intra-arterial and intravenous administration. Journal of the American Society of Echocardiography, 15(5): 396-403.

19. Porter TR, F Xie, K Kilzer (1995) Intravenous perfluoropropane-exposed sonicated dextrose albumin produces myocardial ultrasound contrast that correlates with coronary blood flow. Journal of the American Society of Echocardiography, 8(5): 710-718.

20. Klibanov AL (2009) Preparation of targeted microbubbles: ultrasound contrast agents for molecular imaging. Medical & biological engineering & computing, 47(8): 875-882.

21. Ferreira TAC, G Fornazari, A Saldanha, B Lunardeli, BA Moore, F Montiani- Ferreira (2018) The use of sulfur hexafluoride microbubbles for contrast- enhanced ocular ultrasonography of the pecten oculi in birds. Veterinary ophthalmology.

22. Song W, Y Luo, Y Zhao, X Liu, J Zhao, J Luo, et al (2017) Magnetic nanobubbles with potential for targeted drug delivery and trimodal imaging in breast cancer: an in vitro study. Nanomedicine, 12(9): 991-1009.

23. Gessner R, PA Dayton (2010) Advances in molecular imaging with ultrasound. Molecular imaging, 9(3):7290.2010. 00022.

24. Sirsi S, M Borden (2009) Microbubble compositions, properties and biomedical applications. Bubble Science, Engineering & Technology, 1(1-2): 3-17.

25. Fucile C, S Marenco, M Bazzica, ML Zuccoli, F Lantieri, L Robbiano, et al (2015) Measurement of sorafenib plasma concentration by high-performance liquid chromatography in patients with advanced hepatocellular carcinoma: is it useful the application in clinical practice? A pilot study. Medical Oncology, 32(1): 335.

26. Fang J-Y, C-F Hung, M-H Liao, C-C Chien (2007) A study of the formulation design of acoustically active lipospheres as carriers for drug delivery. European Journal of Pharmaceutics and Biopharmaceutics, 67(1): 67-75.

27. Hung C-F, J-K Chen, M-H Liao, H-M Lo, J-Y Fang (2006) Development and evaluation of emulsion-liposome blends for resveratrol delivery. Journal of nanoscience and nanotechnology, 6(9-10): 2950-2958.

28. Liang LPTHJ, TW Chung, YYHDZ Liu (2007) Liposomes incorporated with cholesterol for drug release triggered by magnetic field. Journal of medical and biological Engineering, 27(1): 29-34.

29. Shang M, K Wang, L Guo, S Duan, Z Lu, J Li (2019) Development of novel ST68/PLA-PEG stabilized ultrasound nanobubbles for potential tumor imaging and theranostic. Ultrasonics, 105947.

30. Talu E, K Hettiarachchi, RL Powell, AP Lee, PA Dayton, ML Longo (2008) Maintaining monodispersity in a microbubble population formed by flow-focusing. Langmuir, 24(5): 1745-1749.

