CHAPTER 5: FABRICATION OF HEPATOCYTE GROWTH FACTOR LOADED NANOPARTICLES EMBEDDED IN GALACTOSE CONTAINING PVA/GELATIN HYDROGEL SCAFFOLDS

5.1 INTRODUCTION

Restoration of functional hepatocytes is a great challenge in hepatic tissue engineering. The success of liver tissue engineering depends on the design of ECM analogue and the continuous availability of tropic factors until complete regeneration. However, diffusion controlled release of growth factors from hydrogels shows limitations such as burst release of factors present near the surface, followed by lesser release of substance at the end as it has initial rapid release rate [1]. Whereas degradation induced release from biodegradable materials follows two-stage release profile: (i) diffusion controlled release during the initial phase until the initiation of degradation and (ii) degradation mediated sustained release. Further, direct incorporation of growth factors into the scaffolds may result in loss of bio-activity due to its fragile nature. This has been overcome by the encapsulation of growth factor loaded microspheres into the scaffolds [2, 3].

Among growth factors such as tumor necrosis factor, IL-6, hepatocyte growth factor (HGF) and epidermal growth factor (EGF), HGF plays a crucial role in the acceleration of tissue repair and regeneration subsequent to acute liver injury [4]. However, exogenous supply of growth factors circulates for less than 5 minutes due to very short half-life [5]. HGF is a heterodimeric form of glycoprotein, which has been found to kindle liver regeneration by increasing the hepatocyte cell number and production of proteins like heparplastin in 70% hepatectomized rats [6]. HGF is a
potent mitogen as it stimulates growth of various epithelial cells; potent motogen for the augmentation of epithelial cell motility; and also a strong morphogen for epithelial tubule formation [7]. Hence HGF has been found to play a vital role in tissue repair, morphogenesis and liver regeneration as cell growth and migration are the premier process in tissue repair and organogenesis [8]. In addition, HGF has been found to promote proliferation of primary hepatocytes [7]. Liver specific mitogen such as HGF is a multi-functional molecule, which interacts with variety of cells and also plays a vital role in the expansion of hepatocytes in vivo [2]. Hence, scaffold with tropic factor like HGF could be a promising substitute to maximize the regenerative potency of liver tissue.

Composite PLGA/PHBV microspheres encapsulated with HGF exhibited sustained delivery and maintained the liver specific function for 40 days while protecting the growth factor from proteolytic damage [2]. De Fail et al., developed microsphere embedded hydrogel system for the sustained release of TGF-β₁ over 21 days and found to show lesser burst release compared to microsphere alone [3].

An attempt was made to develop HGF encapsulated PLGA smooth surfaced nanospheres and to embed on P/G hydrogel scaffolds, to attain the sustained release of growth factor in order to maintain functional hepatocytes for extended period. PLGA (50:50), which is a copolymer of poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) is a FDA-approved synthetic polymer that has been extensively studied for drug delivery application [9].
The present study involves the development of HGF encapsulated PLGA nanocarrier system through double emulsion technique by optimizing PLGA concentration, sonication time, and amplitude. The degradation of PLGA nanospheres, encapsulation efficiency and release of HGF from PLGA nanospheres were evaluated. The developed HGF loaded PLGA nanospheres was embedded into the galactose containing 8:2 and 9:1 P/G hydrogel scaffolds and characterized for evaluating the release kinetics.

5.2 MATERIALS & METHODS

5.2.1 Materials

PLGA (50:50) ($M_w$: 118 kDa and intrinsic viscosity: 0.79 dL/g) was procured from Lakeshore Biomaterials, USA. Dichloromethane (DCM) was purchased from Merck, India. Recombinant human HGF ($\alpha$-chain: 463 amino acid residues and $\beta$-chain: 234 amino acid residues) were purchased from PeproTech, USA and PVA ($M_w$: 80 kDa), BSA ($M_w$: 66 kDa) from Sigma Aldrich, India. HGF ELISA kit was purchased from Ray Bio, USA.

5.2.2 Preparation of PLGA Nanospheres

Different concentrations of PLGA (1, 2 and 4% w/v) were prepared in DCM. About 1% PVA solution was used as surfactant. PLGA solution and surfactant was taken in the ratio of 1:3 and sonicated using Probe ultrasonicator (Vibra-cell, Sonics, USA) at varying time intervals (1, 2 and 3 minutes) and different amplitude (60 % & 70 %) under cold bath to obtain hollow nanospheres. The solvent was removed from
nanospheres by a rotary evaporator, washed with deionised water thrice and centrifuged. The nanospheres were lyophilized overnight for further characterizations.

5.2.3 Preparation of HGF Encapsulated PLGA Nanospheres

HGF encapsulated PLGA nanospheres were prepared by double emulsion technique. Briefly, PLGA solution was mixed with 1 µg/mL HGF and 2 mg/mL Bovine serum albumin (BSA) and was probe sonicated for 45 seconds under ice cold bath. Surfactant was added to this solution in 3:1 ratio and probe sonicated for 2 minutes under ice cold bath. The solvent was then removed from the nanospheres using rotary evaporator, washed with deionised water thrice and centrifuged. The obtained HGF loaded nanospheres were lyophilized and characterized.

5.2.4 Morphological Characterization

The morphology of growth factor loaded PLGA nanospheres and nanosphere embedded hydrogel systems was analyzed using Field Emission Scanning Electron microscopy (FE-SEM, JSM 6701F, JEOL, Japan) at an accelerating voltage of 3 kV. The samples were sprinkled on carbon tape placed on stub and were sputter-coated with gold prior to imaging.

5.2.5 Dynamic Light Scattering Characterization

Hydrodynamic size of the HGF encapsulated and blank PLGA nanospheres were determined by dynamic light scattering (Zetasizer, NANO-ZS - Malvern, UK). Briefly, about 2 mg/mL of nanospheres were dispersed in water using probe sonicator
for 30 seconds. Standard operating protocol (SOP) was created considering the refractive index of PLGA (1.33). Measurements were carried out in triplicates at an operating temperature of 25 °C.

5.2.6  *In vitro Degradation of PLGA Nanospheres*

Phosphate buffered saline (PBS - pH 7.4) was added to the blank nanospheres in eppendorf tubes and kept under constant shaking in water bath shaker (Labline, India) at 37 °C and the PBS solution was changed for every two days. At pre-determined time points (1, 2, 3, 4, 5, 6, 7 & 8 weeks) the samples were centrifuged at 4000 rpm for 5 minutes and pellets were lyophilized to collect the degraded nanospheres. The surface morphology of degraded nanospheres was characterized using FE-SEM and molecular weight (M_W) was quantified using gel permeation chromatography (GPC, Agilent 1200 series, USA) using the formula:

\[
\text{Mass loss percentage(%) = } \frac{M_W \text{ of nanospheres} - M_W \text{ of degraded nanospheres}}{M_W \text{ of nanospheres}} \times 100
\]

5.2.7  *Determination of Encapsulation Efficiency*

Encapsulation of HGF in the nanospheres was quantitatively determined by enzyme linked immunosorbent assay (ELISA) using HGF ELISA kit (Ray Bio, USA). Briefly, HGF encapsulated PLGA nanospheres were treated with DCM under vigorous shaking until the spheres dissolved completely. About 5 mL of PBS was added to extract HGF and centrifuged for 5 minutes at 3000 rpm. The supernatant was
carefully removed and quantified by ELISA to determine the amount of HGF encapsulated in nanospheres.

5.2.8 Embedding HGF loaded PLGA nanospheres onto P/G Hydrogel

Growth factor loaded PLGA nanospheres were embedded onto the 8:2 & 9:1 P/G hydrogel systems in order to control the trans-differentiation of hepatocytes. Briefly, known quantity of HGF loaded PLGA nanospheres were incorporated on the P/G solution and subjected for two F/T cycles. The nanosphere embedded scaffolds were then lyophilized and taken for further characterization.

5.2.9 HGF Release and its Kinetics

Lyophilized HGF encapsulated nanospheres (2 mg) and nanosphere embedded P/G hydrogel were characterized for HGF release using ELISA. In brief, the lyophilized samples were incubated with PBS (pH 7.4) under constant shaking using water bath shaker at 37 °C over a period of 30 days. At different time points the nanospheres were centrifuged at 3000 rpm for 5 minutes in order to collect the HGF and stored at -70 °C to measure the HGF released. Mathematical modelling for the release of HGF from lyophilized samples was carried out with various kinetic models listed in table 5.1 to elucidate the release mechanism using DD Solver 1.0 software.
5.3 RESULTS & DISCUSSION

5.3.1 Fabrication of PLGA (50:50) Nanospheres

5.3.1.1 Effect of Polymeric Concentration

Figure 5.1 shows the effect of various polymer concentrations [1 %, 2 % and 4 % (w/v)] on the development of homogenous nanospheres while keeping other parameters such as surfactant concentration (1 % w/v), ratio of organic to aqueous phase (1:3), sonication time (2 minutes) and sonication amplitude (70 %) constant. There was no proper development of nanospheres at 1 % (w/v) PLGA concentrations (Figure 5.1 A). This may be due to the fact that the viscosity was not sufficient to form spherical particles. As the concentration was increased from 1 to 2 % (w/v), homogenous nanospheres of 250±50 nm were obtained. However, further increase of PLGA concentration to 4 % (w/v) led to the formation of spheres with wider dimension in the range 0.3-1.2 µm (Figure 5.1 C). This may be attributed that the increase in the polymer concentrations increases the viscosity, which in turn reduces the dispersability of PLGA solution in the aqueous phase leading to the development of coarse emulsion [10]. Thus higher polymer concentrations (4 %) in the internal phase of emulsion lead to insufficient dispersion, which in turn increases the particle size with wider distributions. Hence 2 % PLGA concentration was chosen as optimized concentration for subsequent experiments.
Figure 5.1: Scanning electron micrographs showing the effect of PLGA concentrations on formation of nanospheres (A) 1% w/v; (B) 2% w/v and (C) 4% w/v. Magnification - 20,000X. Scale bar - 1 µm
5.3.1.2 Effect of Amplitude on Formation of Nanospheres

Effect of different sonication amplitude (60 and 70 %) on the formation of nanospheres was evaluated under constant conditions of PLGA concentration (2 % w/v) and sonication time (2 minutes) (Figure 5.2 A & B). Heterogeneous distribution of spheres (250 to 800 nm) was observed at the sonication amplitude of 60 % (Figure 5.2 A), suggesting that this amplitude was not sufficient to form smaller droplets during the emulsification process. Increase in sonication amplitude to 70 %, showed homogenous distribution of nanospheres between 200 and 260 nm (Figure 5.2 B). The reduction in the size of spheres with increase in sonication amplitude may be due to the effect of stronger disintegration of droplets at higher amplitude during the emulsification process. Hence sonication amplitude of 70 % was taken as an optimised condition for further experimentation.

Figure 5.2: Scanning electron micrographs showing the effect of sonication amplitudes on formation of nanospheres (A) 60% amplitude; (B) 70% amplitude. Magnification - 20,000X. Scale bar - 1µm
5.3.1.3 Effect of Sonication Time on Formation of Nanospheres

Influence of various sonication times (1, 2 and 3 minutes) on nanosphere size and shape was evaluated while maintaining the other operating parameters such as PLGA concentration and sonication amplitude constant (Figure 5.3). During sonication, on/off cycle was maintained as 45/15 seconds for all conditions. Spherical particles were obtained in all experimental conditions except at 1 minute of sonication (Figure 5.3A). At 1 minute sonication, more particles agglomerated which altered the spherical morphology with wider distribution of dimension from 270 to 900 nm. As the sonication time was increased to 2 and 4 minutes, spherical particles were obtained in the range of 230-330 nm (Figure 5.3 B & C). Higher sonication time releases higher energy to allow complete dispersion of organic phase, which in turn reduces the size of the particles in emulsion [10].

As the carrier system has been designed for the loading of tropic factors, higher sonication time may lead to functional loss due to excess heat generated during prolonged sonication. Since there was no significant change in morphology and size distribution of nanospheres observed at 2 & 4 minutes of sonication, 2 minutes was chosen for further study.
5.3.2 *In vitro* Degradation of PLGA Nanospheres

Degradation of PLGA nanospheres was qualitatively evaluated based on the morphological change and quantitatively by measuring the molecular weight loss percentage. Figure 5.4 shows the scanning electron micrograph of the degradation of PLGA nanospheres at different time points. There was an obvious change in the morphology of the PLGA nanospheres at the end of first week. Swelling of the spheres was observed after the first week, which may be due to water penetration that directly increases the volume of the spheres. The swelling of PLGA 50:50 spheres may lead to the formation of water insoluble low molecular weight oligomers in inner
layers and water soluble oligomers in outer layers through water penetration [11]. After 5 weeks, the spherical morphology was slowly disrupted, which may be attributed the initiation and propagation of hydrolytic reactions between the water molecules and PLGA nanospheres [11]. Further, with the increase in the time periods from 6 to 8 weeks, the nanospheres aggregated and formed clusters losing their spherical morphology (Figure 5.4). The change in the morphology at the end of first and eighth week has clearly indicated the bulk degrading nature of the spheres, which was further confirmed with the molecular weight determination using GPC at various time points (Figure 5.5).

Figure 5.4: Scanning electron micrographs represent the degraded morphology of PLGA nanospheres. Magnifications - Initial: 20,000X, degraded samples: 30,000X. Scale bar - 1μm
After a week of degradation, molecular weight of the nanospheres was lost to about 52±0.8 %, which clearly indicates the burst release behaviour of nanospheres [12]. By four weeks, the molecular weight loss was about 80±0.07 % and increased to nearly 91±0.43 % by eight weeks (Figure 5.5). Bulk degradation pattern of nanospheres was observed from the GPC data. The result obtained were in good agreement with the literature, which highlights that PLGA (50:50) exhibits faster degradation, due to the higher content of PGA that leads to accelerated rate of degradation [12].

Figure 5.5: In vitro degradation of PLGA nanospheres in PBS shown as percentage degradation over eight weeks
5.3.3 Encapsulation of HGF into PLGA Nanospheres

HGF encapsulated PLGA nanospheres were prepared using double emulsion technique by keeping concentration of PLGA, sonication amplitude and sonication time as constant. Briefly, first emulsion was prepared by sonicating the growth factors in organic PLGA solution for 45 seconds under ice cold bath. The second emulsion was developed by sonicating first emulsion in PVA surfactant solution. The scanning electron micrographs of both HGF loaded and unloaded PLGA nanospheres reveal comparable morphology (Figure 5.6). Dynamic light scattering analysis reported the hydrodynamic size of both the HGF loaded and unloaded sphere as 241±56 nm and 254±50 nm respectively.

Figure 5.6: Scanning electron micrographs images of [A] blank PLGA nanospheres; [B] HGF loaded PLGA nanospheres. Magnification - 20,000X. Scale bar - 1µm
5.3.4 Determination of Encapsulation Efficiency

The amount of HGF loaded into the nanospheres was measured using ELISA-HGF kit. The present study involves the co-encapsulation of HGF with Bovine serum albumin (BSA) in order to prevent the functional loss of HGF during fabrication. BSA is a carrier protein with similar molecular weight as that of HGF and it reduces the shear stress during emulsification process and functions as a stabilizer to prolong the bioactivity of HGF [2]. Hence, the loading content of BSA and HGF was measured individually for determining the encapsulation efficiency.

Figure 5.7 shows the effect of various concentrations of BSA (1-50 mg/mL) on encapsulation efficiency while loading into the PLGA nanospheres. At 1 mg/mL initial loading of BSA, the encapsulation efficiency was found to be 15±0.9 %, which may be attributed to the poor availability of BSA during entrapment (Figure 5.7). However, at 2 mg/mL loading of BSA the encapsulation efficiency was significantly increased to 62±2.3 % when compared to 1 mg/mL ($p<0.05$). With increasing BSA concentration (3 mg/mL) the encapsulation efficiency significantly reduced to 31±2.6 % from 62±2.3 % ($p<0.05$). Further increasing BSA concentration to 5, 10, 20, 40 & 50 mg/mL, gradually reduced the percentage of encapsulation. These results demonstrated that the concentration of BSA beyond the threshold (2 mg/mL) drastically reduces the encapsulation efficiency. This may be due to the difference in osmotic pressure between the internal and external aqueous phases, which directly controls the entrapment of BSA into the polymeric spheres [13]. Hence 2 mg/mL of BSA was taken as optimized condition for co-encapsulation of HGF.
Figure 5.7: Encapsulation efficiency of BSA in PLGA nanospheres at different loading concentrations (* indicates statistical significance with concentration at $p<0.05$)

Unlike BSA, lesser concentration of HGF (0.01-1 μg/mL) is sufficient for primary hepatocytes to maintain its phenotype and metabolic functions in vitro [14, 15]. Hence for the present study, maximum concentration of 1 μg/mL was taken as initial loading concentration as reported in literature, which showed the encapsulation efficiency of about 57±2.9%.
5.3.5 Embedding Growth Factor loaded PLGA Nanospheres in P/G Hydrogel

HGF loaded nanospheres were embedded into the 8:2 and 9:1 P/G hydrogel scaffolds and were characterized using a scanning electron microscope to elucidate the incorporation as well as distribution of PLGA nanospheres on P/G hydrogel scaffolds (Figure 5.8). PLGA nanospheres were uniformly distributed into both 8:2 and 9:1 P/G hydrogel scaffolds. Further, nanospheres were dispersed more onto the porous structures thereby it integrated well with the hydrogel scaffolds and did not alter the porous morphology of the hydrogels.

Figure 5.8: Scanning electron micrograph showing the presence of PLGA nanospheres on [A] 9:1 P/G hydrogel scaffold at 5000X; [B] 9:1 P/G hydrogel scaffold at 20,000X; [C] 8:2 P/G hydrogel scaffold at 5000X; and [D] 8:2 P/G hydrogel scaffold at 20,000 X. Scale bar - 1µm
5.3.6 Release of HGF from PLGA nanospheres embedded in P/G hydrogels

Release of encapsulated HGF from PLGA nanospheres as well as nanosphere embedded P/G Hydrogels (8:2 and 9:1) was quantified for 30 days to determine the release kinetics. For hepatic tissue engineering, sustained release of HGF without any initial burst from carrier is advantageous to retain the phenotype and function of primary hepatocyte for longer duration, as the hepatocytes lose their phenotype and function and trans-differentiate to other type of cells rapidly on TCPS [16].

Figure 5.9A shows the release of HGF from PLGA nanospheres was about 1.02±0.006 % after 24 hours, which increased to 4.44±0.03 % after 48 hours. Nanosphere embedded hydrogel systems of 8:2 and 9:1 ratio released equal amount of HGF (0.87±0.013 %) at the end of 24 hours, which was similar to that of HGF release from nanospheres (Figure 5.9B). However, the release from nanosphere embedded hydrogels was found to be lesser (1.9±0.032 %) after 48 hours when compared to the nanospheres. Release of HGF up to 48 hours for all three systems (PLGA nanosphere; nanosphere embedded P/G hydrogel of 8:2 and 9:1) demonstrated the absence of initial burst release, which may be due to the negligible presence HGF on the carrier surface [17].

Figure 5.9B shows the cumulative release of HGF from the nanospheres and nanosphere embedded hydrogel systems for both 8:2 and 9:1 ratio for 30 days. Cumulative release of HGF from the nanosphere embedded 8:2 and 9:1 hydrogel scaffold was 9.8±0.45 % and 8.3±0.65 % respectively after eleven days, which was found to be higher (15.8±0.65 %) when compared to nanosphere mediated delivery (Figure 5.9B). After 21 days, the HGF release from 8:2 and 9:1 nanosphere embedded
hydrogel scaffolds was 17.6±1.45 % and 16.3±1.41 % respectively, while the HGF delivery from the nanospheres was more than two fold (37±1.41 %) (Figure 5.9 B). After 30 days, around 22.2±1.78 % and 21.3±2.28 % of HGF was released from the nanosphere embedded 8:2 and 9:1 hydrogel scaffolds. HGF released from the nanosphere aided delivery after 30 days was around 51±2.28 %, which was found to be higher than the nanosphere embedded hydrogel system. Though the release of HGF from both the systems was comparable after day 1, it was observed that the release was significantly higher in plain nanosphere system when compared to nanosphere encapsulated hydrogel system after day 30. Higher release profile from the HGF nanospheres may be due to the faster degradation behaviour of PLGA (50:50). On the contrary, HGF release from nanosphere embedded hydrogel system encounter two-stage release profile (degradation aided release from nanosphere; diffusion controlled release from hydrogel). This may result in the slower release of HGF throughout the study period. Hence, nanosphere embedded hydrogel could be a promising substitute for liver regeneration as it delivers HGF in a sustained and controlled manner up to 30 days.
Figure 5.9: Cumulative release percentage of HGF from delivery systems for [A] 48 hours and [B] for 30 days
5.3.7 Mathematical Modelling

Modelling of HGF release kinetics for nanosphere and nanosphere embedded hydrogel delivery system was carried out for various kinetic models using DD Solver 1.0 software in order to elucidate the release mechanism. The kinetic equations are represented as follows (Figure 5.10).

Table 5.1 shows the regression coefficient values of various kinetic models for nanosphere and nanosphere embedded hydrogel systems. HGF release pattern for both the carrier systems does not fit into zero order kinetics, which was confirmed by lesser $R^2$ values of 0.8866 to 0.8800, suggesting the non-linear pattern [18]. *In vitro* release data for nanospheres was found to fit the Baker-Lonsdale and Weibull model. The Baker-Lonsdale model was developed in 1974 for water-soluble drug release from matrix carrier systems [19, 20]. The Weibull model is pertinent for the release of factors from matrix delivery systems [19-21]. Hence, this study confirmed that the release of HGF from the nanospheres was governed by the spherical PLGA matrix system. However, release data for nanospheres embedded in a hydrogel matrix for both the ratios (9:1 and 8:2) fitted best with Makoid-Banakar model with the resultant $R^2$ value of 0.9978 and 0.9985 respectively, which suggests diffusion controlled growth factor release from nanosphere loaded hydrogel [21]. Further, the release through nanosphere embedded hydrogels also fitted with Baker-Lonsdale, Weibull as well as Korsmeyer-Peppas with the regression coefficient of greater than 0.99. The release exponent ($n$) in Korsmeyer-Peppas model was 0.5, suggesting the Fickian diffusion controlled delivery. This has clearly suggested that the release of HGF from nanospheres embedded hydrogel systems followed the combination of diffusion and erosion controlled release mechanism from spherical matrix [18, 22].
Table 5.1: Mathematical Modelling for delivery systems

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<th>Kinetics model</th>
<th>HGF encapsulated in PLGA nanospheres ((R^2))</th>
<th>HGF encapsulated PLGA nanospheres embedded in 9:1 P/G hydrogel scaffold ((R^2))</th>
<th>HGF encapsulated PLGA nanospheres embedded in 8:2 P/G hydrogel scaffold ((R^2))</th>
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Figure 5.10: Release kinetic models with equations
5.4 CONCLUSIONS

Operating parameters such as polymeric concentration, sonication amplitude and sonication time were optimized for the development of smooth plain PLGA nanospheres (230-330 nm) using single emulsion technique. The size of spherical particles was found to be increase with increasing polymer concentration, decreasing sonication amplitude and time. In vitro degradation of plain PLGA nanospheres showed 91 % degradation in 8 weeks. In the present study, HGF was loaded into the PLGA nanospheres with the encapsulation efficiency of 57±2.9 % using double emulsion technique. The growth factor encapsulated nanospheres were successfully embedded onto the 8:2 and 9:1 P/G hydrogel scaffolds. Release of HGF from the nanosphere embedded hydrogel delivery systems was found to be slow and sustained when compared to HGF nanospheres. This was also confirmed with the various mathematical models for release kinetics in which nanosphere embedded hydrogel system followed the two-stage release kinetics – diffusion and degradation. The developed nanosphere embedded hydrogel system could be a promising to deliver HGF for prolonged periods, which may control the trans-differentiation of primary hepatocytes.

5.5 REFERENCES


