Development and physico-chemical characterization of PLGA-Casein core/shell nanoparticles encapsulating Ptx and EGCG (hydrophobic-hydrophilic) combination
2.1 Introduction

In cancer, the deregulation of multiple signaling pathways makes it essential to treat cancer with multiple drugs. Combination therapy using multiple drugs has been acknowledged as a viable option to achieve superior response for disease treatment, owing to their effectiveness in individually modulating specific molecular mechanisms. However, patient non-compliance and multiple schedules of administering combination drugs in addition to possible cross-resistance and complexity in controlling the pharmacokinetics and pharmacodynamics of the combination are practical difficulties. Hence, to address these concerns, a rationally engineered therapeutic vehicle that could transport desired dosages of drugs in combination, in a sequential, yet controlled manner, would be an ideal strategy. Such a sequential therapy could substantially improve efficacy, potentially mitigating the toxicity risks associated with individual drugs. Core/shell nanocarriers have gained impetus in the field of combination cancer medicine where they act as candidates to carry multiple drugs to the site of action, thereby enabling improved therapy. Such co-delivery modes have several inherent advantages that include enhanced synergistic therapeutic efficacy, better drug-resistance management, and the capacity to temporally regulate drug release. Despite these benefits, entrapping drugs of distinct chemical nature, solubility and molecular weight is indeed challenging, especially with regard to the modulation of key parameters such as entrapment efficiency and release kinetics.

The present chapter discusses the strategy adopted to encapsulate two anticancer drug molecules, namely paclitaxel (Ptx) and epigallocatechin gallate (EGCG), of hydrophobic and hydrophilic nature respectively into a single core/shell nanocarrier. The rationale for the choice of this drug combination was to address the issues of Ptx adversities by the use of the multifaceted natural polyphenol EGCG, which has chemotherapeutic and chemopreventive properties. This chapter focuses on the synthesis of the core/shell nanomedicine and the characterization of its size, size distribution, colloidal stability, viscosity, casein
quantification, casein degradation, drug entrapment (qualitative and quantitative) and in vitro drug release profile.

2.2 Major research questions

Based on the available literature, the following are the major research questions for this part of the thesis work:

RQ-1 • How can two drugs of chemically distinct nature (hydrophilic/phobic) be optimally accommodated into a single nanocarrier?

RQ-2 • What would be the influence of strong ionic strength solutions and serum conditions on the colloidal stability of the core/shell nanoparticles?

RQ-3 • How would the core/shell design of the nanocarrier influence the release pattern of the two drugs entrapped within it?

2.3. Materials and Methods

2.3.1 Materials

Paclitaxel was procured from LKT laboratories, USA and EGCG from Chengdu Biopurify Phytochemicals Ltd., China. PLGA (75/25, Mw 5,000) with uncapped carboxyl ends was purchased from Wako, Japan. Polyvinyl alcohol (PVA, Mw 10K Da) was purchased from Sigma, USA. Casein sodium salt, acetone and dichloromethane were purchased from Sigma, USA. MilliQ water was used for synthesis purpose and all chemicals were used without further purification.

2.3.2 Preparation of dual drug-loaded PLGA-PVA nanoparticles

Oil-in-water single emulsion route was adopted to prepare PLGA-PVA nanoparticles encapsulating Ptx and EGCG. Briefly, the oil phase consisted of 5 mg PLGA dissolved in acetone alone or in acetone:dichloromethane (1:1) solvent mix with 1mg Ptx dissolved in this oil-phase. The oil-phase was then emulsified
in water-phase containing 1% PVA and 2mg EGCG, under probe sonication (VibraCell, Sonics, USA) for 1 min. The emulsion was allowed to stir for 1 hr at 1000 rpm for solvent evaporation. This was then centrifuged at 12,000 rpm for 10 min (Avanti J26 XP, Beckmann Coulter, USA) and washed using MilliQ water. The pellet was lyophilized (Alpha 2-4 LD plus LT, Martin Christ, Germany) in 10% sucrose as cryoprotectant and stored for further experiments.

2.3.3 Preparation of void and drug-loaded PLGA-Casein core/shell nanoparticles

PLGA-Casein core/shell nanoparticles were prepared through a novel route of emulsion-precipitation. Briefly, 10 mg of PLGA was dissolved in acetone/dichloromethane binary solvent (2:1 v/v) (oil-phase) and emulsified into 3 mg/mL casein aqueous phase (water-phase) under probe sonication for 1 min. A uniform precipitation of casein on PLGA was achieved by the drop-wise addition of 1N HCl into the emulsion under sonication. The organic and acid phases were removed by repeated centrifugation and washing at 12000 rpm for 10 min. For preparing drug loaded nanoparticles, the two drugs viz., Ptx and EGCG were incorporated into the core/shell nanoparticles by mixing Ptx in the oil-phase and EGCG in the water-phase respectively. Varying ratios of Ptx:PLGA (wt:wt = 1:1, 1:2, 1:5) and EGCG:Casein (wt:wt = 1:1, 1:2, 1:10) were analyzed to study the influence of drug and polymer/protein contents on entrapment efficiency. The nanosuspension that yielded the optimum particle size and entrapment was finally lyophilized for further experiments. For comparison, empty casein nanospheres (without PLGA core and no drug entrapped) were also synthesized by acid precipitating casein solution (3 mg/mL concentration) under 1 min probe sonication.

2.3.4 Characterization of drug-loaded nanoparticles

The hydrodynamic particle size, size distribution and zeta potential of the prepared nanoparticles were analyzed using NanoZS Zetasizer (Malvern, USA). Aqueous dispersion of the samples was used for the measurements in triplicates. Size and morphology of the nanoparticles were assessed using scanning electron
microscope-SEM (JOEL, JSM-6490LA, Japan) and transmission electron microscope-TEM (Jeol JEM-2100 LaB6, Japan). The colloidal dispersion was diluted in MilliQ water, drop casted on an aluminum stub and sputter-coated with gold before SEM imaging. For TEM imaging, the diluted sample was dropped on a carbon-coated copper grid and imaged at an operating voltage of 200 kV. To measure the viscosity of the nanoparticles suspension, lyophilized nanoparticles were re-suspended in saline and viscosity was measured using a programmable viscometer at 25°C and 100 rpm (DV-II+Pro, Brookfield, USA). Stability of the nanoparticles in saline, PBS (pH 7.4) and PBS containing 20% serum (pH 7.4) was evaluated by hydrodynamic size and zeta potential analysis. Drug incorporation in the nanomatrix was also confirmed qualitatively using Fourier Transform Infra-red (FTIR) spectroscopy (Spectrum RX-1, Perkin Elmer, USA). The spectral scan of samples prepared with KBr was carried out in the frequency range from 400 to 4000 cm$^{-1}$ at a resolution of 4 cm$^{-1}$.

Raman measurements on various samples were carried out using a confocal microscope system (Model: WITec alpha 300RA, GmbH, Germany). It comprised of a spectrograph [UHTS 300] (600 lines/mm grating, 30 cm focal length) coupled to a Peltier cooled CCD detector. A 488 nm laser source provided excitation via an optical fiber (50 μm) that was focused onto the sample by an objective lens (100X, NA: 0.9) and the scattered light was collected for analysis.

### 2.3.5 Drug encapsulation efficiency and in vitro release by HPLC

The encapsulation efficiency and in vitro release of Ptx and EGCG were quantified by HPLC (Prominence UFLC, Shimadzu) fitted with a UV-VIS detector (SPDM20A). Measurements for both drugs were carried out with Qualisil gold C-18 column (4.6x250mm, particle size 5µm) maintained at 30°C. The optimized mobile phase used was a mixture of methanol (80% v/v) and 0.02M KH$_2$PO$_4$ buffer (pH 2.5, adjusted with H$_3$PO$_4$, 20% v/v) at a flow rate of 0.5 mL/min and an injection volume of 20 μL. The UV detection of the drugs was carried out at 273 and 227 nm for EGCG and Ptx respectively. The total analysis
time for this method was set at 15 min. Drug loading efficiency was calculated using the formula:

\[
\text{Drug loading (\%)} = \frac{\text{Mass of drug in nanoparticles}}{\text{Mass of nanoparticles}} \times 100
\]

To calculate the drug entrapment efficiency, the following mathematical expression was used:

\[
\text{Encapsulation efficiency (\%)} = \frac{\text{Mass of drug in nanoparticles}}{\text{Mass of drug used in formulation}} \times 100
\]

The in vitro release was done by resuspending lyophilised drug-loaded nanoparticles in phosphate buffered saline (PBS) at pH 7.4, 37°C under shaking at 50 rpm on a dual action shaker (PolySciences, USA) for a period of 10 days. The release medium containing drug-loaded nanoparticles was centrifuged at predetermined time intervals and the respective concentrations were derived using HPLC. The release data was fitted to the various drug release kinetic models viz., zero order, first order, Higuchi and Hixon-Crowell models and the corresponding linear regression coefficients (R²) were determined. The model giving an R² value closest to unity was considered the best fit. The data was also fitted to Korsmeyer-Peppas model to determine the release mechanism.

2.4 Results and Discussion

2.4.1 Dual drug-loaded nanoparticle synthesis and characterization

The precision in delivering combinations of drugs in a sustained, selective and sequential manner qualifies core/shell nanoparticles as exceptional drug-carrying tools. The present work was aimed at developing a novel core/shell nanocarrier system for intravenous sequential drug delivery applications.

Initially, a simple oil-in-water emulsion method was adopted to prepare PLGA-PVA nanoparticles encapsulating Ptx and EGCG. PLGA being a well established hydrophobic polymer for encapsulating hydrophobic drugs [1], was
considered as the matrix for Ptx which is hydrophobic in nature [2] (Figure 2.1 A). It is accepted biocompatible and biodegradable polyester which makes them suitable candidates for pharmaceutical purposes [3]. The use of PLGA nanoparticles as carriers for paclitaxel has been published by several groups who have confirmed high entrapment efficiencies for the drug [4-6]. In this study, PVA, a hydrophilic polymer [7], was used as a matrix for EGCG which is hydrophilic in nature [8] expecting non-covalent interactions to occur between the two especially hydrogen bond formation between hydroxyl moieties present in both EGCG and PVA (Figure 2.1 B).

Solvents used for PLGA were also altered since reports have claimed the influence of organic solvents on particle size [9, 10]. The solvents used were acetone and acetone:dicholoremethane 1:1 mix. In our case, using acetone alone as the solvent, rendered particles of size ~440 nm with a very high polydispersity index (PDI-0.6) indicating largely polydispersed nanoparticles (Figure 2.2 A). However, for the binary solvent system used (acetone:dicholoremethane 1:1 mix), the particle size was ~250 nm with a narrow range of particle size distribution as indicated from the PDI (Figure 2.2 B). This could be because dichloromethane is a better solvent for PLGA than acetone [11] and has a very quick evaporation rate unlike that of acetone [12], which probably must have led to the formation of compact particles of smaller size. Further, it was now essential to calculate the
drug entrapment efficiency of Ptx and EGCG for both the above prepared systems. It was found that the entrapment efficiency of Ptx was 83 and 87% for particles synthesized in acetone and the binary solvent mix respectively. However, the entrapment of EGCG was not appreciable in either case with ~32% in both. Also, on evaluating the drug release profile it was observed that EGCG showed a very large burst release with >80% of the entrapped drug releasing just with 10 hrs and Ptx showing no significant release at all in 24 hrs (Figure 2.2 C). These results prompted to an unsuccessful attempt to entrap EGCG within PVA in addition to the uncontrolled release of the entrapped EGCG which was undesirable for our purpose.

In an attempt to seek out for an appropriate matrix system for EGCG, literature revealed the ‘naturally’ occurring interactions of EGCG, a tea polyphenol, with the largely found milk protein, Casein [13-16]. Therefore, the next attempt was to replace PVA with casein as the carrier matrix for EGCG. On emulsifying the oil-phase (PLGA and Ptx in the binary solvent mix) into aqueous-phase containing 3% casein and EGCG, a milky white appearance is revealed. This was further magnetically stirred for solvent evaporation, following which the pellet was collected by repeated centrifugation and washing and then lyophilized. Here, it was interesting to see that the entrapment efficiency of EGCG had increased to 48% as opposed to the low values obtained for the PLGA-PVA systems, although not very appreciable when compared to that of Ptx which had improved to 91% in this case. On examining the TEM image, it was revealed that the particle size was ~200 nm and was found that casein had not formed any layer around the PLGA core, possible also not stabilizing the core (Figure 2.2 D). The marginal increase in entrapment efficiency for EGCG could be due to this very feeble amount of casein that has deposited around the PLGA core.
Figure 2.2: Characterization of dual drug-loaded nanoparticles. SEM images of PLGA-PVA nanoparticles prepared using (A) acetone and (B) Acetone: Dichloromethane (1:1) solvent mix as the oil-phase, (C) Drug release obtained for Ptx and EGCG. (D) TEM image of drug-loaded nanoparticles prepared using Casein in the aqueous phase instead of PVA.

At this juncture, it was necessary to further enhance the EGCG entrapment with a denser shell structure of casein containing EGCG over PLGA core and therefore, the present strategy of simple emulsion was slightly modified to include an additional step of casein precipitation. Here, soon after the oil-phase was emulsified in the casein aqueous phase, acid precipitation was carried out to deposit casein over PLGA core. The schematic of the synthesis strategy is represented pictorially in Figure 2.3.
Since Ptx is extremely hydrophobic with a partition coefficient (log $K_{o/w}$) favoring hydrophobic phases [17, 18], it is expected that the loaded Ptx would be associated with the PLGA phase. On the contrary, EGCG being hydrophilic with a very low partition coefficient (log $K_{o/w}$) of 1.46 ± 0.2 [19], has less probability of interacting with PLGA. However, its interaction with casein via non-covalent bonding and the affinity of polyphenols towards proline in casein is reported [13-15]. This implies that the hydrophilic drug EGCG would retain mostly within the casein shell. Recently, Haratifar et.al have reported the use of casein as a carrier for EGCG with the goal to improve encapsulation of the polyphenol [16], considering the natural interactions between EGCG (green tea) and casein (milk protein).

As already mentioned, the core/shell nanoparticles were synthesized through the combination of two distinct methods viz., emulsion and precipitation, wherein casein precipitation onto PLGA core occurred only at an acidic pH below its reported isoelectric point of 4.6 [20]. Figure 2.4 A depicts the SEM and DLS analysis of drug loaded core/shell nanoparticles revealing nearly monodisperse particles with a polydispersity index of 0.18. DLS data of PLGA core loaded with Ptx showed a hydrodynamic particle size of 190±21 nm. The TEM images of PLGA-Casein system (Figure 2.4 B and D) displayed a core/shell morphology for both void and dual drug loaded nanoparticles with diameters of 152±8 and 190±12 nm respectively. This increase in size could be attributed to the
encapsulation of two drugs together into the core/shell system as discussed earlier. A clear contrast was observed in the core and shell of the nanoparticle when imaged using TEM. This contrast can perhaps be attributed to the difference in electron density between the polymeric core and protein shell materials respectively. The shell thickness was measured to be typically 10-15 nm as shown in Figure 2.4 C. It was observed that dispersing PLGA nanoparticles into casein aqueous solution (neutral pH) did not lead to any shell formation (Figure 2.4 E). However, upon reducing the pH to acidic range (below isoelectric point), the net charge of casein becomes positive due to the rapid protonation of its amine functional groups. This observation is supported by the positive surface charge (+20 mV) of bare casein nanospheres (~ 135nm) prepared by acid precipitation (Figure 2.4 F). Evers et.al recently reported a simulation study stating that β-casein adsorbs to hydrophobic and charged surfaces not only due to direct electrostatic and hydrophobic interactions, but also due to structural rearrangements and charge regulations caused by proton uptake and release [21]. In the present case, we suppose that the availability of protonated amines in casein at pH <4 facilitates its interactions with the free carboxyl groups on PLGA core, yielding a distinctive and stable core/shell morphology for the nanoparticles as evident from the TEM images.
Figure 2.4: Size and morphological characteristics of core/shell nanoparticles. (A) SEM image of PLGA–casein dual-drug-loaded nanoparticles; inset gives the size distribution of the nanoparticles from DLS measurements. (B) TEM image of PLGA–casein dual-drug-loaded core/shell nanoparticles showing distinct core/shell morphology 190 ± 12 nm in size. (C) High-magnification TEM image depicting the thickness of casein shell to be ~10–15 nm. (D) TEM image of empty PLGA–casein core/shell nanoparticles 152 ± 8 nm in size. (E) PLGA–asein nanoparticles prepared without acid precipitation of casein aqueous phase, yielding no core/shell morphology. (F) SEM image of casein nanospheres (~135 ± 15 nm) prepared through simple acid precipitation alone.

Figure 2.5 depicts a clear difference in the hydrodynamic sizes of Ptx encapsulated PLGA core alone, dual drug loaded and void core/shell nanoparticles. The shell formation on PLGA as well as drug loading could have been an attributing factor which led to the increase in size for the PLGA-Casein core/shell. Also the difference in size between drug loaded and void nanoparticles could be due to the presence of drugs which interact with the respective compartments leading to an increased size. Similar results on increased particle size upon drug encapsulation have been reported in literature for various kinds of nanoparticulate systems [22-24].

![Figure 2.5: Hydrodynamic diameter measured using DLS](image)

The stability of the nanomedicine is widely regarded as a critical attribute in determining the overall performance of the formulation and in this regard, the particle size and charge of the nanoparticle have become increasingly important.
Understanding the changes in particle size and charge of nanoparticles suspended in biologically relevant media is vital for the commercial success of nanoformulations. Therefore, in our study, stability monitoring was included and was done for both void as well as drug-loaded PLGA-Casein core/shell nanoparticles in high ionic strength media and serum conditions. Stability studies of the bare core/shell carrier in high ionic strength solutions such as PBS (pH 7.4, Ionic strength: 162.7 mM) and saline (0.9 % NaCl, Ionic strength: 154 mM), and 20 % serum containing phosphate buffered saline (PBS) were carried out. The particle size and zeta potential of the suspension at predetermined time intervals was analysed usingNanoZS Zetasizer (Malvern, USA). The hydrodynamic size of the bare nanocarriers in saline evaluated by DLS varied from 192-195 nm, while for PBS and PBS containing 20 % serum (pH 7.4) it ranged from 228-230 nm and 229-301 nm respectively (Figure 2.6 A). Nanoparticles suspended in both saline and PBS did not show any significant enhancement in size. However, in serum containing PBS, the hydrodynamic diameter of the particles increased with time, which may be due to the adsorption of serum proteins to the particle surface.

The zeta potential values of the nanoparticles suspended in PBS containing 20% serum showed a shift from -25.5 to -19.2 mV in 48 h, while the particles in saline and PBS showed almost negligible variations in surface charge (-24 to -27.3 and -30.4 to -33.4 mV respectively) (Figure 2.6 B). It is previously reported that proteins do not significantly adsorb onto particles with negative zeta potential values [25]. This could be the reason why the core/shell nanoparticles have not exhibited a drastic variation in zeta potential. It is important to mention that the nanoparticles did not coagulate or even flocculate in any of the media even after 48 h.
Figure 2.6: (A) Hydrodynamic diameter variations of the void core/shell nanoparticles in various media viz., saline, PBS and PBS containing serum at different time intervals (0, 24, 48h). (B) Zeta potential variations of the void core/shell nanoparticles in saline, PBS and PBS containing serum at 0, 6, 24 and 48 h.

Additionally, the hydrodynamic diameter and zeta potential of the dual drug loaded nanomedicine was also measured in PBS (pH 7.4), saline, and PBS containing 20% serum for 0, 24 and 48 h (Figure 2.7 A). The trend was very similar to that exhibited by the bare core/shell nanocarriers. The particles suspended in serum containing PBS showed a slight increase in size with time (230 to 300 nm) while those suspended in saline (192-195 nm) and PBS (228-230 nm) showed negligible change even after 48h.

Zeta potential was also recorded for the drug loaded nanoparticles and it was noticed that the presence of drugs influenced the surface charge of nanoparticles. In saline, the particles showed a zeta potential of -42.5, -39.2 and -39.6 mV, while for particles suspended in PBS it was -35.8, -34.3 and -37.4 mV respectively for
0, 24 and 48 h (Figure 2.7 B). In both these media, the surface charge increased for the drug loaded nanoparticles (-35 to -40 mV) when compared to the void nanocarriers (-24 to -33 mV), perhaps owing to the charge contributed by the surface resident EGCG (EGCG is negatively charged at physiological pH conditions) as previously reported for EGCG coated layer-by-layer films and microcapsules [26].

**Figure 2.7:** (A) Hydrodynamic diameter variations of dual drug-loaded core/shell nanoparticles in various media viz., saline, PBS and PBS containing serum at different time intervals (0, 24, 48h). (B) Zeta potential variations of the same in saline, PBS and PBS containing serum at 0, 24 and 48 h.

The zeta potential value of -23±6.5 mV obtained for void core/shell nanoparticles can be attributed to the negative charge rendered by the existence of –COO⁻ of casein on the particle surface when finally dispersed in MilliQ water whose pH (6.9) is above the isoelectric point of casein (4.6). However, upon drug loading, the core/shell particles showed a higher zeta potential of -41±3.4 mV, indicating that the presence of the catechin EGCG which has a negative charge, would have contributed to the overall charge since paclitaxel has no net charge.
For any nanocarrier suggested for intravenous (IV) drug delivery, the viscosity of the formulation is an essential attribute. To assess the applicability of PLGA-Casein nanomedicine for IV administration, its viscosity was measured at a relatively high concentration of 1 mg/mL. For the void and drug loaded nanocarriers dispersed in saline, the viscosity was measured to be 1.14 cP and 1.13 cP respectively. These values are very similar to that of saline control (1.05 cP), implying its suitability for intravenous injection.

Raman spectroscopy was carried out to analyze the formation of the polymer-protein core/shell nanoparticles as well as its drug encapsulation. Figure 2.8 A depicts the Raman spectrum of PLGA, Casein, PLGA-Casein core/shell nanoparticles and Figure 6B that of the drugs Ptx, EGCG and dual drug-loaded nanoparticles. The presence of bands at ~870 and 1760 cm\(^{-1}\) due to C-O-C and C=O stretches respectively are the major Raman peaks for PLGA [27]. This is reflected in the spectrum of PLGA-Casein core/shell nanoparticles. The protein amide bands (Amide I, II and III) of casein which appear at ~1664, 1451 and 1249 cm\(^{-1}\) [28] were found slightly shifted to 1677, 1463 and 1267 cm\(^{-1}\) respectively in the core/shell system. Additionally, a peak at 1016 cm\(^{-1}\) corresponding to P-O-C stretch of the phosphoprotein casein [29] has also appeared in the core/shell nanoparticle spectrum. To confirm the presence of drugs viz. Ptx and EGCG in the core/shell nanoparticles, a comparative analysis of the Raman spectra of dual drug-loaded PLGA-Casein nanocarrier and the bare drugs was done. The most significant Raman peaks of Ptx occurred at 631, 1012 and 1612 cm\(^{-1}\) corresponding to the in-plane ring deformation, C-O-C vibration of carbon in cyclic ring and C=C stretch in the benzene ring of the aromatic compound respectively [30]. The CH\(_2\) scissor vibration band at 1459 cm\(^{-1}\) in Ptx was evident in the core/shell nanoparticles. An evaluation of the spectra depicted in Figure 2.8 B reveals that many of the peaks which are diagnostic of functional groups in Ptx are clearly present in the spectrum of the drug loaded matrix too. The peaks corresponding to C-O-C vibration of carbon in cyclic ring and C=C stretch in benzene ring of aromatic compound are also seen for EGCG, which is a
polyphenol with aromatic ring structure. A major peak at 754 cm\(^{-1}\) corresponding to the aromatic OH out-of-plane deformation is found in the spectra of both EGCG as well as drug-loaded nanoparticles, indicating entrapment of EGCG. A comparison of the spectra (green colour) of void and drug-loaded core/shell nanoparticles showed considerable differences, particularly in the range of 600 – 1700 cm\(^{-1}\) where major peaks of both drugs have appeared, implying drug encapsulation. Bands at 1240 and 1764 cm\(^{-1}\) indicating casein and PLGA are also reflected in the spectrum of drug-loaded nanocarriers.

Figure 2.8: Qualitative evaluation of core/shell and drug-loaded core/shell nanoparticles. (A) Raman spectra of PLGA, casein and PLGA–casein core/shell nanoparticles. (B) Raman spectra of EGCG, Ptx and dual-drug-loaded core/shell nanoparticles.

FTIR spectroscopy was also performed to qualitatively evaluate PLGA-Casein particle formation and drug loading, as shown in Figure 2.9. The prominent carbonyl stretch of Ptx occurs at 1734 cm\(^{-1}\), while that of PLGA is at 1762 cm\(^{-1}\). After encapsulation, these peaks in the drug loaded nanoparticle broadened and appeared with reduced intensity, with a shift to 1768 cm\(^{-1}\), implying the interactions between PLGA and the drug. As already reported by Maiti et.al. [15] conformational changes occur to proteins upon complexation with EGCG, which was reflected in the shift of the amide peak I from 1650 to 1647 cm\(^{-1}\). A similar trend was noticed in the FTIR spectrum of drug loaded
nanoparticles, wherein the amide I band of casein at ~1650 cm\(^{-1}\) widened and shifted to ~1670 cm\(^{-1}\). The aromatic ring C=C stretch peak of EGCG evident at 1532 cm\(^{-1}\) for the bare drug was also found broadened and shifted to 1542 cm\(^{-1}\) upon encapsulation. The broad band between 3600-3400 cm\(^{-1}\) indicating O-H stretching in EGCG became sharper at 3652 cm\(^{-1}\), perhaps owing to the interactions between casein and EGCG.

![FTIR spectra showing characteristic peaks of PLGA, Casein, EGCG, Ptx and dual-drug loaded PLGA-Casein nanoparticles.](image)

**Figure 2.9:** FTIR spectra showing characteristic peaks of PLGA, Casein, EGCG, Ptx and dual-drug loaded PLGA-Casein nanoparticles.

### 2.4.2 Drug encapsulation efficiency, in vitro release and drug release kinetics

Drug entrapment within nanomatrices is governed by the hydrophobicity/philicity of the drugs as well as the drug-carrier affinity. In the present study, the use of PLGA and casein enabled loading of both Ptx and EGCG at relatively high efficiencies upon optimizing the polymer, protein and drug concentrations as depicted in Table 2.1. The use of casein for encapsulating EGCG can be justified by the well understood non-covalent interactions between EGCG and casein, and the affinity of polyphenols like EGCG towards proline, a major amino acid of casein [13-16]. The drug entrapment of the optimized
nanoformulation estimated using HPLC for Ptx and EGCG was 95.7±7.3% and 76.8±9.1% respectively. The hypothesis that the entrapment of hydrophilic EGCG within hydrophobic PLGA matrix would be less, was supported by our experiments yielding an entrapment efficiency of only about 4% for EGCG in PLGA. Precipitating casein over PLGA did not alter the entrapment efficiency of Ptx. However, the entrapment of EGCG was significantly altered upon varying casein concentration (Table 2.1). At an optimal EGCG:Casein ratio of 1:10, the nanoparticles showed good entrapment efficiency for the drug (76.8 ± 9.1%). However, for equal proportions of EGCG and casein (1:1), an instantaneous coagulation of casein was observed which could possibly be due to the heavy crosslinking promoted by the addition of high amounts of EGCG.

Table 2.1. Effect of drug/polymer ratios on drug encapsulation efficiency

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<th>Ptx (% entrapment)</th>
<th>EGCG (% entrapment)</th>
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<tbody>
<tr>
<td><strong>Core alone</strong> Ptx:PLGA = 1:5</td>
<td>96.9 ± 5.2</td>
<td>–</td>
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<tr>
<td><strong>Core/shell</strong> Ptx:PLGA = 1:5</td>
<td>95.7 ± 7.3</td>
<td>–</td>
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<tr>
<td><strong>Core/shell</strong> Ptx:PLGA = 1:2</td>
<td>96.3 ± 9.2</td>
<td>–</td>
</tr>
<tr>
<td><strong>Core/shell</strong> Ptx:PLGA = 1:1</td>
<td>94 ± 5.2</td>
<td>–</td>
</tr>
<tr>
<td><strong>Core/shell</strong> EGCG:Casein = 1:1</td>
<td>–</td>
<td>Precipitated</td>
</tr>
<tr>
<td><strong>Core/shell</strong> EGCG:Casein = 1:2</td>
<td>–</td>
<td>62 ± 6</td>
</tr>
<tr>
<td><strong>Core/shell</strong> EGCG:Casein = 1:10</td>
<td>–</td>
<td>76.8 ± 9.1</td>
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In vitro drug release kinetics for the dual drugs from the core/shell nanocarrier was measured individually by HPLC. The cumulative drug release profile (in Phosphate Buffered Saline (PBS), pH 7.4, 37°C) revealed a sequential and controlled release of the drugs with ~28% of Ptx and ~60% of EGCG released in 10 days (Figure 2.10). EGCG showed an initial burst (7.6±4%) owing to the release of the superficially entrapped EGCG, which eventually released at a controlled rate after 48h, while Ptx showed a delayed release. PLGA particulate delivery systems typically show a release profile with an initial burst followed by a near-zero order phase [31].
Figure 2.10: *In vitro* release of EGCG and Ptx showing a sustained release for 10 days in PBS at 37 °C, pH 7.4.

To assess if the presence of casein shell influenced Ptx release, drug release experiments (PBS, pH=7.4, 37°C) were also conducted for Ptx encapsulated PLGA-Casein core/shell nanoparticles without EGCG, as well as bare PLGA nanoparticles encapsulating Ptx (Figure 2.11 A). It was observed that the presence of a shell over PLGA core influenced the release of Ptx from the core, though not very significantly.

We also compared the release profiles of both the drugs individually encapsulated in the core and shell respectively (Figure 2.11 A). An interesting observation made was that the presence of EGCG in the shell enhanced the release of Ptx from PLGA core at pH 7.4. In the light of literature reporting enhancement of PLGA degradation in acidic medium [32], we have assessed the pH of the release medium at different time points [0, 4, 6, 24, 48, and 72 h]. It was noted that the pH of the release medium gradually shifted to the acidic range (Figure 2.11 B) which could be attributed to the release of EGCG. Therefore, it can be understood that the release of Ptx is due to the slow and steady degradation of PLGA core which possibly could be happening after the initial degradation of casein. The possibility of casein swelling at pH 7.4 [33] is also very likely, as this can also lead to EGCG release and accelerate Ptx diffusion from core. Such a
release profile would benefit drug delivery schemes wherein prolonged release of one drug followed by another is deemed fit. This would be better substantiated by the \textit{in vivo} pharmacokinetics of the two drugs in the subsequent sections.

Figure 2.11: (A) Release profiles from nEGCG (core/shell nanoparticles with only EGCG), nPtx (core/shell nanoparticles with only Ptx), cEGCG (profile of EGCG from dual-drug loaded core/shell nanoparticles), cPtx (profile of Ptx from dual-drug loaded core/shell nanoparticles), CF\_nPtx (casein free PLGA core with Ptx) (B) pH variation with time recorded in the release medium containing dual-drug loaded core/shell nanoparticles.

To understand the kinetics and mechanism of drug release, the data for each drug was separately fitted with various kinetic equations like zero order, first order, Higuchi and Hixon-Crowell models (Table 2.2). The best fit with highest correlation was found with Higuchi model for both EGCG and Ptx with $R^2$ values of 0.9604 and 0.9271 respectively, indicating a diffusion governed release. In the present case, the drug release mechanism due to the swelling of casein at physiological pH of 7.4 coupled with the gradual erosion of PLGA matrix cannot be accounted for by the Higuchi equation. Hence, the data was fitted to the well
known exponential equation (Korsmeyer-Peppas model) and the release exponent value (n), a kinetic constant dependent on the transport mechanism [34], was determined. Ptx and EGCG showed a non-Fickian release profile with n= 0.579 and 0.517 respectively, indicating that drug release from this multicompartmental system occurs through a combination of swelling, diffusion and/or erosion.

Additionally, to evaluate if the presence of serum proteins influenced the release of the drugs, in vitro drug release was performed in 20% serum containing PBS (pH 7.4) at 37°C and the data is shown in Figure 2.12. It was observed that in serum conditions the trend of release more resembled a controlled release profile. The release of EGCG was slightly lower and controlled in serum condition (i.e. ~49 % vs 53%) by 72 hours when compared to serum-free condition, while Ptx release was more or less similar (~26% vs 25%) by 72 hours. This alteration in the release of EGCG might indicate presence of serum proteins on nanoparticle surface which eventually would have interacted with EGCG present on the surface of the casein shell. Aggarwal et.al have reported that negatively charged particles (such as the present PLGA-Casein nanoparticles) generally adsorb IgG-like proteins and the interaction of EGCG with such serum proteins is previously reported [35].
2.5 Summary

- A polymer-protein hybrid PLGA – Casein core-shell nanocarrier of size 190±12nm entrapping a combination of chemically distinct (hydrophobic/hydrophilic) drugs viz., Ptx and EGCG, was prepared by a simple emulsion-precipitation route.
- High ionic strength media (saline, PBS) did not significantly alter the diameter or surface charge of the nanoparticles, but serum conditions slightly altered both its diameter and surface charge.
- PLGA-Casein nanoparticles rendered high entrapment efficiency for both Ptx and EGCG and displayed a temporally controlled and sustained release profile for both drugs in PBS and serum conditions.