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Influence of surface passivation of 2-Methoxyestradiol loaded PLGA nanoparticles on cellular interactions, pharmacokinetics and tumour accumulation

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Research Highlights:
1. Efficient nanoe encapsulation of 2-ME2 in Casein/PEG stabilized PLGA
2. Particles of similar size and surface charge developed by emulsification process
3. PEGylation improved the pharmacokinetics of 2-ME2 over Casein stabilization
4. Differential protein binding onto nanoparticles might be responsible for this effect

ABSTRACT

In the present work, 2-Methoxyestradiol [2ME2] loaded PLGA nanoparticles [NPs] were stabilized with Casein or poly(ethylene glycol) [PEG] and evaluated for its cellular interactions, pharmacokinetics and tumour accumulation. Surface stabilized PLGA nanoparticles prepared through a modified emulsion route possessed similar size, surface charge, drug loading and release characteristics. Particle-cell interactions as well as the anti-angiogenesis activity were similar for both nanoformulations in vitro. However, in vivo pharmacokinetics and tumour accumulation of the drug were substantially improved for the PEGylated nanoformulation. Reduced protein binding was observed for PEG stabilized PLGA NPs. Thus, it was demonstrated that nanoe encapsulation of 2-ME2 within PEGylated PLGA nanocarrier could improve its half-life and plasma concentration and thereby increase the tumour accumulation.
Keywords: 2-Methoxyestradiol, PLGA Nanoparticles, Drug delivery, Breast Cancer, Pharmacokinetics, Tumour accumulation, Protein binding

1. Introduction

The biggest challenge in the treatment of cancer is mostly not the inefficiency of drugs, but our inability to use them to the fullest potential, owing to their high off-target toxicity and low bioavailability. Several physico-chemical characteristics such as hydrophobicity of the drug, its rapid clearance, enzymatic conversion, accumulation in undesired anatomical regions, etc. contribute to these limitations. 2-methoxyestradiol [2-ME2] is one such experimental drug with combined anti-tumour and anti-angiogenic activity, which showed highly promising results for breast cancer therapy in preclinical studies [1,2]. 2-ME2, a metabolite of estradiol, destabilizes the microtubule by binding to its colchicine binding region, thereby preventing the nuclear translocation of HIF-1α [3]. Thus, the production of VEGF and other angiogenic factors are inhibited by 2-ME2 [3]. However, because of its hydrophobicity and rapid clearance in vivo, the drug showed little efficacy in clinical trials[2,4–6]. Moreover, 2-ME2 was shown to undergo rapid conversion by liver enzymes in vivo. Even a therapeutic dose of 1g every 6 hours orally did not result in an effective plasma concentration[5].

Recent scientific strategies to overcome the aforementioned problems include chemical and physical modifications of such drugs or its nanoencapsulation [7–9]. The use of biodegradable and biocompatible polymers such as Poly(lactic-co-glycolic acid) (PLGA), Poly Vinyl Alcohol (PVA), Polycaprolactone (PCL), etc for drug encapsulation is a widely accepted strategy [8,10–13]. However, the benefits of nanoencapsulation are limited by its stability, thereby demanding appropriate stabilization strategies to prevent agglomeration/aggregation of the particles [14–16]. Various categories of molecules such as lipids (phosphatidylcholine), proteins (albumin, Casein), polymers (PEG, PVA & PEI) and artificial polymeric compounds (Pluronics) are widely employed in the stabilization of nanoparticles [17]. Amongst them, PEG is the most studied and applied surface passivating molecule, and there are currently about 35 US-FDA approved nanoparticles incorporating PEG either for imaging or therapy [15,18]. Surface passivation of nanocarriers using PEG facilitates decreased RES uptake, thereby offering prolonged circulation, reduced opsonisation, liver accumulation and clearance. Additionally, PEGylation also provides
functional groups for bio-functionalization [19–23]. Recently, for the first time, our group reported the preparation and pre-clinical analysis of nanocarriers made of PLGA and Casein [24,25]. Casein, a milk protein, when used to stabilise PLGA nanoparticles (PLGA:Casein), could enable entrapment of dual drugs with hydrophobic and hydrophilic characters and provide prolonged in vivo circulation. This suggested a potential nanocarrier stabilizer combination which can be used for a large number of clinically important drugs with unfavourable physico-chemical properties. Therefore, the current study analysed how well encapsulation of 2-ME2 within differently stabilized PLGA nanoparticles would improve its pharmacological profile. Temporal plasma concentration and tumour/tissue accumulation of 2-ME2 entrapped within PLGA:Casein (2ME2:PLGA:Casein) and PLGA:PEG (2-ME2:PLGA:PEG) were evaluated and compared with 2-ME2 in molecular form.

2. Material and Methods

2.1 Materials

Poly(lactic-co-glycolic acid) (PLGA), [75:25, MW:5000 Da] was purchased from Wako Pure Chemical industries, Japan. Poly(ethyl glycol) (PEG), Sodium Caseinate salt from bovine milk, Fluorescein Isothiocyanate (FITC) were purchased from Sigma-Aldrich, USA. Solvents and other reagents used for the study including dichloromethane (DCM), Hydrochloric Acid (HCl), etc were of analytical grade and procured from Merck Chemicals, India and Sigma-Aldrich, USA respectively and used without any further purification.

2.2 Preparation of empty and 2-Methoxyestradiol loaded PLGA:Casein and PLGA:PEG nanoparticles

Preparation of PLGA:Casein nanoparticles was done by modifying a previously reported emulsion–precipitation route [25]. Briefly, 10mg PLGA was dissolved in dichloromethane (oil phase) and emulsified into 0.4 % (w/v) Casein aqueous phase (water phase) under probe sonication and magnetic stirring for 1 min (VibraCell, Sonics, USA). Surface passivation of Casein on PLGA was achieved by the drop-wise addition of 1 N HCl into the emulsion under sonication. The organic and acid phases were removed by repeated centrifugation and washing at 12000 rpm for 15 min (Beckmann Coulter, USA). PLGA: PEG nanoparticles were also prepared using the same method, but without HCl mediated precipitation. Different combinations of surfactant (Casein or PEG) to PLGA ratio (0 to 0.4% Casein and 0 to 0.5% PEG) were used to obtain nanoparticles of desired characteristics. For preparing drug-loaded nanoparticles, 2-ME2 was incorporated into PLGA core of the
particles by dissolving it into the PLGA-DCM mixture prior to emulsification. Varying ratios of PLGA and 2-ME2 (1:1 to 1:25) were analysed to study the influence of drug to polymer ratio on drug entrapment. Drug loaded and bare particles were then lyophilized with 5% sucrose as a cryoprotectant and stored under refrigeration until further analysis. For the preparation of fluorescent nanoparticles, FITC was mixed with PLGA solution in DCM followed by emulsification into the surfactant-containing water phase.

2.3 Characterization of drug-loaded nanocarriers

The hydrodynamic particle size, size distribution and zeta potential of the prepared nanoparticles resuspended in distilled water were analysed by dynamic light scattering (Nano ZS Zetasizer, Malvern, USA). Morphological analysis was carried out by scanning electron microscopy (SEM; JEOL, JSM-6490LA, Japan) and Transmission electron microscopy (TEM, FEI-Technai G230, Germany). Drug incorporation and chemical composition of the nanocarriers were analysed qualitatively using Fourier Transform Infrared spectroscopy (FTIR; IR Infinity-IS, Shimadzu, Japan). 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\(^{-1}\).

2.4 Assessment of drug encapsulation efficiency and \textit{in vitro} drug release

The encapsulation efficiency and \textit{in vitro} release of nanoparticle encapsulated 2-ME2 was quantified by high-performance liquid chromatography (HPLC; Prominence UFLC, Shimadzu) fitted with a UV–VIS detector (SPDM20A). Qualisil gold C-18 column (4.6 X 250 mm, particle size 5 µm) maintained at 40°C was used as stationary phase. The optimised mobile phase used was a mixture of methanol (25% v/v), water (25% v/v) in acetonitrile at a flow rate of 1 ml/min and an injection volume of 20 µl. Drug quantification was monitored at a wavelength of 205 nm. A typical efflux time of 8 min was observed for the drug.

To calculate the drug encapsulation efficiency, the following equation was used

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

The \textit{in vitro} release was performed after resuspending lyophilized drug-loaded nanoparticles in PBS at pH 7.4. These samples were then aliquoted to several eppendorf tubes and incubated at 37°C under shaking in a dual-action shaker (Polysciences, USA). Eppendorf tubes containing the release medium with drug-loaded nanocarriers were centrifuged at predetermined time intervals and the drug concentration in the supernatant was derived using
HPLC. The release data was fitted to various drug release kinetic models such as zero-order, first-order, Higuchi, Hixon–Crowell and Korsmeyer–Peppas.

2.5 Assessment of cell-nanoparticle interactions

Concentration-dependent cytotoxicity studies of 2ME-2 bare and nanoformulations were done on breast cancer cell lines MCF-7, T47D (procured from National Centre for Cell Sciences, Pune), and HUVECs (isolated from human umbilical cord) using Alamar blue assay. Briefly, 8 x 10^3 cells/well were seeded in 96 well plates containing 100 μL cell culture media (α-MEM for MCF-7 and T47D, IMDM with growth factor supplements HUVEC) and incubated for 24 hours at 37°C. Further 100 μL media containing different 2-ME2 formulations at varying equivalent concentrations of 2-ME2 ranging from 10nM to 250μM were added to the wells and incubated for 24 hours. Cells cultured in media containing 10% FBS and 1% Triton X100 served as the negative and positive controls respectively. After incubation, wells were washed with PBS twice, and 100 μL media containing 10% Alamar Blue (Invitrogen, USA) was added and again incubated at 37 °C for 4 hours. The optical density was recorded at 570 nm, with 600 nm set as the reference using a microplate spectrophotometer (Biotek Powerwave XS, USA). Percentage cell viability was calculated by measuring the optical density values at 570nm of particle-treated and control cells.

(Human umbilical vein endothelial cells (HUVECs) were isolated as described elsewhere [26] from the umbilical cord samples that were collected from female donors with the approval of the institutional review board and with appropriate informed patient consent. Briefly, the cords were obtained in sterile phosphate buffered saline (PBS) and washed thoroughly, cannulating the vein to wash out the blood inside the lumen with PBS. Subsequently, the vein was incubated with 0.1% Type I collagenase (Invitrogen, USA) in serum-free IMDM (GIBCO, Invitrogen, USA) for 15 min at 37 °C. The detached cells were washed in serum-free IMDM and suspended in complete IMDM (containing 20% fetal calf serum, GIBCO, Invitrogen), 100 U ml⁻¹ pen/strep antibiotic solutions (GIBCO, Invitrogen, USA) and ECGF (Sigma Aldrich, USA). The HUVECs were grown on 2% gelatin (Sigma Aldrich, USA) coated tissue culture plates in complete IMDM in standard cell culture conditions. Confluent cells in the third passage were used for the experiment. For quiescent HUVEC culture alone, the cells were grown to a monolayer with 100% coverage (typically 3
days for a 96-well plate) before incubation with 2-ME2 formulations, followed by assessment of cytotoxicity as previously explained.

For Boyden Chamber assay, 6-well transwell inserts, 8 µm (Costar, Corning Inc, USA) tissue culture plates were used. Active HUVECs in log phase were seeded onto the upper chamber of the inserts at a seeding density of 20,000 cells/insert along with different formulations of 2-ME2 and incubated for 6 hours at 37°C in 5% CO₂. After this incubation period the upper chamber was removed and the lower chamber incubated further for 24 hours under the same condition, followed by Alamar blue assay to quantify the number of cell infiltrated. Endothelial cells cultured on a chamber after sealing the filtration pores were used as control.

The cellular uptake of nanoparticles was analysed using flow cytometry (FacsAria, BD, USA). The cell culture conditions were kept as described previously. Fluorescent nanoparticles (FITC encapsulated nanoparticles) were added to the cell suspension at a concentration of 100 µg/ml and incubated at 37°C for different time intervals (15 min, 2, 4, and 8 hours). Cells were washed to remove the surface bound and free particles at each time point and subjected to flow cytometry measurement. Data was recorded for each 10000 events.

2.6 Animal Experiments

All animal experiments were conducted after ethical approval by the Institutional Animal Ethics Committee of Amrita Institute of Medical Sciences and Research Centre, Kochi, India. Animal experiments were done to evaluate the temporal plasma concentration and the differential tumour and organ accumulation of different drug formulations.

2.7 Assessment of in vivo temporal plasma concentration of 2-ME2

Pharmacokinetics of 2-ME2 formulations was analysed in adult Sprague-Dawley rats of both sex, (n=6, weight ~250 gms). Briefly, 2-ME2 loaded PLGA:Casein or PLGA: PEG nanoparticles were re-suspended in saline and administered intravenously at a dose equalling to 30mg/kg of 2-ME2. For the injection of bare 2-ME2, the drug was dissolved in DMSO and suspended in 10% Tween-20 containing saline under vortexing. Blood was collected retro-orbitally at pre-determined time points (5 min, 15 min, 30 min, 45min, 1,2,3,4,6,7,8 and 24 hours post injection) in heparinized vacutainers. Plasma was collected and precipitated using
a Methanol/ammonium acetate/DMSO mixture and the drug content was evaluated using HPLC as described above. Pharmacokinetic parameters such as concentration maximum (C$_{\text{max}}$), time of maximum concentration (T$_{\text{max}}$), the area under curve (AUC), mean resident time (MRT), elimination rate constant (K$_{\text{el}}$) and half-life (t$_{1/2}$) were determined using a Microsoft Excel add in tool PKSolver.

2.8 Tumour model development

Tumour models for the study were developed by subcutaneous injection of 10$^6$ 4T1 breast cancer cells (procured from ATCC, USA) into the neck region of healthy 6-8 weeks old female Balb/c mice (approx. 20 gm weight). Prior to cell inoculation, animals were immunocompromised by whole body gamma ray irradiation (600 rad) and kept under aseptic conditions. Animals were used for the study after 10 to 12 days of tumour initiation when tumours reach a palpable mass.

2.9 Tumour/Tissue accumulation of nanoformulations

For the organ/tissue distribution & tumour accumulation studies, the nanoformulations and bare drug were administered intravenously to the tumour bearing Balb/c mice. Animals were euthanized to quantify the drug content indifferen organs, namely lungs, kidney, liver and dissected tumour tissue after 24 hours of the drug injection. Drug content assessment in organs and tumour tissue were done using analytical HPLC as described previously.

2.10 Estimation of protein binding on nanoparticle surfaces

Nanoparticles were dispersed in 50% FBS solution at a concentration of 5 mg/ml and incubated at 37°C in a shaking incubator (Polysciences Inc, USA). To analyse the change in particle size and surface charge of nanoparticles suspended in PBS and plasma, hydrodynamic diameter and zeta potential were measured at three time points, viz., 6, 24 & 48 hours. Protein binding on nanoparticles was quantified using bicinchoninic acid (BCA) assay (after 1, 6 & 24 hours incubation) and Polyacrylamide gel electrophoresis (PAGE) after retrieving the nanoparticles from the solution. For BCA assay, surface stabilised nanoparticles incubated in PBS without plasma served as the control and absorbance of the test samples (Biotek Powerwave XS, USA) was normalised with reference to this control. 1-dimensional PAGE was performed in 25% gel against BioRad precision plus markers, based
on a protocol described elsewhere [26]. Briefly, nanoparticle-protein mixtures were centrifuged at 18,000 g for 15 min at 4°C. Pellets were resuspended in 1.5 ml PBS (10 mM phosphate, pH 7.5, 0.15 M NaCl, and 1mM EDTA) and vortexed. This step was repeated 3 times and the pellets were suspended in PBS. Based on the absorption from BCA assay, all the samples were now diluted with PBS in varying dilution factors to obtain the same protein loading quantity, followed by gel loading using equal volumes of samples in SDS-page loading buffer. Bands were visualised by silver staining and recorded using a gel documentation system (GelDoc XRS, BioRad, USA).

2.11 Statistical analysis

Statistical significance of the results was analysed using GraphPad Prism 6. One-way-ANOVA and students “t”-test were used to confirm significant differences or association between the observed results.

3. Results and discussion

3.1 Nanoparticle preparation and drug loading

PLGA:PEG nanoparticles were prepared by a simple oil-in-water emulsification and PLGA:Casein nanoparticles by a slightly modified emulsion/precipitation process. The surfactant concentration (PEG, Casein) in the water phase was varied from 0 to 0.5 % (w/v) by keeping PLGA concentration in the oil-phase constant (10mg/ml). Size and surface zeta potential variations with varying concentrations of Casein and PEG were as shown in table 1. PLGA particles synthesised through emulsification method without any surface passivation (0% PEG or Casein) were polydispersed and precipitated soon after synthesis. An optimum concentration of 0.4% Casein containing water phase yielded particles of size range 138±38nm, with a colloidal stability of -28.4 mV and PDI of 0.32 (Table 1, Figure 1 (A), and Supplementary Table 1). For Casein concentrations exceeding 0.4%, empty Casein micelles were formed. The negative surface charge of PLGA:Casein nanoparticles could be attributed to the excess COO- groups exposed on the nanoparticle surface in an aqueous solution [24]. Similarly, an increase in PEG concentration in water phase also lead to a decrease in particle size up to a concentration of 0.05%, beyond which the hydrodynamic diameter increased, with concurrent decrease in colloidal stability. Here, a PEG concentration of 0.05% in water phase was found optimal for preparing PLGA:PEG nanoparticles of size 160±32 nm and zeta potential -20.7mV, with a PDI of 0.3 (Table 1, Figure 1 (B), and Supplementary Table 1). PEG chains are highly hydrophilic and align water molecules around itself which prevents
hydrophobic aggregation of nanoparticles, conferring thermodynamic stability [18,27]. Temporal stability and aggregation of both nanoparticles were also analysed in different media (distilled water, 0.9% saline, and cell culture media). PEGylated and Caseinated nanoparticles maintained colloidal stability even after 48 hrs with negligible aggregation (supplementary figures 1 and 2).

Further, 2-methoxyestradiol, a highly hydrophobic oestrogen derivative with a partition coefficient of 2.8 was encapsulated within PEG and Casein stabilized PLGA nanoparticles. It is already well established that PLGA can interact with hydrophobic drugs and entrap them inside a precipitated network in water medium [28]. In the current study, 2-ME2 was mixed along with PLGA in oil phase before the emulsification process. Different drug-to-polymer ratios were examined with the concentration of 2-ME2 fixed at 1 mg/ml and PLGA concentrations varying from 0.1 to 20 mg/ml. A direct incremental relationship was observed between drug loading and the drug-to-polymer ratio of 1:12, beyond which PLGA aggregation effects were seen, resulting in reduced drug entrapment (Table 2).

FTIR spectroscopy of PLGA (Figure:1(C)) nanoparticles confirmed the presence of prominent functional groups of PEG, Casein and 2-ME2. The carbonyl stretch of PLGA at 1762 cm$^{-1}$ was observed in all the nanoparticle samples. PEGylation was confirmed by 3441 cm$^{-1}$(O-H) stretching and 2878 cm$^{-1}$(C-H) stretching [29]. Likewise, Caseinated nanoparticles displayed the characteristic amide I band of Casein at 1650 cm$^{-1}$. Presence of 2-ME2 was confirmed by the presence of peaks at 1600 cm$^{-1}$ and 2861 cm$^{-1}$corresponding to amide C=O stretch and alkyl C-H stretch interactions respectively [30].

Drug encapsulation behaviour of Caseinated and PEGylated nanoparticles measured by HPLC analysis were comparable, with a drug encapsulation efficiency of 80±6% and 76±5% at a drug-to-polymer ratio of 1:12. The drug loading efficacy of Caseinated and PEGylated particles at this optimized drug to polymer ratio were 7.24±1 and 9.80±1 respectively (Supplementary Fig S3). These results suggest that surface passivation with PEG or Casein did not significantly affect 2-ME2 – PLGA interactions. Furthermore, for the same 1:12 ratio, variations in PEG and Casein concentrations (Supplementary table S2) did not influence its particle size or stability.

3.2  

**In vitro** drug release
The \textit{in vitro} drug release profile (in PBS, pH 7 at 37°C) revealed a sustained release pattern, with ~80% of the entrapped drug being released in 48 hours for PEGylated and Caseinated systems. Both formulations showed an initial burst release of ~ 20%, owing to the presence of loosely bound or superficially adsorbed 2-ME2, beyond which the release was slow (Figure 2(B)). Drug loaded PLGA showed a typical burst release profile followed by a near zero order sustained release [31].

The release of active ingredient loaded within a polymeric nanoformulation may be governed by several factors including the degradation of the polymer or diffusion of the drug across a concentration gradient [8]. This can follow various kinetic models that fit to zero-order, first-order, Higuchi, Hixon–Crowell models and the mechanism of drug release [32] can be deciphered from the Korsmeyer–Peppas model. Supplementary table S3 depicts the theoretical fit obtained using the above models and it was noted that the best fit with the highest correlation was observed in the Higuchi model for 2-ME2 loaded in both PLGA:Casein and PLGA:PEG with $R^2$ values of 0.9417 and 0.9612 respectively, indicating a diffusion-governed release. However, in the current scenario, owing to the fast drug release observed, water mediated swelling of PLGA matrix also could not be ruled out. Hence, the data was fitted to the well-known exponential equation (Korsmeyer–Peppas model) and the release exponent value (n), was determined [33]. Caseinated and PEGylated nanocarriers showed a non-Fickian release profile with “$n$” = 0.537 and 0.514 respectively, indicating that drug release occurs through a combination of swelling of PLGA matrix and diffusion of the drug. Not much variation in the drug release pattern was noted for PEGylated and Caseinated nanoparticles, suggesting that surface passivation did not interfere with the properties of drug release.

3.3 \textit{In vitro} cellular interactions of PEGylated and Caseinated PLGA nanoparticles

Cellular interactions of PEGylated and Caseinated nanoformulations were assessed on cultured breast cancer cell lines MCF-7 and T47D as a function of 2-ME2 concentration. 2-ME2 is a well-studied anti-cancer agent which exhibits excellent toxicity profile against a large library of cancer cell lines [34]. Here, varying concentrations of 2-ME2 (100 nM to 250 μM) in free form and its equivalent nanoform were tested for 24 and 48 hours. The anti-cancer property of 2-ME2 nanoformulation was more or less similar to that of the bare drug for both the formulations examined (Figure 3(A)). However, the IC$_{50}$ values of 2-
ME2:PLGA:PEG (10 µM and 6 µM for MCF-7 and T47D respectively) and 2-ME2:PLGA:Casein (8 µM and 6 µM respectively) were slightly higher than that of the bare drug. 2-ME2 is a microtubule destabilizer, which binds to the cholchicine binding sites of the microtubule and initiates apoptosis by inhibiting MAPK pathway [35]. Cytotoxicity mechanism of 2-ME2 is reported to be similar for all cancer cells, with its efficiency being limited by the availability of the drug inside the cells. Hence, any nanocarrier designed for 2-ME2 should facilitate its accumulation in the tumour vicinity by EPR effect and thereby its uptake by the cells. To ascertain this, time dependent cell internalisation profile of PEGylated and Caseinated nanoparticles was analysed using flow cytometry in model cell lines MCF-7 and T47D. Cell uptake of PLGA:PEG nanoparticles was comparatively lesser than PLGA:Casein nanoparticles. It was observed that after 8 hours of incubation, 40.2% and 55.9% of MCF-7 and T47D cells internalised PLGA:Casein particles, while only 30.2% and 29.4% PLGA: PEG nanoparticles were uptaken by MCF-7 and T47D cells. The same scale of difference was observed at all time points examined (Figure 3(B)). This slow uptake of PLGA:PEG nanoparticles by the cells followed by the sustained release of 2-ME2 from the nanocarriers may be the reason for its slightly enhanced IC_{50} value. However, it is important to note that the overall anti-cancer effect of 2-ME2 is retained even after nanoencapsulation.

Tumour associated endothelium is highly active and rapidly replicating, while the normal endothelium of a blood vessel is quiescent [36]. 2-ME2 was shown to inhibit the growth of actively proliferating endothelium, while sparing the non-proliferating endothelial cells [37]. The property of 2-ME2 to distinguish between these two makes it an attractive anti-angiogenesis agent. We further examined whether nanoformulation of 2-ME2 could exert its toxicity differentially on active and quiescent endothelium (Figure 3(A) bottom panel). HUVEC monolayers and actively growing HUVECs were treated with different concentrations of 2-ME2 in bare and nanoforms. All the formulations tested (upto 250 µM) were non-toxic to HUVEC monolayers (quiescent endothelium), while it showed significant toxicity towards actively growing endothelium. The dose dependent toxicity profile of the nano and bare formulations remained the same.

2-ME2 being an active anti-angiogenesis agent can inhibit endothelial cell proliferation, migration and infiltration [3]. In order to examine the effect of nanoformulation on the inhibition of endothelial migration by 2-ME2, Boyden chamber assay was performed (Figure 3(C)). After 6 hours of infiltration and 24 hours of incubation, there was a considerable reduction in cell number in the bottom chamber for samples treated with 2-ME2
formulations compared to the negative control. However, there was no significant difference between the anti-migratory activity of bare 2-ME2, PLGA:PEG and PLGA:Casein formulations. Cumulatively, experiments assessing cellular interactions of nanoformulation suggest that the in vitro toxicity and anti-angiogenesis activity have not been significantly altered upon nanoencapsulation of 2ME2.

3.4 In vivo pharmacokinetics of 2-ME2 and its nanoformulations

PLGA:PEG and PLGA:Casein nanoformulations of 2-ME2 had comparable drug loading, release pattern and other physico-chemical characteristics and toxicity profile in vitro. In order to understand their interactions with the biological system in vivo, pharmacokinetics analysis of both formulations were performed in adult Sprague-Dawley rats and compared with that of the bare drug (Figure 4(A)). A rapid clearance was observed for the bare 2-ME2 formulation, with a plasma half-life of 6.2±1.3 min and a mean retention time (MRT) of 12.6±2 min (Table 3). In contrast, both nanoformulations showed considerable advancement in the pharmacological profile and parameters. Plasma half-life increased to 50.12±7.2 min and 70.21±5.2 min for 2-ME2:PLGA:Casein and 2-ME2:PLGA:PEG respectively. MRT increased to 55.28±7.8 min (2-ME2:PLGA:Casein) and 125.11±22 min (2-ME2:PLGA:PEG). A complete clearance of 2-ME2 was observed within 1 hour for PLGA:Casein formulation, while PLGA:PEG formulation sustained up to 8 hours. As can be noted from Table 3, the bioavailability was increased for the nanoformulations (1.8 fold for PLGA:Casein and 4.33 fold for PLGA:PEG) when compared to bare 2-ME2.

To assess the biodistribution of 2ME2 in tumour bearing mice, adult female BALB/c mice bearing subcutaneous 4T1 breast tumour cells were injected intravenously with bare and nanoformulations of 2-ME2 and the intra-tumoural concentration was assessed at the 24th hour. As evident from Figure 4(B), tumour accumulation of 2-ME2 nanoformulation was significantly improved over its bare forms. After 24 hours, intra-tumoural concentration of 2-ME2 in the bare drug-treated animal group was insignificant, while animals that received PLGA:Casein and PLGA:PEG formulations retained 16ng/mg and 38ng/mg respectively. Such concentrations of drug accumulation for a period of over 24 hours have been reported to yield therapeutic benefits [38]. This improved drug accumulation of the nanoformulations in comparison to the bare form of 2ME2 in the tumour can be attributed to its increased retention and circulation. Additionally, the amount of drug quantified in other organs such as liver, kidney and lungs post 24 hour injection is depicted in figure 4(B). As expected,
pharmacokinetic profiles and tumour accumulation of 2-ME2 nanoformulations were improved in comparison to the drug in its free form. The non-specific accumulation of the drug was however less for the nanoformulations in liver, kidney and lungs. Notably between the two nanoformulations, PLGA:PEG formulation showed improved half-life, retention time and AUC, with reduced clearance rate compared to PLGA:Casein nanoformulation. The interesting fact is that although both preparations had similar sized nanoparticles of comparable surface charge, drug loading and release characteristics, their pharmacokinetic profiles were quite distinct.

These results suggest that there are multiple parameters which affect the interaction of a nanoparticle with biological systems. One such important difference in the current study is the surface passivation of PLGA nanoparticles. PEG is a widely used surfactant that aids the preparation of long circulating nanocarriers. It is well established that PEG-passivated nanosurfaces attract less proteins than many other surface passivating agents [39]. In a recent study by our group, it was noted that Casein functionalized PLGA nanoparticles attracted less protein corona, in comparison to positively charged particles [40]. Here, the cellular uptake of nanoparticles was found to vary proportionally to its surface protein adsorption. It is already well established that nanoparticle-biological interactions are mainly governed by the differential protein adsorption onto particle surfaces [41–44]. Nanoparticle recognition and clearance via reticular-endothelial system occur mainly by opsonisation, followed by macrophage uptake. Hence, it is possible that the reduced cell uptake and increased circulation observed in this study is because of the reduced protein binding on to PLGA:PEG surfaces.

3.5 Evaluation of differential protein binding on PLGA:Casein and PLGA:PEG surfaces

To understand the variation in protein binding between PLGA:Casein and PLGA:PEG nanoparticles, both formulations were incubated in 50% plasma in PBS for different time intervals. In order to quantify the protein binding onto nanoparticles, the increase in hydrodynamic diameter was recorded and compared with that of respective nanoparticles in PBS [41]. As evident from figure 5(A), PLGA:Casein nanoparticles showed a significant increase in size (172±18nm to 315±30nm in 24 hours), while the hydrodynamic diameter variations of PLGA-PEG nanoparticles were only marginal. Further, the increase in
hydrodynamic diameter was correlated with surface protein binding by BCA assay and 1-D agarose gel electrophoresis (Figure 5(B)). As evident, the quantity of protein bound to PLGA:Casein nanoparticle surfaces were ~ 6-fold higher than on PLGA:PEG (figure 5(C)). Thus, we confirmed that reduced protein binding was responsible for the enhanced pharmacokinetics of PLGA:PEG particles.

4. Conclusion

In the current study, nanoencapsulation of 2-ME2 within a polymeric PLGA nanocarrier stabilized by PEG or Casein has aided in improving its half-life and plasma concentration to a value where it might result in a therapeutic activity. Although PLGA:PEG and PLGA:Casein formulations possessed similar physico-chemical characteristics, PLGA:PEG nanocarriers were found to be pharmacologically beneficial because of its low protein binding, increased retention and circulation.

5. Acknowledgements

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Figure 1: Electron micrographs (TEM images and SEM in inset) of (A) PLGA: Casein 2-ME2 and (B) PLGA: PEG 2-ME2. (C): FTIR spectra of 2-ME2 formulations and PLGA

Figure 2: *In vitro* drug release profile of 2-ME2 loaded in PLGA:PEG and PLGA:Casein nanocarriers in PBS @ pH 7 and 37°C. (n=3, Error bars :SD)
Figure 3: Cytological evaluation of 2-ME2 loaded PLGA:PEG and PLGA:Casein nanoparticles. (A) Top panel: Cytotoxic activity of bare and nanoformulations of 2-ME2 on MCF-7 and T47D breast cancer cell lines for 24 hours. Bottom panel: Differential toxicity of 2-ME2 formulations towards quiescent and active endothelial cells in vitro. (B) Time dependent uptake of PLGA:Casein and PLGA:PEG nanoparticles by MCF-7 and T47D breast cancer cells. (C) Top panel: Inhibition of endothelial migration to the bottom chamber of a Boyden chamber by free and nanoformulations of 2-ME2. Bottom Panel: Representative bottom panel images of 2-ME2 bare and nanoparticle treated Boyden chambers.

Figure 4: (A): In vivo pharmacokinetics of bare 2-ME2 and nanoformulations. (Sprague-Dawley rats, n=6, error bars: SD). (B): In vivo tumour accumulation and biodistribution of bare 2-ME2 and nanoformulations. (Tumour bearing Balb/c mice, n=3, error bars: SD, 2-Way ANOVA, *: Significant (P>0.5) **: Highly Significant (P>0.005)).
Figure 5: Differential plasma protein binding to PLGA:PEG and PLGA:Casein nanoparticles. (A) Temporal increase in hydrodynamic diameter of nanoformulations incubated in 50% FBS Vs PBS. (B) Normalized protein binding on to PLGA:PEG and PLGA:Casein nanoparticles for varying incubation with 50% FBS. Gel loading concentrations were normalized by diluting the samples to obtain measurable band intensity. (C) Quantitative measurement of differential protein binding on PLGA:Casein and PLGA:PEG nanoparticles (n=3, Error bars :SD).
**Table 1**: Variations in size, surface charge and polydispersity index of PLGA:Casein and PLGA:PEG nanoparticles with varying surfactant concentration in water phase (n=3, Error : SD)

**Casein Concentration (% w/v)**

<table>
<thead>
<tr>
<th>Casein Concentration (%)</th>
<th>Diameter (nm)</th>
<th>Zeta (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Precipitated</td>
<td>8.72±2</td>
<td>1</td>
</tr>
<tr>
<td>0.1</td>
<td>1378±300</td>
<td>-6±0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>0.2</td>
<td>698±200</td>
<td>-8.38±7</td>
<td>0.78</td>
</tr>
<tr>
<td>0.25</td>
<td>565±250</td>
<td>-24.69±9</td>
<td>0.5</td>
</tr>
<tr>
<td>0.3</td>
<td>209±6</td>
<td>-26.5±6</td>
<td>0.52</td>
</tr>
<tr>
<td>0.4</td>
<td>138±30</td>
<td>-28.4±5</td>
<td>0.32</td>
</tr>
<tr>
<td>0.5</td>
<td>28±16</td>
<td>-31.3±7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**PEG concentration (% w/v)**

<table>
<thead>
<tr>
<th>PEG concentration (%)</th>
<th>Diameter (nm)</th>
<th>Zeta (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Precipitated</td>
<td>8.72±2</td>
<td>1</td>
</tr>
<tr>
<td>0.01</td>
<td>2563±500</td>
<td>-12.8±1.5</td>
<td>0.82</td>
</tr>
<tr>
<td>0.02</td>
<td>300±65</td>
<td>-23.5±5</td>
<td>0.48</td>
</tr>
<tr>
<td>0.05</td>
<td>160±32</td>
<td>-28.7±9</td>
<td>0.3</td>
</tr>
<tr>
<td>0.1</td>
<td>240±10</td>
<td>-26±3</td>
<td>0.42</td>
</tr>
<tr>
<td>0.15</td>
<td>422±168</td>
<td>-21.56±4.8</td>
<td>0.41</td>
</tr>
<tr>
<td>0</td>
<td>Precipitated</td>
<td>8.72±2</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2: Variation in entrapment efficiency (%) with varying 2-ME2 to PLGA ratio. (n=3, Error: SD).

2-ME2 to PLGA Ratio

<table>
<thead>
<tr>
<th>Ratio</th>
<th>PLGA:PEG</th>
<th>PLGA:Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:01</td>
<td>26.5±1.5</td>
<td>18.4±6.9</td>
</tr>
<tr>
<td>1:05</td>
<td>29.5±0.7</td>
<td>24±5.2</td>
</tr>
<tr>
<td>1:09</td>
<td>55.8±4.4</td>
<td>56.9±7.6</td>
</tr>
<tr>
<td>1:10</td>
<td>62.21±1.5</td>
<td>68.6±3.5</td>
</tr>
<tr>
<td>1:12</td>
<td>80.3±6.2</td>
<td>76.5±5</td>
</tr>
<tr>
<td>1:15</td>
<td>76.9±1.1</td>
<td>72.7±6.9</td>
</tr>
<tr>
<td>1:20</td>
<td>44.5±7</td>
<td>-----</td>
</tr>
</tbody>
</table>

Table 3: Comparison of pharmacokinetics parameters of bare 2-ME2 and nanoformulations (P>0.05, Students t-test *: vs Bare 2-ME2, #: vs 2-ME2:PLGA:Casein)

<table>
<thead>
<tr>
<th></th>
<th>Bare 2-ME2</th>
<th>2-ME2:PLGA:Casein</th>
<th>2-ME2:PLGA:PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>T½ (min)</td>
<td>6.2±1.3</td>
<td>50.12±7.2 *</td>
<td>70.21±5.2 * #</td>
</tr>
<tr>
<td>T Max (min)</td>
<td>5</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>C max (µg/ml)</td>
<td>1588.13</td>
<td>656.82</td>
<td>840.21</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>12.6±2</td>
<td>55.28±7.8 *</td>
<td>125.11±22 * #</td>
</tr>
<tr>
<td>AUC (µg/ml*min)</td>
<td>32231.26±157</td>
<td>61756.65±302</td>
<td>144606.1±283 * #</td>
</tr>
</tbody>
</table>
