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OPTIMIZATION OF POLOXAMER 188 CONCENTRATION AS MEDIA FOR NANOPARTICLE DISPERSION: EFFECT OF CONCENTRATION, NANOPARTICLE SIZE AND IN VITRO PENETRATION THROUGH BLOOD BRAIN BARRIER

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ABSTRACT

One percent w/v of various surfactants have been used for the dispersion of nano particles in the dispersant media and their size being maintained at nanometer range.While investigating a nanoparticle based project and dispersing PLGA nanoparticles as synthesized based on previous literature with modification in Poloxamer 188 (PLX188) at a concentration of 1% w/v as dispersion media, a massive change in size is observed. Hence a systematic titration study regarding the concentration effect (0.1-4% w/v) of PLX188 on PLGA nanoparticles has been designed using acoustic spectroscopy to study the parameters of size and its distribution. Supporting experiment using dynamic light scattering (DLS) technique-using concentration ranging 0.01% to 0.1% w/v PLX188 has been carried out to compare and validate the trend using different techniques. In this experiment 1% PLGA dispersion system was prepared in water and parameters of size and distribution were measured. Then the system was successively titrated with addition of PLX188 starting from 0.1 to 4% to observe the effect of surfactant concentration on size. The nanoparticle size increased from 27 nm (in case of system without PLX188) to 1.58 µm (in case of 0.1% w/v presence of PLX188) and continued to grow bigger with the increase in PLX188 concentration. DLS results showed similar trend of enlargement in size of the particles even at the presence of 0.01% to 0.1% w/v PLX188 and the morphology further confirmed the findings of the change in size of the PLGA nanoparticles. The size distribution is also affected. The PLGA nanoparticles uptake using of 0.01% w/v PLX188 as dispersion media was a success using Human Brain Microvascular Endothelial Cells (HBMEC) as a blood brain barrier model and hence the above concentration is optimized as the suitable amount of surfactant to be used during the nanoparticles dispersion.

KEYWORDS: *PLGA nanoparticle, Poloxamer 188 (PLX188), acoustic spectroscopy, nanoparticles size distribution, DLS, TEM*

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INTRODUCTION

Over the last few decades' nanoparticle-based therapeutic systems have gained immense popularity due to their ability to overcome biological barriers, effectively deliver hydrophobic therapies, and preferentially target disease sites. Surfactant (also known as surface-active agent or emulsifier) forms the critical component of the nanoparticle formulation.[1] It plays an important role in the nanoparticulate dispersion system by lowering the surface tension and minimizing their aggregation.[1] In the field of pharmaceutical sciences, surfactants play a crucial role in the successful formulation of biphasic dosage forms.[2] The presence of surfactants as a coating material for the nanoparticles have been reported to reduce their uptake by the reticuloendothelial system (RES) with cells in the liver, spleen, and bone marrow resulting in improved bioavailability of the active pharmacological ingredient (API).[3,4]Previous research has also demonstrated the reduction of potential toxicity of nanoparticles because of the surfactant presence.[5] Surfactant plays a major role in promoting endocytotic uptake of nanoparticles thereby enhancing the penetration through the blood brain barrier.[6] Kreuter *et al*., [7]; have reported polysorbate-80 coating enhances uptake of nanoparticles by human and bovine primary brain capillary endothelial cells. It has been reported that the modification of the surface of nanoparticles is one of the common approaches to achieve novel drug delivery to the brain.[8] Polysorbate 80 with Apo-B or Apo-E-mediated transport at the BBB has been demonstrated as targets for nanoparticle delivery to the brain. However, it has been suggested that the polysorbate 80 induces change in the BBB permeability.[7,8] There are many types of surfactant used in the formulations of nanoparticles, and one of the commonly used one is Poloxamer.[9] Poloxamer is a non-ionic triblock copolymer and a member of the poloxamer family that is widely used as surfactants in many applications. It comes with trade names, as Supronic, Pluronic or Tetronic.[9] It is soluble in both water and organic solvent. It has been introduced in the 1950, and since then very famously used in diverse pharmaceutical applications such as drug delivery, medical imaging, pore forming agent, and also drug release enhancer.[9-11] The FDA has approved the use of Poloxamer 188 in various medical fields ranging from drug delivery and medical imaging as well as treatment of disease such as vascular disease and disorders.[11] It is reported that Poloxamer 188 (PLX188) can interact with multidrug resistant cancer cells resulting in drastic sensitization of the cells to various anticancer agents because of its involvement in easy incorporation of the nanoparticles into membrane and the subsequent translocation into cells.[9 -11] In one study, Poloxamer 188 has shown to have the ability to inhibit P-gp and CYP3A4 in *in vitro* study. This study suggested the potential for Poloxamer 188 to modify the pharmacokinetics of orally administered drugs that are P-gp and CYP3A4 substrates *in vivo* study.[12] Zhang *et. al*[13]; have proved Poloxamer 188 as a potent adjunct agent in anticancer drugs. In this study, Paclitaxel/Poloxamer 188 nanoparticle formulations is able to overcome multidrug resistant human breast cancer cell line MCF-7/TAX in comparison with paclitaxel alone as there was an increased level of uptake by the cells with paclitaxel/Poloxamer 188 nanoparticles formulation. Therefore Poloxamer 188 could have considerable therapeutic potential for breast cancer.[13] In this pretext Poly (lactic-*co*-glycolic acid) (PLGA) has been extensively used as drug carrier due to its biodegradability and biocompatibility.[12,13] Previous studies have evaluated the synthesis of PLGA with many different stabilizers including PLX188.[12,14,15,22] It has been widely reported that dispersion in 1% surfactant solution leads to the nanoparticulate dispersion in nanometer (nm) size range.[6, 8,10,14,19,20,24,26,27] BBB restricts entry of many potentially therapeutic agents (PTA) into the brain.[15] But recently, several neuroactive proteins of potential therapeutic value have highlighted the crucial need for effective and safe transcapillary delivery methods to the brain.[15] Brain derived neurotrophic factor (BDNF) was found to be neuroprotective following delayed intravenous administration in either regional or global brain ischemia. For example, BDNF is reformulated to enable BBB transport. Our group is involved in BDNF reformulation to enable BBB transport. We have successfully prepared the PLGA nanoparticles as well as BDNF containing PLGA nanoparticles with the due characterization. During experimentation while preparing the nanodispersion, the literature value of surfactant concentration (1% w/v) was followed and a massive size enlargement from nanometer to micrometer was observed. Hence we attempted to investigate the impact of surfactant concentration upon the nanoparticle size in a systematic way. Higher concentration of surfactant ranging from 0.1% to 4% w/v and 0.01% to 0.1% as well have been investigated using electroacoustic spectroscopy and dynamic light scattering (DLS) technique respectively. The size of small molecules can be measured by Dynamic Light Scattering (DLS) technique as the size of the molecules can be used as a quality control measure.[16] Characterization of the small molecules in a colloidal dispersion using DLS utilizes the illumination of a suspension of particles or molecules undergoing Brownian motion by a laser beam.[17] DLS measures time-dependent fluctuations in the scattering intensity arising from particles undergoing random Brownian motion. Diffusion coefficient and particle size information is obtained from the analysis of these fluctuations.[17] The speed of Brownian motion is measured and provides the translational diffusion coefficient D. This diffusion coefficient is converted into a hydrodynamic diameter (DH) using the Stokes-Einstein equation

$$D_H = \frac{kT}{3\,\pi\,\eta D}$$

where k is the Boltzmann constant, T is the temperature and g is the dispersant viscosity.[17] Hence in this communication, we intend to present the systematic study on the effect of the concentration of surfactant in this case PLX188 upon the size and size distribution of 1% of PLGA nanoparticles dispersion. The

concentration of PLX188 has been varied from 0.1% to 4% and utilized in a titration experiment. Initially we used the acoustic spectroscopy for this range of concentration. Further experiments we choose to use dynamic light scattering (DLS) in which the concentration varied from 0.01% to 0.1% as to strengthen our findings in conjunction with the use of Transmission Electron Microscopy (TEM) and Environmental Scanning Electron Microscopy (ESEM) to further confirm the particle size distribution and morphology. Conversely, the Electro Acoustic system is superior compared to DLS because of the ability of the former to characterize concentrated disperse system avoiding dilution as in case of the latter.[18] The optimal formulation using the least concentration of PLX 188 has been tested on HBMEC as a blood brain barrier model.

MATERIALS AND METHODS

Materials

The poly (lactide-*co*-glycolide) polymer Resomer H RG 502H (PLGA, lactide/glycolide=50:50, i.v. 0.16–0.24 dl/g) was obtained from Boehringer Ingelheim, Germany. Poloxamer 188 (PLX188) (Pluronic H F68), poly (vinylalcohol) (PVA, MW 30–70 kDa, 88% hydrolyzed), was purchased from Sigma (Steinheim, Germany). All other chemicals and solvents were of analytical grade.

Experimental

Preparation of PLGA nanoparticles and ElectroAcoustic assessment

PLGA nanoparticles have been synthesized as previously described method with modifications.[19,20,24] The effect of PLX188 upon the nanoparticle size and its distribution has been studied using a combined acoustic and electroacoustic Spectrometer (DT 120) developed by Dispersion Technology Inc. USA. The instrument is equipped with separate sensors for measuring the acoustic and electroacoustic signals both using pulse technique. However, in our experiment we used the acoustic spectroscopy for the size measurement and its distribution. Briefly acoustic sensor has two pieces of crystal transducers. The gap between transmitter and receiver usually vary in steps. The default setting is from 0.15mm to 20mm, which happens in 18 steps; from 3 to 100MHz. The number of pulses collected for each gap and each frequency adjust automatically in such a way that the target signal to noise ratio is reached. The acoustic sensor also measures the speed of sound at one chosen frequency by using the time of arrival of the pulse at the receiver. Then instrument automatically adjusts pulse sampling depending on the speed of sound that eliminates possible artifacts such as excess attenuation at low frequencies. All experimental data are stored in an access database. The special analysis programmed calculates particle size distribution (PSD) from attenuation spectra testing the log normal, bimodal, and modified log normal particle size distributions. Also it uses an error analysis to search for the best PSD. The sample well can accommodate around 100ml and is fitted with a magnetic stirrer in order to prevent sedimentation and mix the chemicals during titration. A conductivity and temperature probe is also can access the sample chamber. Acoustic spectroscopy has been used for our study to access the effect of PLX188 on the nanoparticles size and distribution at higher concentration involving titration experiment. The blank system has been prepared with 1% w/v of PLGA nanoparticles, with continuous stirring at 12rpm and is denoted as S_0. This system indicated the one without PLX 188. The density of particles was measured from the density of the dispersion system using software fitted with the experiments. The density of the dispersion system was determined using an AntonPaar DMA 35N Density Meter. After the first measurement of S_0, 0.1% w/v of PLX188 was added to the sample chamber and the system was equilibrated for 5 minutes before further analysis. . Successively each time 0.1% w/v of PLX188 was added to the sample chamber for the measurement of attenuation spectra so to to reach 1% w/v and these samples were denoted as S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, S_9, and S_{10} respectively. After that the PLX188 amount was increased successively by 0.5% w/v so to reach the concentration by 1.5, 2, 2.5, 3, 3.5 and 4% w/v. These systems were denoted as S_{11}, S_{12}, S_{13}, S_{14}, S_{15}, and S_{16} respectively. The above mentioned titration experiment was performed in triplicate.

Dynamic light scattering (DLS) of nanoparticles

DLS for characterization of particles size of the PLGA nanoparticles in solution was performed on a Malvern Instruments Zetasizer 1600 (Malvern UK). Briefly, measurements of all PLGA nanoparticles were made on a Malvern Zetasizer 1600 (Malvern Instruments Ltd., UK) with a detection angle of 173^0. All measurements in this study were taken at a temperature of 25^0C. At least 3 repeat measurements on each sample were taken to check for result repeatability. PLGA nanoparticles were weighed on an analytical mass balance, suspended in deionized water at a concentration of 10 mg/ml. The dispersions were then divided into six groups denoted as S_0, S_1, S_2, S_3, S_4, S_5, S_6 depending on the PLX188 concentrations ranging from 0.01% to 0.1% (w/v) respectively. The samples were transferred to a clear disposable zeta cell for DLS measurements. Subsequently the samples were then further diluted to 10 times and 200 times more.

TEM and ESEM of nanoparticles

Transmission electron microscopy (TEM) characterization was performed to obtain nanoparticle size and morphology on a FEI TECNAI G2 20S TWIN instrument at an accelerating voltage of 200 kV. Nanoparticles were examined after suspension in water and subsequent deposition onto formvar/carbon-coated TEM grids. The samples were preceded for imaging after negative staining of nanoparticles with 2% w/v uranyl acetate (UVA). The AMT software for the digital TEM camera was calibrated for size measurements of the nanoparticles. Information on mean size and SD was calculated from measuring over 100 nanoparticles in random fields of view in addition to images that show general morphology of the nanoparticles. Environment scanning electron microscopy (ESEM) characterization and morphology obtained using FEI QUANTA 450 FEG. Briefly, The ESEM incorporates a cold cathode field

emission gun, with a low vacuum and sophisticated digital technique for the high-resolution high quality imaging of microstructure. The FEI QUANTA 450 FEG is able to handle samples up to 8 inches in diameter. The specimens were coated with a Pd-Au film by an Emitech Magnetron Sputter Coater before imaging in order to avoid electric charge build up.

Materials for invitro cell culture

Human Brain Microvascular Endothelial cells (HBMEC / ACBRI 376) and the corresponding cell culture media were purchased from Cell System Corporation (USA). Phosphate buffered saline (PBS), Dimethylsulfoxide (DMSO), and paraformaldehyde (PFA), were purchased from Sigma-Aldrich (USA). Additionally, 4,6-diamidino-2-phenylindole (DAPI), and Prolong® Gold were purchased from Invitrogen (USA). The Nunc™ Lab-Tek™ II Chamber Slide™ System (Collagen IV coated Lab Tek II was bought from ThermoFisher Scientific (USA).

Cell culture

HBMEC were cultured in T-25 Flasks (Orange Scientific, Belgium) containing CSC complete media. The cells were grown at 37^0C with 5% CO_2 and confluent monolayers of HBMEC were passaged every 7-8 days. Passage No 8 was used for all experiments.

Cellular uptake and intracellular distribution of the PLGA nanoparticles

The permeability of the cells for the nanoparticles was studied using confocal laser scanning microscopy (CLSM). HBMEC (1 x 10^5 cells/well) were cultured on collagen IV coated Lab Tek II 2 wells chamber slides and incubated with 2µg/ml PLGA nanoparticle formulations for 4 hours at 37^0C. Subsequently, the cells were washed twice with 2ml PBS and fixed with 1% PFA for 10 minutes. Following that, the cells were washed twice with PBS and counterstained with DAPI (300 nM in PBS) for 30 min. Finally, the chambers were removed from the slides, and cover slips were mounted with Prolong® Gold Antifade reagent to minimize photobleaching. A total number of 50 cells (n=50) were analysed per sample/treatment using Leica QWin software. The fluorescence intensity of the coumarin 6 (excitation: 458nm, emission: 497nm) and DAPI (excitation: 358 nm, emission: 461nm) were measured according to the wavelengths.

RESULTS AND DISCUSSION

In this present research Acoustic Spectroscopy has been opted as superior method rather than Light scattering method, because of its ability to characterize concentrated disperse systems. Furthermore Acoustic Spectroscopy based on the attenuation measurement does not require any assumptions regarding electrosurface properties. As can be seen in Figure 1, the PLGA nanoparticle size drastically increased from 27nm (S_0) to 1.58µm (S_1) in the presence of 0.1% w/v of PLX188 and continues with the trend, with an increase in PLX188 concentration.

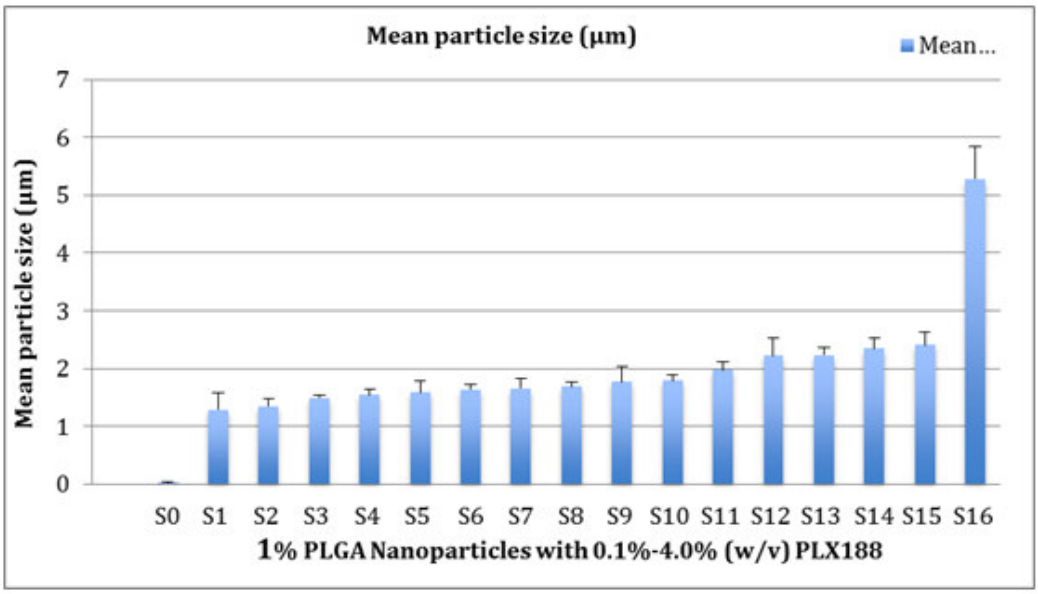

Figure 1

PLGA nanoparticles size distribution during the system titration with PLX 188

For the easy of comparison, the S_0, S_1, and S_{10} have been compared and represented in term of weight basis (Figure 2) and cumulative weight basis PSD plots (Figure 3). It is indicated clearly that the size distribution is narrow and unimodal in case of S_0 than that of the bimodal and widely distributed pattern in case of S_1 and S_{10} respectively. This clearly indicates that PLX 188 at a concentration of even 0.1 w/v % increases the PLGA nanoparticles size to a micrometer range with a wide size distribution.

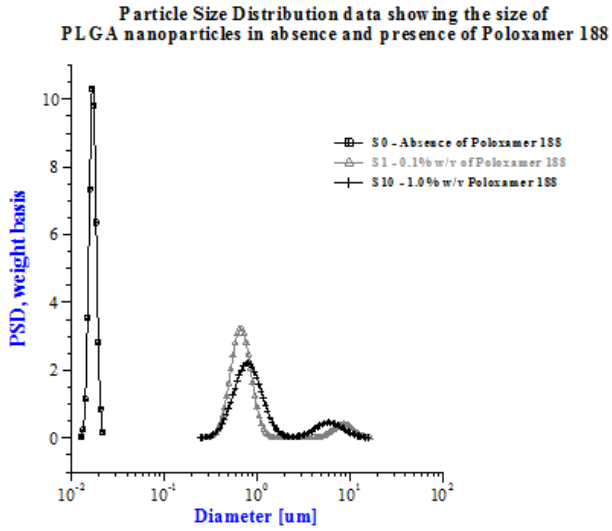

Figure 2

Particle size distribution data showing the size of PLGA nanoparticles in absence and presence of PLX188.

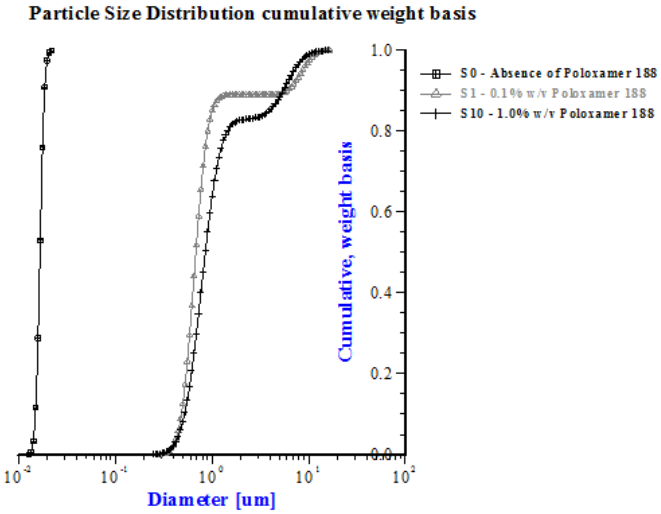

Figure 3

Particle size distribution cumulative weight basis of samples S0, S1, and S10.

This increase in particle size is further reflected in the attenuation Spectra (Figure 4). Attenuation spectra is a plot of attenuation (y = axis) versus frequency (x = axis). It is seen clearly that with an increase in size increases the attenuation and spectral pattern.

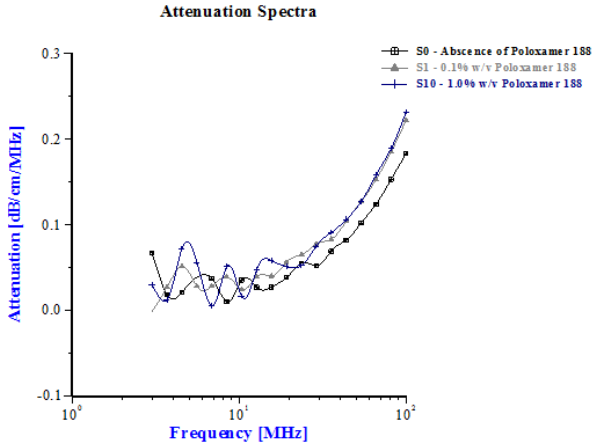

Figure 4

Attenuation spectra of samples S0, S1, and S10.

It is evident from Figure 5 that the size distribution and mean size is higher than that of the case of S_0. The attenuation spectra (Figure 6) also compliment the data as represented in Figure 5.

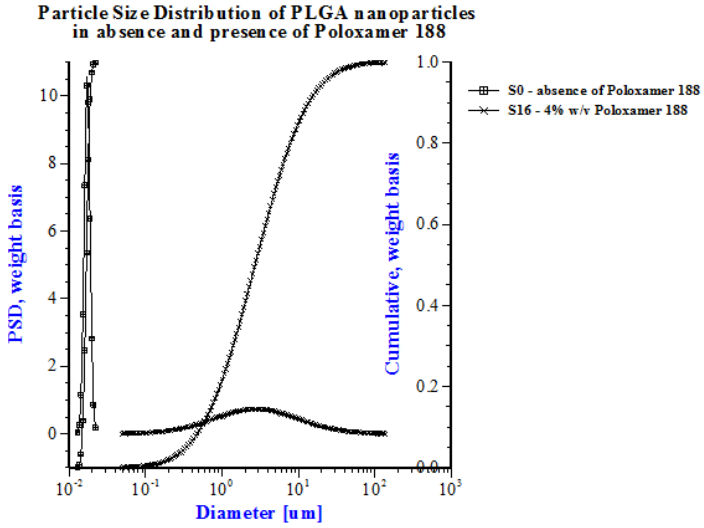

Figure 5
Particle size distribution of PLGA nanoparticles in absence and presence of PLX188

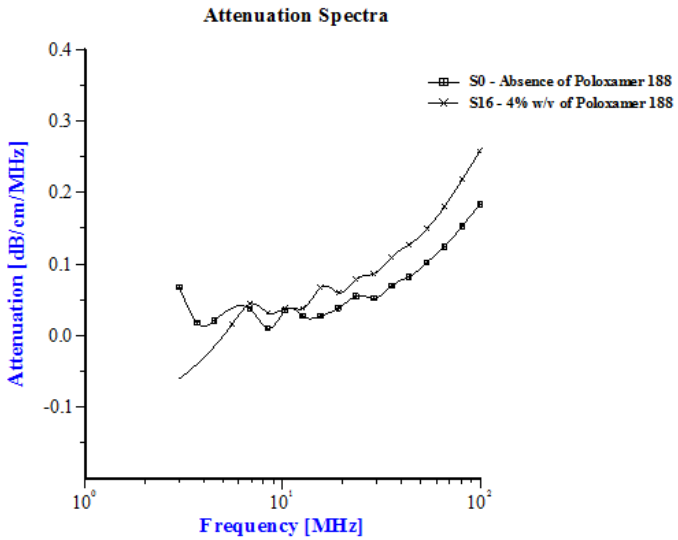

Figure 6
Attenuation spectra of samples S0 and S16.

Previous study reported by Gao et al[25]; nanoparticles have been coated using 0.5% - 1.2% w/v PLX188 concentrations and the results were 87nm – 203nm respectively. Other study on nanoparticles surface coating with surfactants such as polysorbate 80, which was done by Wilson et al[26]; resulted in particles size of 40.5nm after coating with 1% polysorbate 80. For drug targeting to the brain, Gelperina et al[27]; have successfully synthesized PLGA coated with 1 w/v % PLX 188 and 1% polysorbate 80 and the particles size were found to be 168.5nm and 166.9nm respectively. As for Petri et al[19]; the particles size of PLGA nanoparticles was found to be 246nm after coating it with PLX 188. B. DLS Particle Characterization in Solution We further strengthen our findings by testing the samples with DLS method. In this experiment we chose to use even smaller concentration of PLX188. In our experiments, we found there is increase in particles size as we increase the PLX 188 concentrations. The samples were denoted as S_0, S_1, S_2, S_3, S_4, S_5, and S_6 as representing variation of PLX 188 concentration from (S_1) 0.01% to (S_6) 0.1%. Even at a very low concentration of PLX 188, 0.01% the particle size noted to be 403.2 nm and it increase up to 1154.4 nm when the concentration of PLX 188 is 0.1% as shown in figure 7. As DLS measurements were done in aqueous or physiological solutions, further complimentary experiments were performed to obtain size under high vacuum conditions that require a dry sample by using TEM and ESEM. The DLS results for particle size in solution for the PLGA and PLX188 are presented in figure 7.

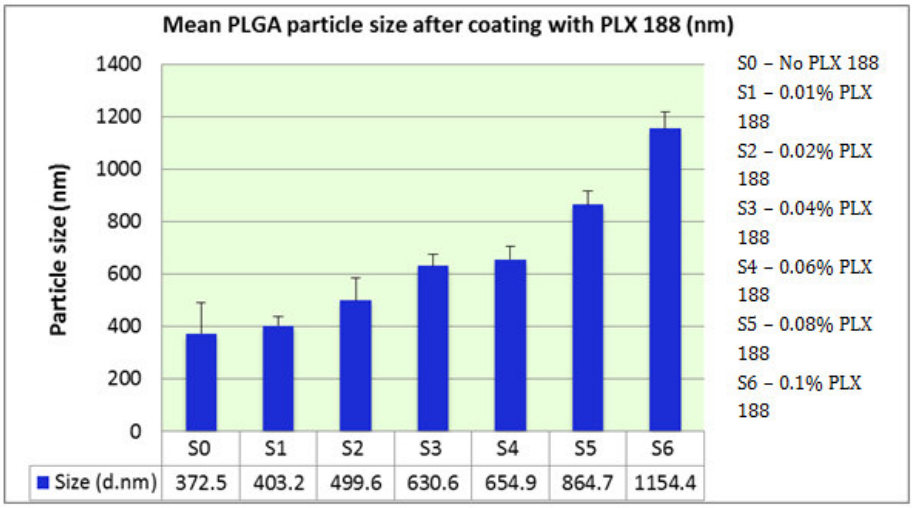

Figure 7

Mean particle size distribution of PLGA nanoparticle with and without PLX 188 coating measured by dynamic light scattering (DLS) 100x dilution (data as mean with S.D; n=3).

TEM and ESEM of Nanoparticles for Particles Size Determination

TEM and ESEM were used to obtain essential information on PLGA nanoparticle sizes and morphologies (Figs. 8A–C and Figs. 9A-C). All the PLGA nanoparticles examined by TEM and ESEM show similarities in their spherical morphologies and sizes

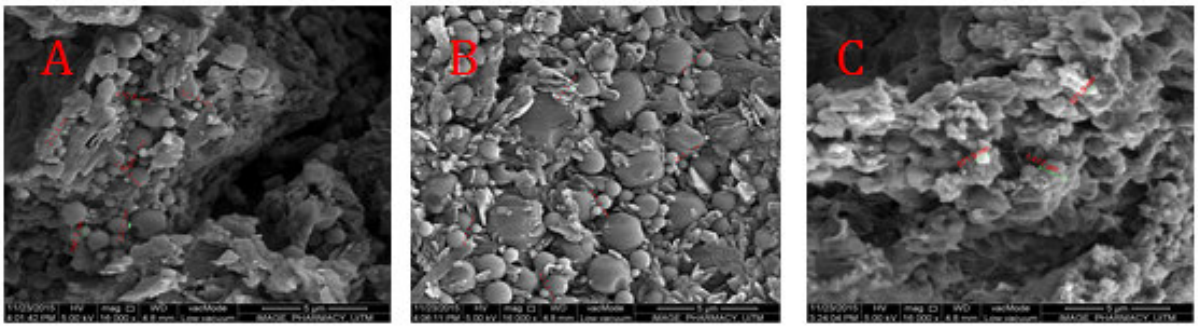

Figure 8

SEM images representing nanoparticles from samples A (S0), B(S1), C(S6)

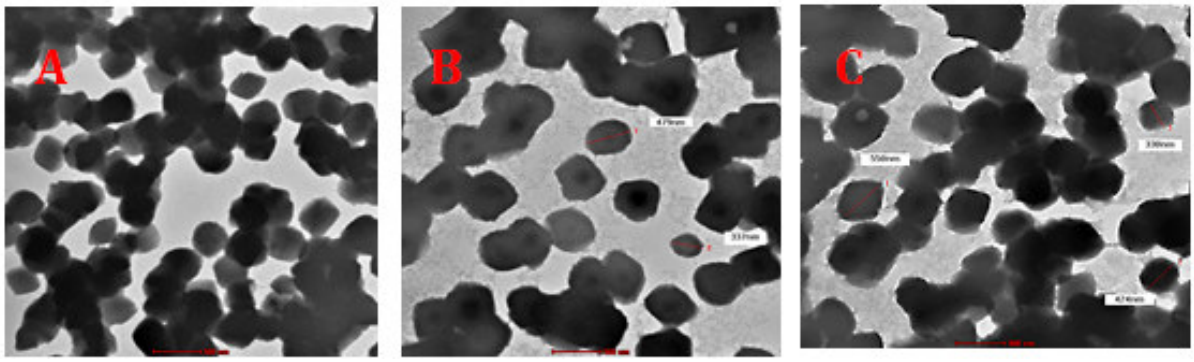

Figure 9

TEM images representing nanoparticles from samples (S0), B (S1), C (S6)

PLGA Nanoparticles uptake by HBMEC The HBMEC were used as an *in-vitro* model of the blood brain barrier to test the efficiency of PLGA nanoparticles to penetrate and carrying bioactive agents inside the cells. The PLGA nanoparticles successfully penetrated the cells after using 0.01% PLX 188 (Figs 10A-C). Several studies have shown that using nanoparticles carrying the bioactive substance have the ability to transport the agents inside the brain and where it exerts its positive effects towards the brain cells [28, 29].

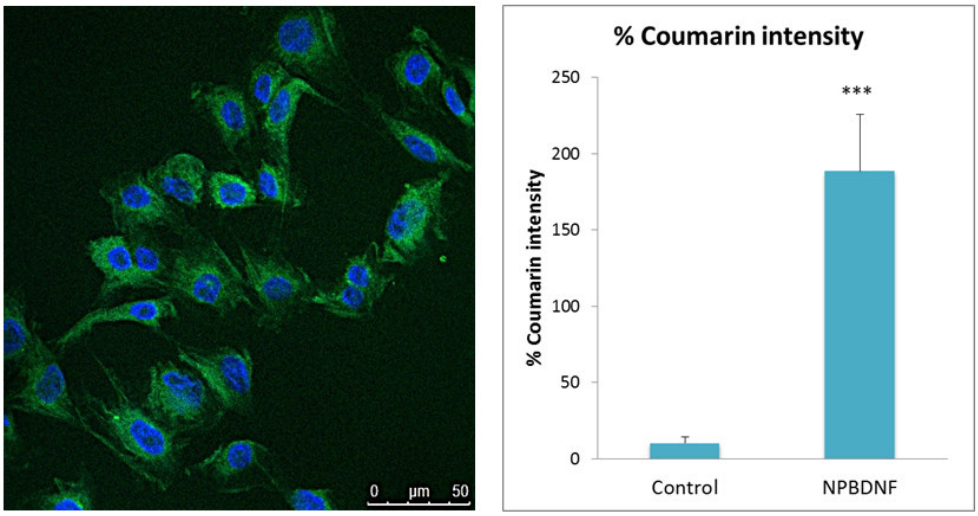

Figure 10

Representing Human Brain Microvascular Endothelial Cells (HBMEC) penetrated with 2µg/ml PLGA nanoparticle coated with 0.01% PLX 188 following 4 h incubation time. Fixed HBMEC was viewed under Confocal Laser Scanning microscope at 40x magnification (scale bar: 50 µm). PLGA nanoparticles are seen as green fluorescence while the blue color represents the DAPI stained nucleus.

% Coumarin intensity inside the HBMEC was analyzed using Leica Qwin Software. Data obtained from 50 cells *(n=50)* is shown as the mean value ±SD of percentage of coumarin intensity inside the cells relative to control using student t test. Results represent the means ±SD of nine replicate samples (n=9): ***p<0.001

CONCLUSION

In this research, it is demonstrated that on the basis of acoustic spectroscopy based measurement PLX188 even at a very low concentration of 0.1% w/v can result in an increased size and as well as distribution. Further study revealed the increase in size with the increase in concentration of PLX 188. This is shown by DLS and complimented by imaging technique using TEM and ESEM. PLGA nanoparticles coated with 0.01% PLX 188 did successfully penetrate HBMEC, a blood brain barrier model. The fluorescence of the nanoparticle which have penetrated HBMEC were found to be high after 4 h incubation with PLGA nanoparticles. In conclusion, our present study demonstrates that concentration of dispersion media as low as 0.01% w/v is sufficient to obtain nanometers range of particles with excellent penetration across the *in vitro* model of blood brain barrier. The 0.01% w/v PLX 188 dispersion media for nanoparticles provided reliable particle size that correlated well with those obtained from particle size with higher (1% w/v) concentration of surfactant. Added to this there is a definite economic advantage of PLX 188 at 1% w/v of the normally used.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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