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Original Article

Preparation, Characterization and Evaluation of Resveratrol Loaded Pegylated PLGA Nanoparticles

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ABSTRACT

Background: This study focuses on the preparation and evaluation of Resveratrol loaded polymeric nanoparticles made of pegylated PLGA. **Materials and Methods:** In this study, both double-emulsion solvent evaporation and single-emulsion solvent evaporation were employed to formulate the Resveratrol loaded pegylated PLGA nanoparticles with PVA as surfactant. For the encapsulation of the hydrophobic drug Resveratrol, formulations were successfully prepared and tested for their particle size, polydispersity index, zeta potential, drug loading, and entrapment efficiency. Scanning electron microscopy was used to observe the morphology and surface characteristics of the nanoparticles. Using a sample and a separate method, an *in vitro* release study of the formulated polymeric nanoparticles was conducted. MTT assay was also used to determine the cytotoxicity of polymeric nanoparticles on breast cancer cells (MCF7). **Results:** The results demonstrated successful fabrication of the Resveratrol loaded pegylated PLGA nanoparticles. PVA was demonstrated in the present study to be a promising surfactant for the encapsulation and delivery of poorly water-soluble compounds as pegylated PLGA nanoparticles with the desired particle size, morphology, drug loading and entrapment efficiency. From the result of *in vitro* release study, it was observed that the optimized formulation showed 92.11% of cumulative percentage drug release which is higher than other formulations. Moreover, the optimized formulation demonstrated significant cytotoxic effect in MTT assay. The optimized nanoformulation showed 42.19% cell viability compared to the free Resveratrol at 66.59% cell viability. **Conclusion:** PLGA nanoparticles encapsulated with Resveratrol had been successfully used to deliver the drug to the target site by pegylated PLGA nanoparticles.

Keywords: Cytotoxicity, Nanoparticles, Polyethylene glycol, PLGA, Resveratrol.

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INTRODUCTION

Nanosized polymeric particles have emerged as a pragmatic method in the formulation of hydrophobic drugs. They are characterized by rapid dissolution rates, which enhance bioavailability after oral administration. Due to their simplicity and advantages over other strategies, they have proven to be highly effective in addressing the problems associated with poorly water-soluble and poorly and lipid-soluble drugs. A suitable method and a suitable stabilizer are used to prepare colloidal dispersions of nanosized drug particles. For the preparation of nanoparticles, Resveratrol was selected as a model drug.

The effects of Resveratrol on cancer are numerous as there is an antiproliferative effect of Resveratrol on prostate and breast cancer cell lines.[1] In breast cancer models, Resveratrol suppresses the expression of cell cycle regulatory proteins such as cyclin D1, E and Insulin-like Growth Factor (IGF-1).[2,3] Its low solubility and stability, limits its potential activity, making it poorly bioavailable and susceptible to metabolism. Our literature review found few studies exploring how to enhance Resveratrol's effectiveness and overcome its related problems. It is indeed a challenge to target natural bioactive specifically.[4,5]

The present research was performed to examine how an amphiphilic polymeric conjugate could be used to create and evaluate nanoformulations that are effective at delivering the poorly soluble model drug Resveratrol. We are investigating the feasibility of synthesizing nanoparticles using pegylated PLGA conjugates to enhance wetting characteristics and decrease nanoparticle agglomeration while evaluating their drug loading, entrapment, polydispersity index, zeta potential and surface morphology characteristics. To overcome the related issues and increase the thrust of drugs in breast tumours, the current study developed a targeted delivery system for Resveratrol.

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The presence of Resveratrol in the food chain, as a dominant bioactive component is unlikely to cause adverse or untoward effects. As a result of its enormous safety margins, it can provide additional preventive and curative benefits. It uses PLGA and Polyethylene Glycol (PEG), which are further conjugated to develop a pioneering system for delivering Resveratrol.[6] Resveratrol could be delivered to tumour cells via intravenous injection in a sustained manner and protected against rapid degradation.

MATERIALS AND METHODS

PEG and PLGA were purchased from Sigma Aldrich, USA. Resveratrol was received from Alpha Remedies, Ambala as a gift sample. Other drugs and chemicals were procured from reputable vendors. Analytical grade chemicals were used for all other experiments.

Drug Excipient Compatibility studies

Fourier Transform Infrared (FTIR) Spectroscopy

There is a possibility that the drug may be degraded as a result of the drug-excipient interaction. In order to produce a stable and effective dosage form, the excipients must be compatible with each other. Therefore, Fourier Transform Infrared (FTIR) spectroscopy (Bruker instrument, Germany) was used to investigate possible drug interactions between the drug and the excipients. FTIR spectra were obtained for a physical mixture of pure drug with excipients and the lyophilized formulation with the drug from wavelength of 4000 cm^{-1} to 400 cm^{-1}. Interactions were observed in the FTIR spectra.

Preparation of pegylated PLGA conjugate

Pegylated PLGA conjugates were successfully synthesized and characterized in the laboratory.[7] FTIR and NMR spectroscopy have been used extensively in laboratory to analyze and characterize the conjugate.

Preparation of Nanoparticles by Double Emulsion Solvent Evaporation (DESE) using PVA as stabilizer

PVA was used as a surfactant in the DESE method to prepare Resveratrol-loaded nanoparticles from pegylated PLGA polymer and designated as S1. In 2.5 mL of dichloromethane, 10 mg of Resveratrol was dissolved and allowed to solubilize. Approximately 20 mg of polymers (pegylated PLGA) were dissolved in a drug-dichloromethane solution after 20-30 min (drug: polymer ratio of 1:2). In order to prepare the first primary emulsion, 2.5 mL of 1.5% w/v PVA was added dropwise and homogenized at high speed 3,000 rpm for 20-30 min until the emulsion became rich and creamy. Using the creamy foam consistency of the primary emulsion as a guide, 25 mL of 0.5% w/v PVA was homogenized at 18,000 rpm for 20-30 min to form the secondary emulsion. A magnetic stirrer was used overnight to evaporate the organic solvent from the secondary emulsion and after it was sonicated for 45 min. The double emulsion was centrifuged at 5,000 rpm for 5 min to discard the large particles formed. Afterwards, the supernatant was centrifuged for 30 min at 7,000 rpm to obtain the nanoparticles, which were then washed three times with distilled water to remove the surfactant and finally freeze-dried. The same concentrations of PVA were used in both primary and secondary emulsions of formulation S2 based on pegylated PLGA. The volume of PVA increased to 5 mL in the primary emulsion and 50 mL in the secondary emulsion.[8,9]

Preparation of Nanoparticles by Single Emulsion Solvent Evaporation (SESE) using PVA as stabilizer

In 2.5 mL of dichloromethane, 10 mg of Resveratrol was dissolved. An addition of 20 mg polymer (pegylated PLGA) was made to the drug: polymer ratio of 1:2. To form an emulsion, 50 mL of 2.5% w/v PVA was added dropwise to the resulting drug-polymer solution and homogenized at 15,000 rpm for 10-15 min. A creamy emulsion is formed. For the removal of the organic solvent dichloromethane, the resulting solution was sonicated for 45 min, followed by gentle magnetic stirring for 12-14 hr.

After centrifugation at 15,000 rpm for 30 min, the nanoparticles were washed repeatedly three times with distilled water to remove the surfactant, then freeze-dried. S3 is the formulation designation. Formulation S4 was prepared by using 1.5% w/v of 50 mL of PVA.[8,9]

Characterization of pegylated PLGA Nanoparticles

Percentage Yield of Nanoparticles: This formula calculates the Percentage Yield of the formulations after nanoparticles have been prepared by both DESE and SESE methods:

$$Percentage\ Yield\ = \frac{\text{Weight of nanoparticles obtained}}{\text{Weight of drug and polymer used for nanoparticles preparation}} x100$$

Drug Loading and Entrapment Efficiency Determination: Two grams of Resveratrol nanoparticles were accurately weighed and put into a centrifuge tube with two mL of dichloromethane to determine drug loading and entrapment efficiency. A shaker was used to continuously shake it for 3-4 hr at 37°C. A centrifuge was used to separate the dispersed phase from the continuous phase. A UV-spectrophotometric measurement at 475 nm was then conducted on the supernatant to determine the amount of drug released. Here are the equations used to calculate drug loading and entrapment efficiency percentages.[10,11]

$$Drug\ loading\ efficiency\ (\%) = \frac{\text{Amount of drug present in nanoparticles}}{\text{Amount of drug loaded nanoparticles}} x100$$

$$Entrapment\ efficiency\ (\%) = \frac{\text{Amount of drug present in nanoparticles}}{\text{Intial amount of drug added}} x100$$

Particle Size Analysis

The particles and the distributions of the sizes of the nanoparticles were measured using an instrument called the Malvern Nano ZS90, which is equipped with a solid-state laser and uses Dynamic Light Scattering (DLS). Before measuring, required amount of dried nanoparticles from each formulation were suspended in double distilled water and sonicated. After the homogeneous suspension was formed, the average hydrodynamic particle size, size distribution and polydispersity index were determined.

Zeta Potential Measurement (ZP)

A calculated amount of dried nanoparticles from each formulation were sonicated for a suitable period before measuring their zeta potential with the Malvern NANO ZS90. The ZP characterizes particle surface charge and provides long-term stability information.

Scanning Electron Microscopy (SEM)

A scanning electron microscope (Hitachi SEM-S-3600N) was used to examine the shape and surface morphology of the nanoparticles. A suitable sample of nanoparticles was mounted on metal stubs using double-sided adhesive carbon tape and a razor blade. Gold was sputter-coated onto the samples for secondary electron emissive SEM and morphology was observed under argon.

In vitro Drug Release Study of the Nanoparticles

Using a sample and a separate method, we conducted an *in vitro* release study of the formulated polymeric nanoparticles. To dissolve and separate the release drug from the nanoparticles, magnetic shaking and orbital shaking were used.[12] Using centrifuged tubes containing 2mg of nanoparticles, phosphate buffer pH 7.4 was used to dissolve the nanoparticles. The samples were incubated at 37°C and shaken regularly at 120 rotations/minute using an orbital shaker. At scheduled intervals, 0.5 mL of the supernatant was taken and tested for drug release at 306 nm using UV-visible spectrophotometry and withdrawal volume is replaced by buffer of same pH. The experiments were performed with three replicates with same conditions to meet the statistical requirements.[13]

Cytotoxicity Evaluation via MTT Assay

As described here, MTT assay was used to determine the cytotoxicity of NPs on breast cancer cells (MCF7).[14,15]

RESULTS

Drug-excipient compatibility studies

By comparing the FTIR spectrum of pegylated PLGA, Resveratrol and PVA with that of a physical mixture of pegylated PLGA-Resveratrol-PVA, it is evident there is no incompatibility between the excipients, suggesting its perfect stability (Figures 1 and 2).

Preparation of Nanoparticles

Table 1 shows the formulation of NPs containing resveratrol using both the Double Emulsion Solvent Evaporation (DESE) and Single Emulsion Solvent Evaporation (SESE) methods. It is possible to obtain formulations with the desired size, encapsulation and surface properties by either of the methods. It is important to complete emulsification of both organic and aqueous phases in emulsion technique. There are different stabilizers used in literature, but PVA is the most common and most suitable. As can be seen in Table 2, both techniques yielded sufficient particles, which indicates a better way to formulate them.

Characterization of Nanoparticles

SEM images revealed smooth surface NPs (Figure 3). As shown in Table 3, the particles loaded with Resveratrol were nano sized and homogeneously distributed based on the polydispersity index. A Zeta Potential (ZP) analysis was performed on nanoparticles loaded with Resveratrol to determine their surface charge. A nanoparticle's zeta potential can also affect its biodistribution and pharmacokinetics. The reticuloendothelial system is more

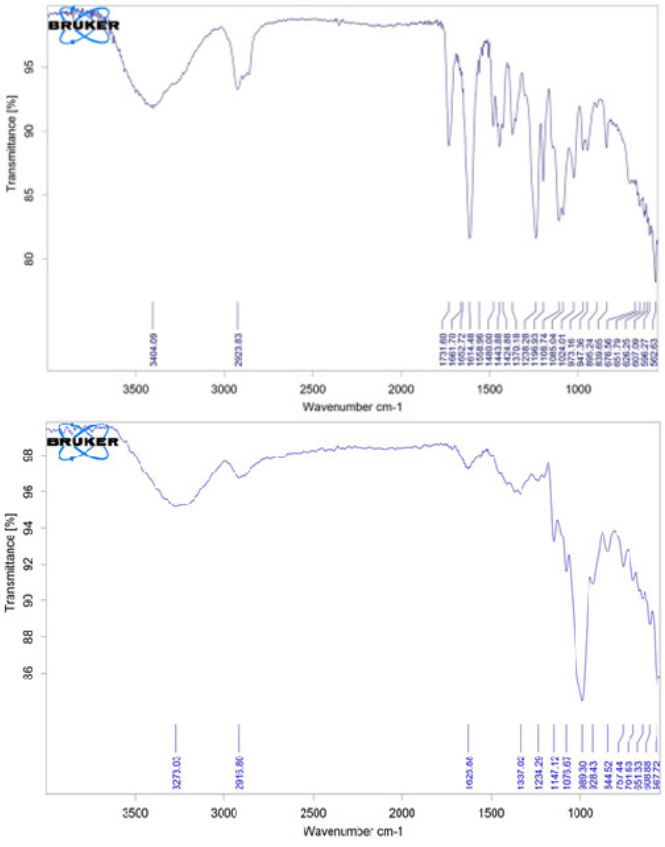

Figure 1: FTIR spectrum of (a) PVA and (b) Resveratrol.

Table 1: Composition of nanoparticles S1-S4.

Formulation code	Resveratrol (mg)	Polymer Used	Amount of Polymer (mg)	Method used	Stabilizer PVA (% w/v) and Volume (mL)	
					Primary	Secondary
S1	10	Pegylated PLGA 85:15	20	DESE	1.5 and 2.5	0.5 and 25
S2	10	Pegylated PLGA 85:15	20	DESE	1.5 and 5	0.5 and 50
S3	10	Pegylated PLGA 85:15	20	SESE	2.5 and 50	---
S4	10	Pegylated PLGA 85:15	20	SESE	1.5 and 50	---

Table 2: Percentage yield of the nanoparticles.

Formulation Code	Yield (%)
S1	74.32
S2	73.56
S3	72.78
S4	73.87

Table 3: Characteristics of Resveratrol Loaded Polymeric nanoparticles using PVA as surfactant.

Formulation code	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Drug loading (%)	Entrapment efficiency (%)
				(Mean ± SD) *	
S1	156.0	0.442	-24.6	35.82 ±0.17	63.45±0.19
S2	230.0	0.421	-29.3	36.44± 0.46	65.86±0.17
S3	139.0	0.421	-32.9	36.06±0.16	66.44±0.16
S4	199.9	0.393	-36.4	37.14±0.17	69.35±0.25

likely to absorb negatively charged nanoemulsions than neutral or positively charged nanoemulsions.

Nanoparticles have a zeta potential or surface charge, which determines the loading efficiency and rate of desorption of drugs in nanoparticles, as well as the types of binding between drugs and nanoparticles. This can also be used to determine if active ingredients/drugs are encapsulated at the centre or adsorb on the surface of nanoparticles. Furthermore, a number of studies have shown that negatively charged nanoparticles clear the bloodstream faster from the bloodstream than positively charged nanoparticles after intravenous administration and remain in the bloodstream for longer periods of time than positively charged nanoparticles.[16] As suggested by studies, nanoparticles with negative zeta potentials or cationic charges have increased cytotoxicity. The reason for this may be due to the fact that nanoparticles interact more readily with oppositely charged cell membranes, causing a destabilizing and destructive effect on the membranes as a result.[17] The ZP values of all the formulations also indicated the stability of polymeric nanoparticles. Hence, it could be concluded that nanoparticles of pegylated PLGA prepared using PVA as surfacting agent could be a successful delivery system for encapsulating hydrophobic drugs like Resveratrol (Figures 4-6).

In vitro drug release from Resveratrol loaded polymeric nanoparticles

The release studies were carried out for selected Resveratrol loaded polymeric nanoparticles (S1-S4) in phosphate buffer (pH 7.4) by orbital shaker method. 2 mg of the sample in 2mL of phosphate buffer pH 7.4 in centrifuged tube were incubated at 37°C in an orbital shaker and were shaken regularly at 90 rpm. It was centrifuged at 10,000 rpm for 20 min and 0.5 mL of supernatant was sampled and analysed for drug release. The cumulative percentage drugs released were calculated and are presented in Table 4. From the result of 168 hr of drug release it was observed that the formulation (S3) showed 92.11% of cumulative percentage drug release which is higher than other formulations.

Cytotoxicity *via* MTT assay

In MCF7 cells, MTT cytotoxicity assays were performed on all nanoparticles in order to check their cytotoxicity, with a blank

Table 4: ***In vitro*** **drug release data of formulations S1-S4.**

Time (hr)	Cumulative percentage drug release (Mean ± SD)*			
	S1	S2	S3	S4
0	0	0	0	0
1	13.82±0.09	15.36±0.14	16.92±0.11	12.29±0.19
3	19.82±0.19	18.38±0.11	19.96±0.09	18.38±0.09
6	23.72±0.16	24.37±0.11	26.91±0.14	23.39±0.06
9	33.14±0.05	30.89±0.15	34.98±0.17	32.35±0.08
12	40.05±0.16	41.41±0.09	43.39±0.18	37.98±0.13
24	50.69±0.10	53.74±0.09	55.95±0.17	53.46±0.08
36	53.62±0.14	56.24±0.11	57.48±0.18	55.18±0.14
48	58.34±0.19	59.14±0.16	60.42±0.12	58.74±0.09
72	63.10±0.12	65.22±0.11	66.99±0.09	65.89±0.11
96	66.17±0.07	68.33±0.08	69.71±0.13	68.88±0.09
120	68.82±0.10	71.11±0.09	76.28±0.15	73.23±0.17
144	72.72±0.13	81.11±0.19	84.42±0.18	78.23±0.16
168	84.22±0.12	85.67±0.16	90.22±0.12	83.75±0.18

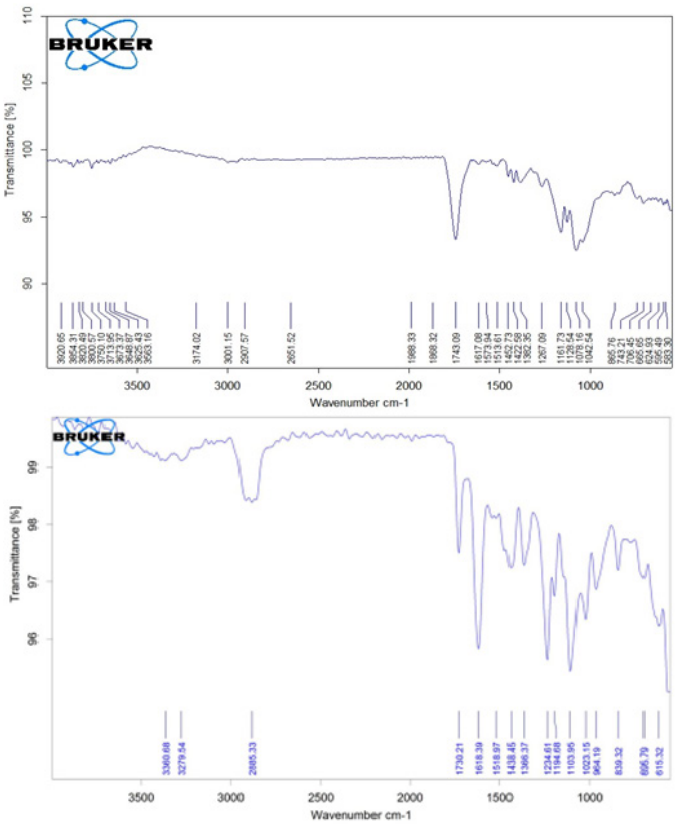

Figure 2: FTIR spectrum of (a) PLGA and (b) Resveratrol, pegylated PLGA and PVA.

nanoparticle serving as a control. Pegylated PLGA and PLGA NPs exhibited a greater cytotoxicity than the free drug. Thus, free Resveratrol at 2.5 M concentrations was found to possess 66.59% cell viability, while PLGA NPs and Pegylated PLGA NPs recorded 26.84% and 42.19%, respectively (Figure 8). In the study of several batches of blank nanoparticles, it was evident that there was no cytotoxicity induction on MCF7 cells based on the results of treating several batches of these blank nanoparticles.

DISCUSSION

Many medicinal and active therapeutic moieties are used in medicine, and nanoparticles made of biodegradable polymers, such as Poly Lactic-co Glycolic Acid (PLGA), are frequently used to increase plasma drug concentration, solve insolubility issues, and improve plasma circulation 1. These polymeric nanoparticles could target genes to tumour tissues in addition to lipophilic or poorly water-soluble drugs. Tumour cells favour a process known as the Enhanced Permeation and Retention (EPR) effect as a result of their common physiological characteristics, such as leaky vasculature and a lack of a lymphatic system. Additionally, nanoparticles can lessen the development of multiple drug resistance because they can enter cells and reduce P-glycoprotein-mediated cell efflux. However, it is challenging to create an effective targeted and active delivery system that can increase the internalisation of medications to the tumour site.[18,19] One of the most rapidly evolving fields of nanotechnology is drug delivery systems based on polymeric nanoparticles. Nanoparticles can be used to deliver a variety of active pharmaceutical as well as nutraceutical ingredients, including hydrophilic and hydrophobic small molecules, biological macromolecules, vaccines and sRNA etc. This strategy has several benefits, including high carrier capacity, high stability, the ability to incorporate both hydrophilic and hydrophobic substances, and the viability of multiple administration methods, such as oral application and inhalation.[20,21] In the current study, PVA was used as a stabilizer while double emulsion-solvent evaporation and single

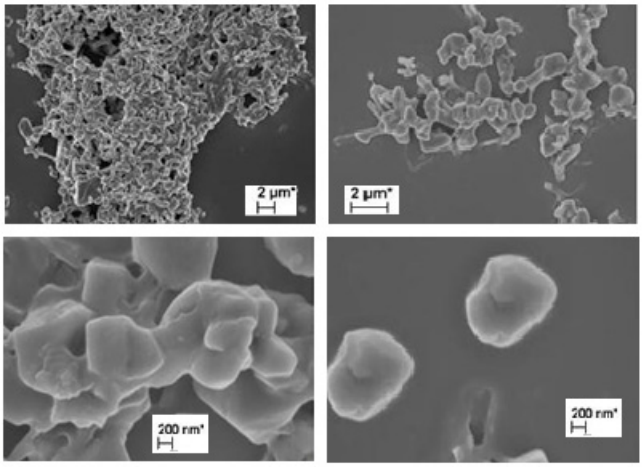

Figure 3: SEM images of prepared nanoparticles.

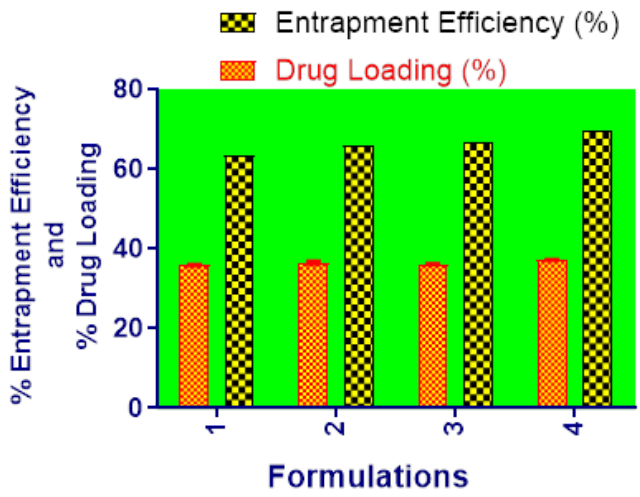

Figure 4: Entrapment efficiency and drug loading of formulations S1 - S4.

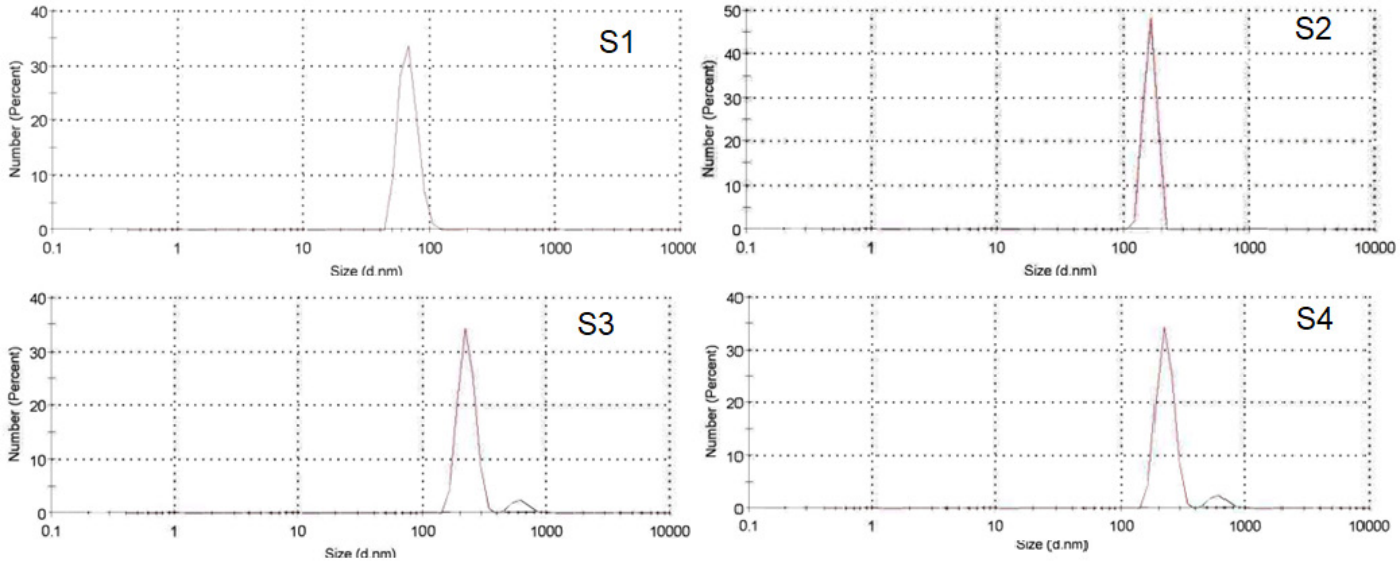

Figure 5: Particle size distribution curve of S1, S2, S3 and S4.

emulsion-solvent evaporation methods were used to prepare Resveratrol-loaded polymeric nanoparticles. Resveratrol-loaded polymeric nanoparticle formulations were examined for their physiochemical characteristics. The most effective formulation was chosen based on characterization. Scanning Electron Microscopy (SEM) was used to further characterize the optimized formulation for their morphological characteristics. The spherical nature of polymeric nanoparticles was revealed by SEM images. At the end of 48 hr, the optimized formulation's cumulative percentage drug release of lyophilized Resveratrol-loaded polymeric nanoparticles was found to be 90.220.12%, which is higher than that of other formulations. The Korsmeyer-Peppas plot and zero order kinetics were found to have more linear R^2 values from the *in vitro* drug release kinetics studies. The Korsmeyer-Peppas plot revealed that the drug release exponent (n value) was less than 0.5, indicating "Fickian diffusion" of the drug from the matrix type nanoparticle formulation. Finally, it can be said that the study's goal was achieved by creating a controlled-release nanoparticulate drug delivery system for resveratrol. The methodology used in this study enabled the rapid and repeatable fabrication of a nanoparticle scaffold with a uniform and spherical morphology. A few studies using the fabrication of PLGA and PLGA-PEG based nanoparticles have reported similar findings in the literature.[22-24] Consequently, it can be said that the formulation created in this study may be regarded as an efficient and promising anticancer drug delivery system for long-term cancer therapy.[25] The objective of the current study was to design and develop a targeted delivery system for resveratrol that would not only address the pertinent issues but also enhance the ingredient's absorption by breast cancer cells. In this study, a

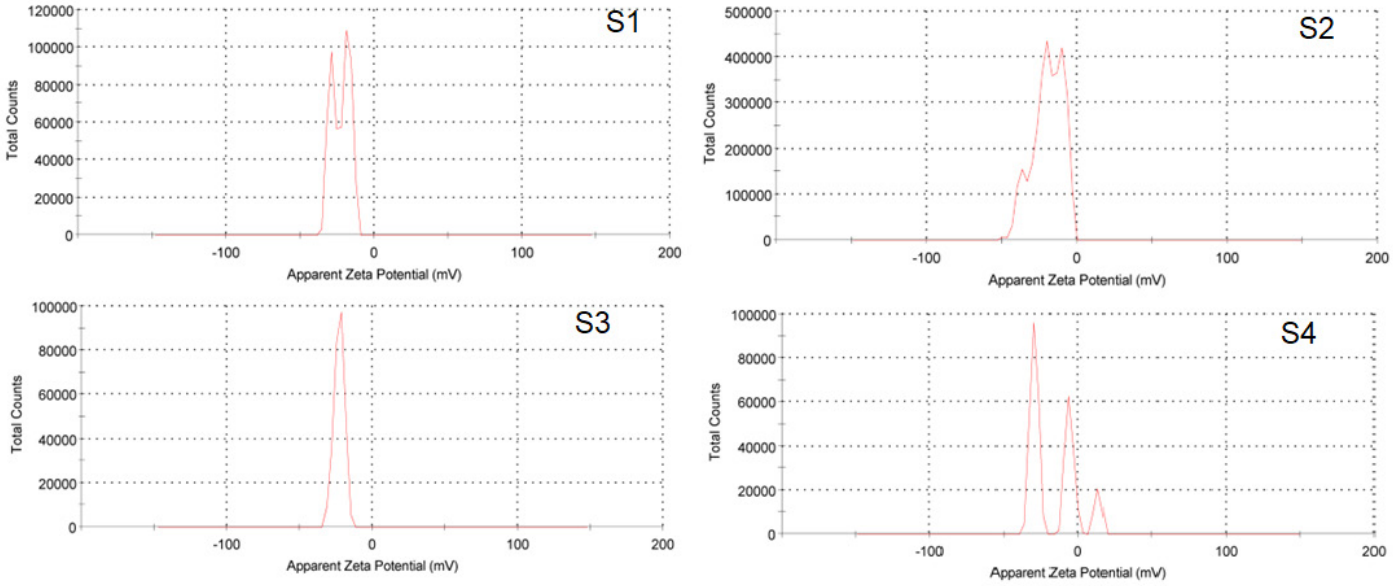

Figure 6: Zeta potential of S1, S2, S3 and S4.

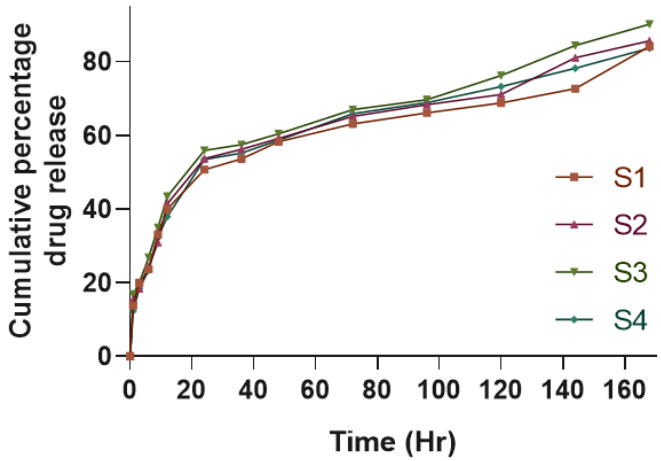

Figure 7: *In vitro* release profile of formulations S1-S4 in optimized condition.

novel drug delivery system for resveratrol is made using pegylated PLGA nanoparticles. It is believed that administering NPs directly into the bloodstream will enable sustained delivery of resveratrol to a specific tumour site while guarding it against rapid deterioration.[26,27] In order to fulfil their function as long-circulating sustained-release drug delivery systems, the pegylated PLGA nanoparticles helped to extend Resveratrol's blood residence time. Resveratrol blood concentration increased as a result of surface modification of nanoparticles, which supports PEG's role in giving these nanoparticles long-circulation properties. In this present study, to deliver resveratrol to a specific target tissue group, biodegradable pegylated nanoparticle were prepared using the Double Emulsion Solvent Evaporation (DESE)[28] and Single Emulsion Solvent Evaporation (SESE) methods.[29] PVA was used as a stabilizer or surfactant in the current study. All synthesized nanoparticles were evaluated for their particle size, zeta-potential, Polydispersity Index measurement, entrapment efficiency, and

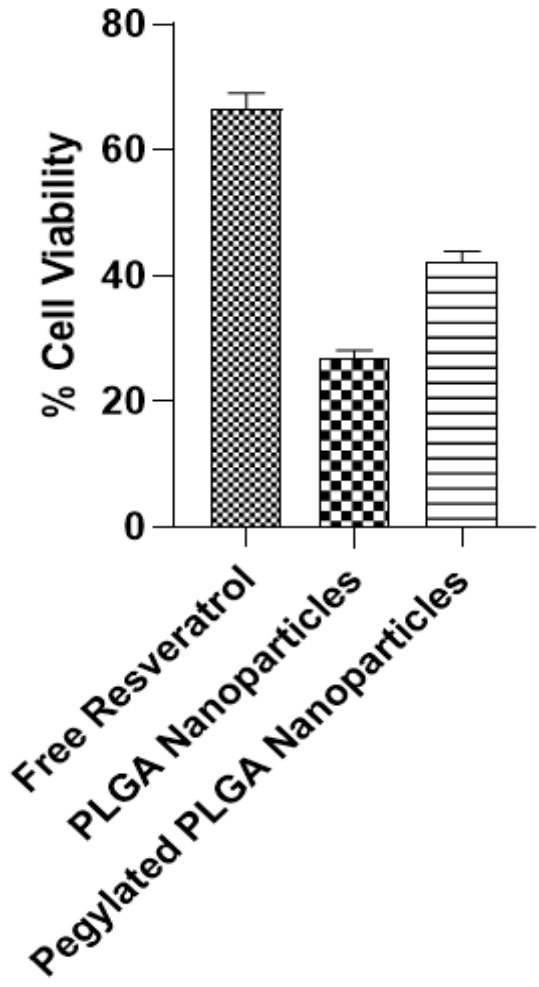

Figure 8: Percentage cell viability for free Resveratrol, PLGA nanoparticles, pegylated PLGA nanoparticles.

drug loading. Given that the PVA-stabilized nanoparticles produced the desired results, it was decided to use Scanning Electron Microscopy (SEM) to look at the surface morphology. The MTT cell proliferation assay was used to evaluate the *in vitro* cytotoxicity effect of pegylated PLGA NPs and blank NPs by assessing the reduction of 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma) to Formazan in MCF7 breast cancer cell lines.[30] According to the findings, pegylated PLGA were more cytotoxic than the free drug.

CONCLUSION

By using PVA as a surfactant, the nanoparticles loaded with Resveratrol were designed and evaluated effectively. As demonstrated in this study, PVA is a promising surfactant for encapsulating and delivering poorly water-soluble compounds as pegylated PLGA nanoparticles with desired particle size, morphology and drug loading. By using pegylated PLGA nanoparticles encapsulated with Resveratrol, the drug was successfully delivered to the target site. PLGA pegylated polymers were synthesized based on the study results to target the desired sites. There are several characteristics that have been observed in nanoparticles prepared by double emulsion solvent evaporation including size, surface morphology, drug loading and encapsulation and the particle size observed for the formulations S1-S4 were 156.0 nm, 230.0 nm, 139.0 nm and 199.9 nm respectively. Entrapment efficiency (%) for the formulations S1-S4 were found to be 63.45±0.19, 65.86±0.17, 66.44±0.16 and 69.35±0.25, respectively. The drug loading (%) of formulations S1-S4 were found to be 35.82 ±0.17, 36.44± 0.46, 36.06±0.16 and 37.14±0.17 respectively. Moreover, the optimized nanoformulation demonstrated significant cytotoxic effect. The free Resveratrol at 2.5 M concentrations was found to possess 66.59% cell viability, while PLGA NPs and Pegylated PLGA NPs recorded 26.84% and 42.19% cell viability, respectively.

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ABBREVIATIONS

PLGA: Poly (lactic-co-glycolic acid); **IGF-1:** Insulin-like growth factor 1; **PEG:** Polyethylene glycol; **FTIR:** Fourier Transform Infrared; **DESE:** Double Emulsion Solvent Evaporation; **SESE:** Single Emulsion Solvent Evaporation; **PVA:** Polyvinyl acetate; **DLS:** Dynamic light scattering; **ZP:** Zeta Potential Measurement; **SEM:** Scanning Electron Microscopy; **CDER:** Center for Drug Evaluation and Research; **MTT:** 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; **MCF:** Michigan Cancer Foundation; **EPR:** Enhanced permeation and retention; **PLGA-PEG:** Pegylated poly (lactic-co-glycolic acid).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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