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Article

Formulation and evaluation of vascular endothelial growth factor loaded polycaprolactone nanoparticles

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In an attempt to increase molecular stability and provide controlled release, vascular endothelial growth factor (VEGF) was encapsulated into polycaprolactone (PCL) nanoparticles. Both VEGF-free and VEGF-loaded PCL nanoparticles were formulated by w/o/w double emulsion of the dichloromethane-water system in the presence of polyvinyl alcohol (PVA) and rat serum albumin. To achieve the optimal formulation concerning particle size and monodispersity, studies were carried out with different formulation parameters, including PVA concentration, homogenization time and rate. Scanning electron microscopy and dynamic light scattering analysis showed respectively that particles had a spherical shape with a smooth surface and particle size varying between 58.68-751.9 nm. All of the formulations were negatively charged according to zeta potential analysis. *In vitro* release study was performed in pH 7.4 phosphate-buffered saline at 37°C and released VEGF amount was measured by enzyme-linked immunosorbent assay (ELISA) method. At the end of the 35th day, 10% of total encapsulated VEGF was released with a sustained-release profile, which fitted the Korsmeyer-Peppas kinetic model. The bioactivation of the nanoparticles was evaluated using XTT and ELISA methods. As a result, the released VEGF was biologically active and also VEGF loaded PCL nanoparticles enhanced proliferation of the human umbilical vein endothelial cells in cell culture.

Keywords: Nanoparticles. Polycaprolactone. VEGF. Double emulsion method. Bioactivity.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is one of the first known and most significant angiogenic agents which has a key role in the proliferation of endothelial cells (Ferrara, Davis-Smith, 1997). In the 70s, the VEGF molecule is isolated and named as vascular permeability factor (VPF) owing to the 50,000-fold increase in vascular permeability compared to histamine (Dvorak, 2006). In addition to the VEGF-A as the main VEGF molecule, the VEGF family also includes placental growth factor (PlGF), VEGF-B, VEGF-C (VEGF-2), VEGF-D, VEGF-E and VEGF-F, and their different isoforms, depending on the molecular weight or protein structure (Ylä-Herttuala *et al.,* 2007). After several VEGF specific tyrosine kinase receptors, which different VEGF types were able to bind to, were defined, different pathways were seen to cause different effects, such as placenta regulation, macrophage activation, angiogenesis and lymphangiogenesis (Shibuya, 2006). Especially the angiogenic effect of VEGF supports cell and tissue regeneration by supplying nutrition and oxygen to tissues and increasing vascular permeability (Fruttiger, 2008). Therefore, the use of VEGF in tissue engineering and diseases related to tissue damage is a reasonable approach. Besides, the neurotrophic properties of VEGF have recently become crucial in neurological disorders (Verheyen *et al.,* 2013). Furthermore, overexpression of growth factors may increase carcinogenicity (French, Frazier, 2011), studies against different types of cancer are being carried out by inhibiting this expression (Ferrara *et al.,* 2004; Tabernero, 2007; Ekentok, Özbaş-Turan,

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Akbuğa, 2017). Therefore, the local administration of VEGF should be preferred to prevent tumor cell growth rather than systemic application (Verheyen *et al.,* 2013).

Polycaprolactone (PCL) is a well-known hydrophobic and semicrystalline polymer, which is frequently used in polymer-based drug carrier systems. The crystallinity of PCL tends to decrease while molecular weight is increasing. Due to its low melting point (59-64°C), low glass transition temperature (-60°C) and miscibility with other polymers, PCL has a wide potential in biomedical areas and varied research studies. Additionally, depending on biological compatibility and biodegradability, plenty of formulation studies as a nanoparticle, microsphere, implant, nanofiber, scaffold, composite, hydrogel and micelle have been carried out using PCL (Sinha *et al*., 2004; Nair, Laurencin, 2007; Dash, Konkimalla, 2012). As a polymer that can degrade in the long term, PCL can provide drug release along several months up to several years, depending on molecular weight and crystallinity (Labet, Thielemans, 2009). This property of PCL brings superiority in sustained-release systems in comparison with other polyester polymers, such as poly (glycolic acid) derivatives (Sinha *et al*., 2004).

Protein structure and instability against enzymatic and chemical degradation of VEGF result in very short half-life and inadequate bioavailability (Sato *et al*., 2001; Eppler *et al*., 2002). Particularly, proteins can be protected against degradation factors by encapsulation into polymeric nanoparticles. Thus, VEGF can reach therapeutic concentration while preserving its bioactivity. Moreover, the drug can be released from nanoparticles in a controlled manner and maintenance dose can be avoided. Previously, different polymeric microparticles and nanoparticles have been fabricated in an attempt to deliver VEGF. For instance, injectable VEGF loaded PLGA nanoparticles resulted in remarkable achievements in a mouse femoral artery ischemia model (Golub *et al.* 2010). Moreover, *in vivo* application of nerve conduits containing VEGF-loaded poly lactic-co-glycolic acid (PLGA) microspheres was shown to accelerate nerve regeneration (Rui *et al.,* 2012; Karagöz *et al.,* 2012; Eren *et al.,* 2015). Notably, De la Riva *et al*. (2009) incorporated VEGF containing alginate nanoparticles into a polymeric scaffold to enhance bone formation. This study aimed to encapsulate VEGF into PCL nanoparticles to protect the bioactivity of VEGF and provide controlled release. To our knowledge, our study is the first study in the literature that is based on the formulation and characterization of VEGF loaded PCL nanoparticles.

MATERIAL AND METHODS

Material

VEGF ($VEGF_{165}$, M_W: ~39 kDa), PCL (M_n: 45 kDa), polyvinyl alcohol (PVA; M_W: 72 kDa) and albumin from rat serum (RSA) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Dichloromethane (DCM) was obtained from Merck (Darmstadt, Germany). Quantikine VEGF ELISA kit was purchased from R&D Systems Inc. (Minneapolis, MN, USA) and used according to the manufacturer's instructions. Pathscan® VEGFR-2 ELISA kit and XTT kit were provided by Cell Signaling Technology Inc. (USA) and Biological Industries Ltd. (Kibbutz Beit-Haemek, Israel), respectively. DMEM, fetal bovine serum and penicillin-streptomycin were received from Gibco (Grand Island, NY, USA). All the reagents used were of analytical grade.

Preparation of the PCL nanoparticles

VEGF loaded PCL nanoparticles were formulated by the modified double emulsion-solvent evaporation method (Golub *et al*., 2010). Briefly, VEGF (5 or 10 µg) and RSA (25 or 50 mg) were dispersed into 20 mL of aqueous PVA solution at various concentrations (Table I). 0.5 mg PCL was dissolved in 10 mL dichloromethane. This organic solution was poured into PVA containing aqueous phase and mixed under homogenizer (IKA, T18 Digital Ultraturrax, Germany) with 9000 rpm for five minutes in an ice bath. Then, the primary emulsion was added to 150 mL of 0.1% (w/v) PVA solution and the secondary emulsion was formed by mixing under homogenizer in an ice bath. Final emulsion was stirred for two hours in a propeller mixture (IKA, Labortechnik RH Basic, Germany) at 750 rpm to evaporate DCM. Hardened nanoparticles were obtained by centrifugation (Sigma 3K30, Germany) at 21000 rpm (41415 RCF) for 15 min

at 4°C, washed three times with distilled water and then lyophilized (Christ Alpha 1-2 LD Plus, Germany) for 24 h.

Characterization of the PCL nanoparticles

Particle size measurement

The particle size of VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles were measured by dynamic light scattering method using Malvern NanoZS Zen 3600 (Malvern Instruments LTD., Malvern, UK). VEGF-free PCL nanoparticles or VEGF loaded PCL nanoparticles were dispersed into distilled water and the average particle size was calculated as the number mean. Particle size distribution was evaluated by PDI values of formulations.

Zeta potential measurement

Zeta potential of VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles was measured in mV using Malvern NanoZS Zen 3600 (Malvern Instruments LTD., Malvern, UK) after suspending the nanoparticles into distilled water. Results were expressed as mean values.

Surface morphology

Morphology of the free and VEGF loaded PCL nanoparticles was displayed by scanning electron microscopy (SEM) (JEOL/JSM-6335F) after coating the nanoparticles with 10 nm of platinum using a sputter coater.

Encapsulation efficiency

The encapsulation efficiency was calculated indirectly. Briefly, the amount of non-encapsulated VEGF in the external aqueous phase was quantified using ELISA according to the manufacturer's protocol. In this manner, encapsulation efficiency was calculated according to equation 1.

$$\%EE = \frac{Total\ VEGF\ amount - Nonencapsulated\ VEGF\ amount}{Total\ VEGF\ amount} x100 \quad (1)$$

In vitro release study

VEGF release from VEGF-loaded PCL nanoparticles was performed in phosphate buffer solution (PBS) (pH 7.4) at 37 ± 0.5°C using Memmert WB 14 shaking water bath (Memmert, Germany). Firstly, 5 mg of VEGF-loaded PCL nanoparticles were placed into Eppendorf polypropylene tubes. 1.5 mL of phosphate buffer was added into the tubes and then incubated with agitation at 100 rpm. At determined time intervals (1, 3, 5, 8, 12, 17, 21, 25, 30 and 35 days), Eppendorf tubes were centrifuged and 200 µL of release medium collected for ELISA analysis. The same volume of fresh buffer was replaced into the release tube immediately after each sampling to maintain the VEGF concentration within sink conditions. Samples were stored at -20°C. The released amount of VEGF was measured using the VEGF sandwich ELISA kit pursuant to the manufacturer's instructions. According to the quantitative sandwich enzyme immunoassay technique, standard solution, control solution and samples were pipetted into the wells, which have been pre-coated with a specific polyclonal antibody to bind the VEGF. After unbound substances had been washed away, the substrate solution was added to the wells and the wells were incubated in darkness for 30 minutes to achieve an enzymatic reaction. Subsequently, the stop solution was added, and the color of the product was turned from blue to yellow. VEGF concentration was determined by measuring the optical density of the wells using a microplate reader (Biotek ELx800, USA) at 450 nm of wavelength. This study was carried in triplicate, and the mean values were calculated with standard deviations.

Release kinetics of VEGF

Based on the *in vitro* release study, this study aimed to identify the proper kinetic release model for VEGF encapsulated into PCL nanoparticles. Thus, VEGF amounts released from the nanoparticles were fitted to various kinetic models, including zero order, first order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas. For the Korsmeyer-Peppas equation, "n" and "K" values were also calculated from the slope of the straight line for the plot,

$$M_t/M_\infty = K.t^n \quad (2)$$

where the "K" is the diffusional constant, and "n" is the exponent that determines the release mechanism of the nanoparticles (Korsmeyer, Peppas, 1981). Each of the plots was compared, and the plot providing linearity with the highest r^2 value was selected.

In vitro bioactivity of the VEGF loaded PCL nanoparticles

The biological activity of VEGF released from the nanoparticles was evaluated *in vitro* by determining its ability to stimulate the growth of cultured human umbilical vein endothelial cells (HUVECs), as mentioned in the literature (De la Riva *et al.,* 2009; Rui *et al*., 2012). HUVECs were cultured in T-75 cell culture flasks in Dulbecco`s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin; 100 µg/mL streptomycin) at 5% CO_2 and 37°C. Cells were subcultured when the culture was reached about 80% confluency. For activity assay, HUVECs were plated into 96-well plates at a density of $5x10^3$ cells/well and allowed to attach for 24 h. After 24 h, the culture medium was removed and incubated with supernatant from VEGF-free nanoparticles (FR_2), VEGF-loaded nanoparticles (FV_3) or free VEGF (at 1, 5, 10 ng/mL) in PBS for 24 h. Supernatant samples were obtained from FV_3 or FR_2 after the release period of three days or defined VEGF standard solutions in PBS.

The cell proliferation was measured using the colorimetric 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-([phenylamino]carbonyl)- 2H-tetrazolium hydroxide (XTT) assay. The principle of the method is metabolization of the tetrazolium salt XTT to formazan dye by mitochondrial dehydrogenases of viable cells (De la Riva *et al.,* 2009). The absorbance of released VEGF from nanoparticles and VEGF was obtained using an Epoch microplate spectrophotometer (BioTek Instruments, USA) at a wavelength of 450 nm/reference 655 nm.

The capability of released VEGF from nanoparticles to induce its major receptor VEGFR-2 in the cultured HUVEC was evaluated to confirm the VEGF bioactivity. VEGFR-2 undergoes autophosphorylation after ligand binding and becomes activated. Thus, the PathScan® Phospho-VEGFR-2 (Tyr1175) sandwich ELISA kit was used to detect endogenous levels of Phospho-VEGFR-2 (Tyr1175) protein in cultured cells. ELISA method was carried out according to the manufacturer's procedure. The absorbance was measured at a wavelength of 450 nm.

Statistical analysis

Studies were performed in triplicate, and the results were calculated with standard deviations. SPSS independent samples t-test was used to reveal statistical significance. Results were considered significantly different at $p<0.05$ and highly significantly different at $p<0.01$.

RESULTS AND DISCUSSION

Preparation of the PCL nanoparticles

Double emulsion-solvent evaporation is a favorable method for the encapsulation of hydrophilic drugs (Iqbal *et al.,* 2015). In this method, the drug is entrapped into the inner aqueous phase of the double-layer emulsion to prevent leakage into the external aqueous phase. Owing to the hydrophilicity of VEGF, free and VEGF loaded PCL nanoparticle formulations prepared employing this method and formulation parameters are given in Table I. Albumin was used to provide VEGF's bioactivity by creating a protective layer around the VEGF (van de Weert *et al*., 2000; Bummer, 2008; Sipahigil *et al*. 2012; Alarçin *et al*., 2018). PVA was chosen to form the aqueous phase due to being a good stabilizer in addition to its biocompatibility and biodegradability (Wisniewska *et al.,* 2015). VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles were prepared with 2%, 2.5% or 5% aqueous PVA solution (w/v) to define the optimal concentration for the internal aqueous phase. Herein, 5% PVA concentration was viscous and resulted in excess foaming and aggregation during preparation (formulation F_5 and F_6). Therefore, 5% of the PVA was not used for the following formulations. Additionally, the same foaming problem was observed with increasing stirring time in F_3 (from 10 to 15 min) and an increasing rate of homogenizer in F_6 (from 15000 to 20000 rpm).

TABLE I - Formulations of the free and VEGF-free loaded PCL nanoparticles prepared by water-in-oil-in-water double emulsion technique

Formulation	Concentration of Primary PVA Solution (Inner Aqueous Phase) (w/v)	RSA (mg)	VEGF (µg)	Homogenization *	
				Rate (rpm)	Time (minute)
F_1	%2	-	-	15000	5
F_2	%2	-	-	15000	10
F_3	%2	-	-	15000	15
F_4	%2.5	-	-	15000	10
F_5	%5	-	-	15000	10
F_6	%5	-	-	20000	10
FR_1	%2.5	50	-	15000	10
FR_2	%2.5	25	-	15000	10
FV_1	%2.5	50	5	15000	10
FV_2	%2.5	50	10	15000	10
FV_3	%2.5	25	10	15000	10

PVA: Polyvinyl alcohol, RSA: Rat serum albumin, VEGF: Vascular endothelial growth factor.
*Homogeniser rate and using time during the preparation of secondary water-in-oil-in-water emulsion.

Characterization of the PCL nanoparticles

Mean particle size, particle size distribution (PDI) and zeta potential values of VEGF-free and VEGF loaded PCL nanoparticles are shown in Table II.

TABLE II - Particle size, PDI and Zeta potential of the VEGF-free and VEGF loaded PCL nanoparticles

Formulation	Particle Size (nm)	PDI	Zeta Potential (mV)
F_1	423.5±89.91	0.721	-9.70±0.405
F_2	339.2±63.75	1.000	-12.3±2.19
F_3	361.1±66.7	0.842	-11.1±0.416
F_4	380±60.27	0.958	-7.09±0.294
F_5	464.6±66.48	0.286	-10.3±0.388
F_6	249±50.07	0.523	-6.36±0.145
FR_1	487.4±61.6	0.509	-16.9±0.321
FR_2	58.68±10.54	0.607	-3.69±0.046

(continues on the next page...)

TABLE II - Particle size, PDI and Zeta potential of the VEGF-free and VEGF loaded PCL nanoparticles

Formulation	Particle Size (nm)	PDI	Zeta Potential (mV)
FV_1	421.8±71.15	0.994	-5.41±0.569
FV_2	751.9±119	0.844	-5.17±0.292
FV_3	79.3±23.16	0.498	-3.38±0.128

The concentration of the primary PVA solution was varied between 2% and 5% to evaluate the effects of the PVA concentration upon formulations. Under the same conditions, formulations were prepared with 2% (F_2), 2.5% (F_4) and 5% (F_5) PVA (w/v) and when these formulations were compared, particle size was increased with an increase in PVA concentration. As the concentration of PVA increases, the particle size may decrease owing to increased stabilization at the emulsion interface. However, due to the increase in viscosity, the mixing efficiency will be reduced and the particle size can be increased correspondingly (Budhian *et al.,* 2007; Miladi *et al.,* 2016). Moreover, in the preparation of PCL nanoparticles, it was reported that the particle size increased in the case of using PVA at a concentration above 2% (Ahmed *et al.,* 2012).

FR coded formulations containing 25 or 50 mg of RSA showed different particle diameters. From the two formulations prepared with the same parameters, the formulation contained 25 mg RSA (FR_2) had 58.68±10.54 nm of particle size, while the particle size of 50 mg of RSA contained formulation (FR_1) was 487.4±61.6 nm.

Based on the pre-formulation studies, VEGF loaded FV_1, FV_2, and FV_3 coded formulations were prepared using 2.5% PVA solution as primary aqueous phase, and 150 mL of 0.1% PVA solution as the secondary aqueous phase. The secondary homogenizer rate was chosen to be 15000 rpm and homogenizer used for 10 minutes. Firstly, 5 µg of VEGF was loaded to FV_1 formulation as a pretesting and formulation was evaluated in the manner of particle size, morphology and zeta potential, and then increased to 10 µg for FV_2 and FV_3 formulations. Regarding that, increasing drug: polymer ratio has resulted in bigger diameters of formulations, which is in agreement with the literature (Bouissou, Van der Walle, 2006). Similarly, for VEGF-free PCL nanoparticles, smaller particle size was obtained when 25 mg of RSA was added to the VEGF loaded PCL nanoparticles. The particle size of the FV_3 coded formulation was 79.30±23.16 nm, while the particle size of FV_1 and FV_2 coded formulations were 421.8±71.15 and 751.9±119 nm, respectively.

All of the VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles were negatively charged according to the literature (Singh *et al.,* 2006; Ribeiro *et al.,* 2013). Herein, the zeta potential of the formulations varied from -3.38±0.128 mV to -16.9±0.321 mV.

Morphological analysis of the formulations showed that both VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles were spherical (Figure 1). RSA containing formulation (FR_2) had a smoother surface than RSA-free formulation (F_4). VEGF loaded PCL nanoparticles had a porous structure on the surface. Moreover, pores were more visible, especially for bigger particles (FV_1). This porosity is generally associated with dissolution and drug release behaviors of the particle (Zhou *et al.,* 2017).

As a result, FV_3 coded formulation found to have a particle size smaller than 100 nm with an acceptable PDI value, spherical shape and smooth surface morphology. Therefore, FV_3 was chosen as the optimal formulation to carry out the *in vitro* release study. Subsequently, encapsulation efficiency (EE%) of the FV_3 formulation was found to be 22.543±2.052%. The hydrophilic-lipophilic character of both the active substance and the polymer used in nanoparticles is a noteworthy criterion affecting EE% (Yeo, Park, 2004). Similarly, in another study, EE% of the water-soluble carboplatin in PCL nanoparticles was calculated as 27.95 ± 4.21% (Alex *et al*., 2016).

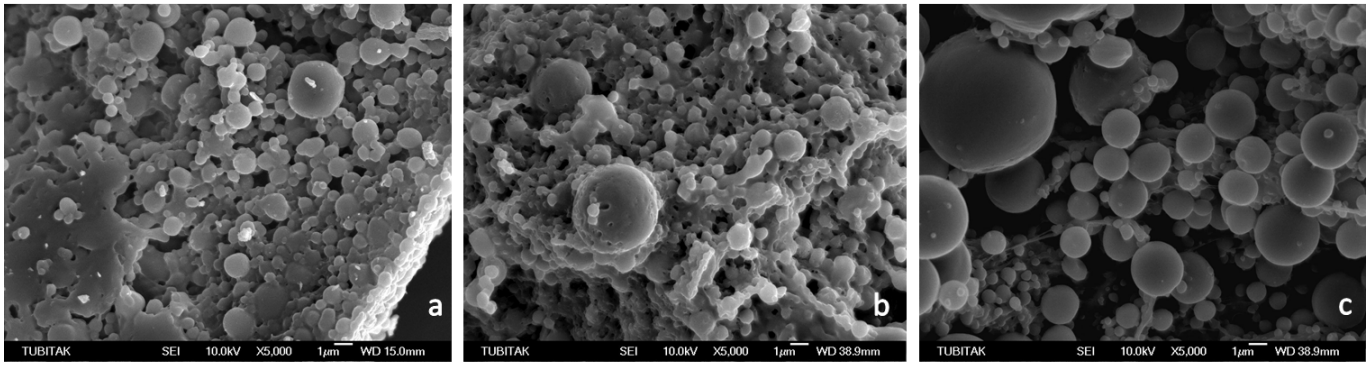

FIGURE 1 - Scanning electron micrographs of a) VEGF-free PCL nanoparticles prepared with 2.5% (w/v) concentration of the PVA as an aqueous inner phase (F_4), b) VEGF-free PCL nanoparticles prepared with 25 mg RSA and 2.5% (w/v) concentration of PVA as an aqueous inner phase (FR_2), c) VEGF loaded PCL nanoparticles prepared with 10 µg VEGF, 25 mg RSA and 2.5% (w/v) concentration of the PVA as an aqueous inner phase (FV_3).

In vitro release study

In vitro release study of VEGF from FV_3 formulation was carried out for 35 days. When the cumulative VEGF release versus time plot was analyzed, first, a rapid release was seen, followed by a sustained release. The initial burst release of the VEGF might be attributed to loosely attached or adsorbed VEGF molecules on the nanoparticle surface. Drug release from the nanoparticles might be achieved by either drug diffusion through polymer or degradation of the polymer. The sustained release observed in the release profile might be due to the slow diffusion of the hydrophilic drug from the highly lipophilic polymer and the long-term degradation of polycaprolactone. After 35 days, 10.036 ± 1.201% of the encapsulated VEGF was released from PCL nanoparticles (Figure 2). Notably, the complete degradation of PCL may vary between 2-4 years, regarding its molecular weight (Labet, Thielemans, 2009). Considering that only 10% of the encapsulated VEGF was released in 35 days, the total VEGF release from nanoparticles prepared with PCL of 45 kDa was predicted to continue several months.

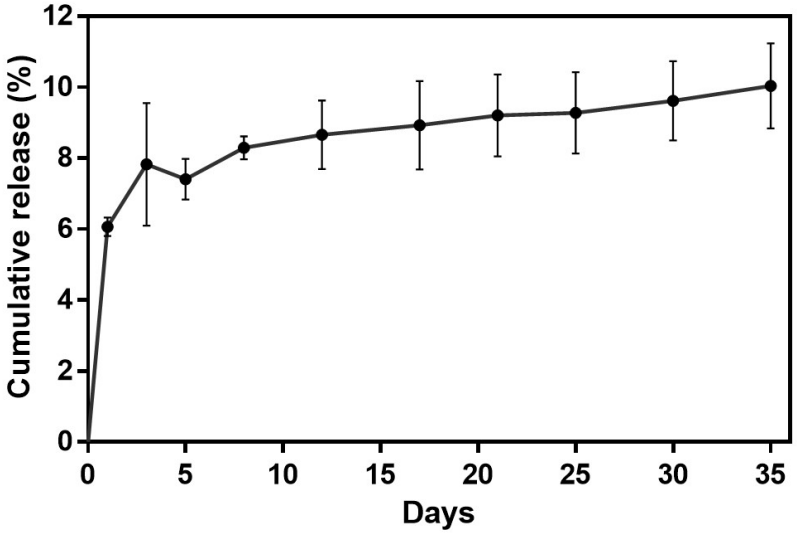

FIGURE 2 - *In vitro* release profile of the VEGF from VEGF loaded PCL nanoparticles prepared with 10 µg VEGF (FV_3 formulation) in pH 7.4 phosphate buffer solution at 37±0.5°C. Cumulatively released VEGF (%) was expressed as mean ± SD (n=3).

Release kinetics of the VEGF

Releasing data of the optimized formulation (FV_3) was fitted to different kinetic models to study the VEGF release kinetics for VEGF loaded PCL nanoparticles. Regression coefficients (r^2) for zero order, first order, Korsmeyer-Peppas, Higuchi and Hixson-Crowell models were compared to achieve the best linearity and r^2 for Korsmeyer-Peppas equation was found to be the highest coefficient as 0.949. Additionally, the release exponent of the equation for the Korsmeyer-Peppas model (n) was 0.127. The Fickian diffusion limit for spherical matrix structures is considered to be n=0.43. Lower n values can be found in particles with high particle size distribution (PDI). In these cases, the release mechanism is considered a quasi-Fickian (Peppas, 1985). Similarly, the release of exemestane (Kumar, Sawant, 2013) and aceclofenac (Kaur, Sharma, Sinha, 2017) from PCL particles was explained by quasi-Fickian kinetics (n<0.45).

In vitro bioactivity of VEGF loaded PCL nanoparticles

The bioactivity of free VEGF, VEGF loaded nanoparticles (FV_3) and VEGF free nanoparticles (FR_2) were assessed to evaluate the potential applications for drug development approaches. For this purpose, the capacity of VEGF released from the nanoparticles to induce the proliferation of HUVECs was determined. According to our results, 10 ng/mL of free VEGF or VEGF loaded nanoparticles (FV_3) increased the proliferation of HUVECs (138.5% and 109.6% of cell viability, respectively) in comparison with control or FR_2. The proliferative effect of free VEGF on HUVECs was significant at 10 ng/mL *($p<0.05$).* No significant differences were observed among lower VEGF concentrations (1 and 5 ng/mL of free VEGF or released VEGF). Figure 3 represents the VEGF bioactivity after the release period of three days from nanoparticles.

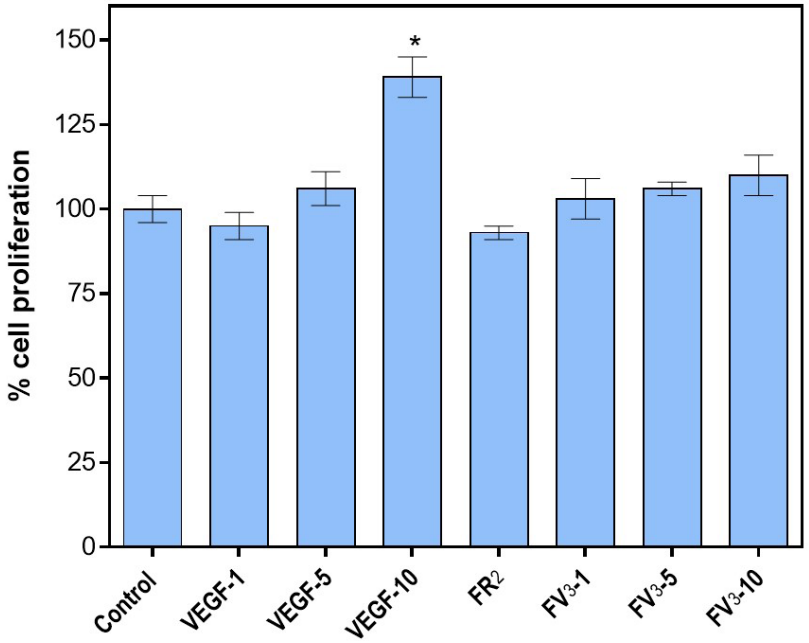

FIGURE 3 - Bioactivity of the VEGF released from nanoparticles by XTT. It was determined that both 1, 5 and 10 ng/mL free VEGF solutions (VEGF-1, VEGF-5, VEGF-10) or VEGF-loaded nanoparticles (FV_3) that released VEGF providing 1, 5 and 10 ng/mL concentration (FV_3-1, FV_3-5, FV_3-10) increased proliferation in HUVEC culture compared to the culture medium as control. VEGF-free PCL nanoparticles (FR_2) did not increase cell proliferation. *$p<0.05$.

FV_3 bioactivity was confirmed by measuring quantifying endogenous levels of Phospho-VEGFR-2 protein, which exhibits VEGF-induced signaling. The obtained results verified the bioactivity of the released VEGF from nanoparticles (Figure 4). VEGF-induced signaling levels in HUVEC treated either with the free VEGF or the FV_3 were similar, and the differences were highly significant when compared with control *($p<0.01$).*

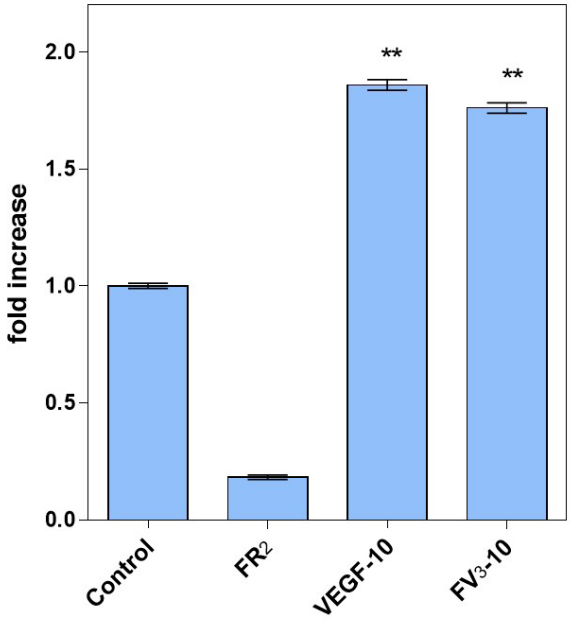

FIGURE 4 - Effects of VEGF-free PCL nanoparticles (FR_2), free VEGF solution at a concentration of 10 ng/ml (VEGF-10) or FV_3 formulation which contains encapsulated VEGF released at a concentration of 10 ng/ml (FV_3-10) in HUVEC culture on inducing VEGFR-2 (KDR) activation by ELISA is shown (y-axis shows fold increase of VEGFR-2 compared to the untreated control group) ** $p < 0.01$.

The process for the formulation of nanoparticles may affect the bioactivity of the growth factors. Therefore, encapsulated VEGF bioactivity was tested HUVECs in this study. Our results showed that the biological activity of the VEGF solutions or released VEGF was found to retain cell viability above 90% at treated concentrations.

In conclusion, spherical VEGF loaded PCL nanoparticles with a diameter smaller than 100 nm were successfully formulated in the present study, and these nanoparticles showed negative zeta potential. To our knowledge, our study is the first study in the literature that has been based on the formulation and characterization of the VEGF loaded PCL nanoparticles. Nanoparticles achieved sustained release for VEGF and it was determined that the release mechanism was dominated by diffusion according to the Korsmeyer-Peppas model. According to *in vitro* cell culture studies, obtained nanoparticles increased proliferation in endothelial cells and did not lose their bioactivity during the formulation process. Furthermore, no other cytotoxicity arising out of formulation was observed. Consequently, the formulation developed in this study was successful in line with the research objectives of this study. The promising results are expected to be supported by *in vivo* studies for tissue engineering by incorporating these nanoparticles into scaffolds to achieve the controlled release of VEGF in the future.

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REFERENCES

Ahmed N, Michelin-Jamois M, Fessi H, Elaissari A. Modified double emulsion process as a new route to prepare submicron biodegradable magnetic/polycaprolactone particles for in vivo theranostics. Soft Matter. 2012;8:2554-64.

Alarçin E, Lee TY, Karuthedom S, Mohammadi M, Brennan MA, Lee DH, et al. Injectable shear-thinning hydrogels for delivering osteogenic and angiogenic cells and growth factors. Biomater Sci. 2018;6(6):1604-1615.

Alex AT, Joseph A, Shavi G, Rao JV, Udupa N. Development and evaluation of carboplatin-loaded PCL nanoparticles for intranasal delivery. Drug Deliv. 2016;23(7):2144-2153.

Bouissou C, Van der Walle C. Poly (lactic-co-glycolic acid) microsphere. In: Uchegbu JF, Schatzlein AG, editors. Polymers in drug delivery. Boca Raton, FL: CRC Press; 2006, p. 81-99.

Budhian A, Siegel SJ, Winey KI. Haloperidol-loaded PLGA nanoparticles: systematic study of particle size and drug content. Int J Pharm. 2007;336(2):367–375.

Bummer PM. Chemical consideration in protein and peptide stability. In: McNally EJ, Hastedt JE, editors. Protein Formulation and Delivery, 2nd edition. New York: Informa Healthcare; 2008, p. 7-42.

Dash TK, Konkimalla VB. Poly-ε-caprolactone based formulations for drug delivery and tissue engineering: A review. J Controlled Release. 2012;158(1):15-33.

De la Riva B, Nowak C, Sanchez E, Herandez A, Schulz-Siegmund M, Pec MK, et al. VEGF-controlled release within a bone defect from alginate/chitosan/PLA-H scaffolds. Eur J Pharm Biopharm. 2009;73(1):50-58.

Dvorak HF. Discovery of vascular permeability factor (VPF). Exp Cell Res. 2006;312(5):522-526.

Ekentok C, Özbaş-Turan S, Akbuğa J. In vitro gene silencing effect of chitosan/shRNA PDGF-D nanoparticles in breast cancer. J Res Pharm. 2017;21(4):793-803.

Eppler SM, Combs DL, Henry TD, Lopez JJ, Ellis SG, Yi JH, et al. A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. Clin Pharmacol Ther. 2002;72(1):20-32.

Eren F, Öksüz S, Küçükodacı Z, Kendirli MT, Cesur C, Alarçin E, et al. Targeted mesenchymal stem cell and vascular endothelial growth factor strategies for repair of nerve defects with nerve tissue implanted autogenous vein graft conduits. Microsurgery. 2015;36(7):578-585.

Ferrara N, Davis-Smith T. The biology of vascular endothelial growth factor. Endocr Rev. 1997;18(1):4-25.

Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rew Drug Discov. 2004;3(5):391-400.

French KJ, Frazier KS. The role of VEGF and VEGF receptors in carcinogenesis. J Carcinog Mutagen. 2011;S2: 002.

Fruttiger M. VEGF Gene Regulation. In: Ruhrberg C, editor. VEGF in Development. Austin, Texas: Landes Bioscience; 2008, p. 30-39.

Golub JS, Kim Y, Duvall CL, Bellamkonda RV, Gupta D, Lin AS, et al. Sustained VEGF delivery via PLGA nanoparticles promotes vascular growth. Am J Physiol Heart Circ Physiol. 2010;298: H1959–H1965.

Iqbal M, Zafar N, Fessi H, Elaissari A. Double emulsion solvent evaporation techniques used for drug encapsulation. Int J Pharm. 2015;496(2):173-190.

Karagöz H, Ülkür E, Kerimoğlu O, Alarçin E, Şahin C, Akakın D, et al. Vascular endothelial growth factor loaded poly (lactic co glycolic acid) microspheres induced lateral axonal sprouting into the vein graft bridging two healthy nerves: Nerve graft prefabrication using controlled release system. Microsurgery. 2012;32(8):635-41.

Kaur M, Sharma S, Sinha VR. Polymer based microspheres of aceclofenac as sustained release parenterals for prolonged anti-inflammatory effect. Mater Sci Eng C. 2017;72:492-500.

Korsmeyer RW, Peppas NA. Effect of the morphology of hydrophilic polymeric matrices on the diffusion and release of water soluble drugs. J Membr Sci. 1981;9(3):211-227.

Kumar A, Sawant K. Encapsulation of exemestane in polycaprolactone nanoparticles: optimization, characterization, and release kinetics. Cancer Nano. 2013;4(4-5):57-71.

Labet M, Thielemans W. Synthesis of polycaprolactone: a review. Chem Soc Rev. 2009;38(12):3484-3504.

Miladi K, Sfar S, Fessi H, Elaissari A. Nanoprecipitaion process: From particle preparation to in vivo applications. In: Vauthier C, Ponchel G, editors. Polymer Nanoparticles for Nanomedicines: A Guide for their Design, Preparation and Development. Switzerland: Springer; 2016, p. 17-53.

Nair LS, Laurencin CT. Biodegradable polymers as biomaterials. Progr Polym Sci. 2007;32(8-9):762–98.

Peppas NA. Analysis of Fickian and non-Fickian drug release from polymers. Pharm Acta Helv. 1985;60(4):110-111.

Ribeiro AF, Rezende R, Cabral LM, Sousa VP. Poly ε-caprolactone nanoparticles loaded with Uncaria tomentosa

extract: preparation, characterization, and optimization using the Box–Behnken design. Int J Nanomed. 2013;8:431-442.

Rui J, Dadsetan M, Runge MB, Spinner RJ, Yaszemski MJ, Windebank AJ, et al. Controlled release of vascular endothelial growth factor using poly-lactic-co-glycolic acid microspheres: In vitro characterization and application in polycaprolactone fumarate nerve conduits. Acta Biomater. 2012;8(2):511-518.

Sato K, Wu T, Laham RJ, Johnson RB, Douglas P, Li J, et al. Efficacy of intracoronary or intravenous VEGF165 in a pig model of chronic myocardial ischemia. J Am Coll Cardiol. 2001;37(2):616-623.

Shibuya M. Vascular endothelial growth factor (VEGF)-receptor2: Its biological functions, major signaling pathway, and specific ligand VEGF-E. Endothelium. 2006;13(2):63-69.

Singh J, Pandit S, Bramwell VW, Alpar HO. Diphtheria toxoid loaded poly- (ε- caprolactone) nanoparticles as mucosal vaccine delivery systems. Methods. 2006;38(2):96-105.

Sinha VR, Bansal K, Kaushik R, Kumria R, Trehan A. Poly-ε-caprolactone microspheres and nanospheres: an overview. Int J Pharm. 2004;278(1):1-23.

Sipahigil O, Alarçin E, Türkoğlu M, Dortunç B, Karagöz H, Ülkür E, et al. Characterization, cell proliferation and cytotoxicity evaluation of vascular endothelial growth factor loaded poly (lactic-co-glycolic acid) microspheres. Nobel Medicus. 2012;8(1):77-82.

Tabernero J. The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents. Mol Cancer Res. 2007;5(3):203-220.

Van de Weert M, Hoechstetter J, Hennink WE, Crommelin DJ. The effect of a water/organic solvent interface on the structural stability of lysozyme. J Controlled Release. 2000;68(3):351-9.

Verheyen A, Peeraer E, Lambrechts D, Poesen K, Carmeliet P, Shibuya M, et al. Therapeutic potential of VEGF and VEGF-derived peptide in peripheral neuropathies. Neuroscience. 2013;244:77-89.

Wisniewska M, Ostolska I, Szewczuk-Karpisz K, Chibowski S, Terpilowski K, Gun'ko VM, et al. Investigation of the polyvinyl alcohol stabilization mechanism and adsorption properties on the surface of ternary mixed nanooxide AST 50 (Al2O3-SiO2-TiO2). J Nanopart Res. 2015;17(1):12.

Yeo Y, Park K. Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch Pharm Res. 2004;27(1):1-12.

Ylä-Herttuala S, Rissanen TT, Vajanto I, Hartikainen J. Vascular endothelial growth factors: Biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol. 2007;49(10):1015-26.

Zhou M, Shen L, Lin X, Hong Y and Feng Y. Design and pharmaceutical applications of porous particles. RSC Adv. 2017;7:39490–39501.

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