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Montelukast Incorporated Poly(methyl vinyl ether-co-maleic acid)/ Poly(lactic-co-glycolic acid) Electrospun Nanofibers for Wound Dressing

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Abstract: The aim of the present study was to prepare nanofibers loaded with montelukast, a cysteinyl leukotrienes (CysLTs) inhibitor, with anti-inflammatory properties effective on wound healing. Polymeric nanofibers containing montelukast were spun by electrospinning method using different ratios of the blend of two biodegradable polymers of poly(methyl vinyl ether-co-maleic acid) (PMVEMA) and poly(lactic-co-glycolic acid) (PLGA) at the total polymer concentration of 37 %, the distance of the needle to rotating screen of 19 cm, the voltage of 12 Kv and the rate of injection of 0.2 ml/h. The ratio of two polymers in the blend and the concentration of montelukast were optimized based on the diameter of the nanofibers, drug loading percent and release efficiency by a full factorial design. The morphology, diameter and diameter distribution of the nanofibers were studied by scanning electron microscopy (SEM). Drug loading percent in the nanofibers was determined by extracting the loaded drug from a specific surface of the nanofibers which was subsequently analyzed spectrophotometrically. The drug release rate from the nanofibers was studied in phosphate buffer solution (pH 7.4) containing 0.5 % Tween 20 at predetermined time intervals until 10 days. The cytotoxicity of the designed nanofibers was evaluated on mouse fibroblast cells using trypan blue method, their platelet adherence property was quantified by measuring the lactate dehydrogenase (LDH) activity and confirmed by SEM micrographs. The optimized ratio of PLGA/PMVEMA was 3:1 with the total concentration of polymers as 37 % loaded with 30 % of montelukast produced nanofibers with a diameter of 157.6 nm, drug loading percent of 43.7 % and release efficiency of 75 % after 10 days. The cell viability was similar in nanofibers and the negative control group. The platelets adhesion to the nanofibers was more than the negative control group ($p<0.05$).

Keywords: Montelukast, Nanofibers, Poly(methyl vinyl ether-co-maleic acid), Wound dressing, PLGA

Introduction

In the past decade, attention to nanofibers for biomedical applications such as wound cover, tissue engineering, and controlled release of drugs has increased. Very thin diameter and porous structure of nanofibers helps the drug to have a more effective exit in the nanofibers than in films. Electrospinning is the most famous and low cost method to fabricate polymeric nanofibers [1]. The process of using electrostatic forces to form synthetic fibers has been known for over 100 years. In the electrospinning process, a high voltage is used to create an electric jet of a polymeric or melting solution that needs to be dried to produce a polymeric fiber. An electrode is coupled to the polymer solution and the other to a collector. The electric field is located at the end of the capillary tube, which contains a polymeric liquid maintained by a surface tension, and produces a charge at the solution surface. An alternating charge burst causes a force directly opposite to the surface tension [2]. Despite the relative ease of electrospinning, a number of processing parameters can greatly affect fiber formation and structure. These parameters include polymer concentration, applied voltage, polymer flow rate, and needle distance to the collector plate. In addition, all four parameters can affect the formation of beads [2].

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Biodegradable polymers are mostly used in biomedical applications by elecreospinning method. Poly(lactic-co-glycolic acid) is one of the most widely used biodegradable polymers used in slow-release drug delivery systems [3]. The reason for this wide use is that PLGA is hydrolyzed to two biocompatible monomers of lactic acid and glycolic acid which are easily cleared from the body [4].

Poly(methyl vinyl ether-maleic acid) (PMVEMA) is an acidic polymer, water soluble, bioadhesive, biodegradable polymer used for drug delivery [5] and widely applied for pharmaceutical purposes due to its low toxicity [6]. Conjugation of this polymer to PEG and antisense has successfully been used to treat inflammation and tumors in mouse models [5, 7]. Moreover, it is used in encapsulation of calcitonin [8], but there is no report of its use in electrospinning.

One of the most popular fields of local and transdermal applications of nanofibers is their use in chronic and acute wound dressings, acne, infections, skin cancers, inflammatory diseases like psoriasis, cosmetics and ophthalmology. Electrospun nanofibers have been used to deliver both hydrophobic and hydrophilic drugs with controlled release capability for a prolonged period of time [9].

Leukotrienes (LT) are powerful inflammatory mediators that are made from arachidonic acid. First, unstable leukotriene A4 is built, then either during hydrolysis turns to leukotriene B4 or combines with glutathione and builds cysteine leukotrienes including leukotriene C4, D4 and E4. Leukotrienes

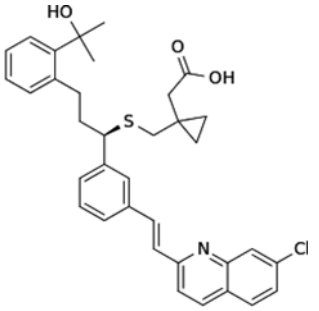

Figure 1. Chemical structure of montelukast.

apply their effect through four receptors including BLT1 and BLT2 for LTB4, and CysLT1 and CysLT2 for CysLTs. Montelukast is a selective inhibitor drug for CysLT1. CysLTs increase the expression of monocyte chemoatractant protein 1 (MCP1) in human macrophages and monocytes by binding to CysLT1 receptor. MCP1 is a strong chemotactic factor for macrophages and monocytes.

Montelukast shows its anti-inflammatory effect by reducing MCP1. During several animal studies on inflammatory diseases, montelukast has inhibited inflammatory cell recall, preserved oxidant-antioxidant balance, and decreased levels of circulating inflammatory mediators including LTB4, TNF-a, IL-1 and IL-6 [10]. In a study conducted by Turtay *et al*. [11] the effectiveness of montelukast was shown on local burn wound healing in rats. The authors showed that the combination of montelukast and bacitracin neomycin sulphate significantly increased angiogenesis and fibroblast proliferation while macrophage infiltration and edema decreased statistically in comparison with the control group ($p<0.05$). Peng *et al*. [12] also investigated the effectivness of montelukast on the contractility of human Tenon fibroblasts to treat the scar formation after surgery of glaucoma. Their results showed that montelukast could be a possible therapeutic agent for inhibition of scar formation after glucoma surgery due to reduction of the expression of MMP-1 and MMP-3 caused by TGF-β1, generation of fibronectin, collagen production, and focal adhesion kinase. Celik *et al*. [13] showed that this drug is effective in wound healing of anastomotic ischemia injury in a reperfused colon rat model by increasing the mean tissue hydroxyproline and decreased tissue caspase-3 activity and malondialdehyde levels without changing the anastomosis bursting pressure and reversal of the oxidative damage [14]. Investigations of Cakıcı *et al*. [15] showed the effectiveness of montelukast on fracture healing in a rat tibia fracture model by enhanced endochondral bone formation during healing of fractures and tissue repair [16]. Among the other reports on the wound healing effects of montelukast is its inhibitory effect on the the wound contraction process and inhibitory effect on collagen maturation in rats [17].

Wound healing is a native process of regenerating dermal and epidermal tissues in which the skin repairs itself after injury. In normal skin, the epidermis and dermis exist in steady state equilibrium, forming a protective barrier against the external environment. Once the protective barrier is broken, a complex biochemical process takes place to repair the damage. Wound healing consists of three phases: inflammation, proliferation, and tissue remodeling. In the inflammatory stage blood vessels are injured, followed by coagulation, and an acute local inflammatory response, with infiltration of leukocytes, macrophages, monocytes and subsequent secretion of inflammatory cytokines and growth factor. In the proliferation phase, the cytokines and factors released in the inflammatory stage stimulate the proliferation of progenitor cells, the recruitment of fibroblasts, keratinocytes, and endothelial cells, and finally cell migration and proliferation. At the proliferative stage, granulation tissue is formed, angiogenesis induced and extracellular matrix secreted. Then a remodeling phase characterized by the epithelial-mesenchymal transition happens where cells migrate to re-epithelialize the damaged tissue in the edges of the wound [18].

Considering the promising reports on the wound healing effects of montelukast, the aim of the present study was production of montelukast loaded polymeric nanofibers of a combination of PLGA and PMVEMA, a novel biodegradable polymer which itself has the anti-inflammatory and bioadhesive effects. This combination of polymers which are both biodegradable and biocompatible has not been used so far for electrospinning of nanofibers specially as the wound dressing with hemostatic and anti-inflammatory effects.

Experimental

Materials

PMVEMA, Triton X-100 and dimethylformamide (DMF) were purchased from Sigma Company (US) and PLGA 50:50 (Resomer® RG 502) from Boehringer Ingelheim (Germany). Fetal bovine serum (FBS) was from GIBCO Laboratories and montelukast was provided from MOrepen laboratory (New Delhi, India). Deionized water freshly purged with nitrogen gas was used in all steps for preparing all aqueous solutions. Mouse fibroblast cells (L929) were supplied by Pasture institute (Iran). Dulbecco's phosphate buffered saline (PBS) from BioIdea (Iran) and 0.25 % trypsin from Sigma (USA). DMEM medium was from GIBCO Laboratories (Scotland). NADH, and pyruvate were provided by Boehringer Mannheim (Germany).

Electrospinning of Nanofibers

In our preliminary studies to optimize the processing parameters of electrospinning of PMVEMA nanofibers, a Taguchi design was used by Design expert software (version 7.2, USA) [19]. At first different conditions of electrospinning of PMVEMA alone were examined to get the best conditions for obtaining nanofibers with the least beads and diameter.

For this purpose only the polymer of PMVEMA was dissolved in DMF at a concentration range of 35-39 w/v%. Solution injection speed was set between 1-0.2 m*l*/h, the voltage applied was 12-16 kV and the needle distance to the rotating surface was 15-23 cm. The polymer solution was stirred for 20 hours on the stirrer at 500 rpm. The final transparent solution was transferred to 1 m*l* syringe attached to a 22 gauge needle [19]. The final fibers were collected on an aluminum surface. All procedures were performed at 25 °C. After optimization of the processing parameters of electrospinning, the optimized nanofibers were prepared with 37 % of PMVEMA containing 20 % of motelukast and the drug release test was carried out as the method mentioned in in vitro drug release studies section. However, quick release of the drug led us to incorporate a more hydrophobic polymer like PLGA with the first polymer. So in the second step, optimization of the formulation of the nanofibers was done by a 3 level full factorial design using mixed electrospinning solutions with a total concentration of 37 w/v% of PLGA/PMVEMA mixture in the ratios of 25:75, 50:50, and 75:25 dissolved in DMF. Montelukast was used in the ratio of 10-30 w/w% compared to the polymers weight. For preparation of the electrospinning solution, the mixture of PLGA/PMVEMA in different ratios as mentioned in Table 1 with the total concentration of 37 % were dissolved in DMF. Then the drug (10-30 % of the polymer weight) was dissolved in the polymers solution according to Table 1. The viscosity of the solutions were determined by BROOKFIELD DV-II+- PRO Rheometer (USA) at 25 °C and 5 rpm. Different studied formulations are seen in Table 1.

Morphological Studies of Nanofibers

Nanofibers were coated with gold under vacuum in an argon atmosphere and their morphology was studied using a scanning electron microscope (LEO 440i, UK) with magnification of ×600-1000k. The average fiber diameter of each sample was measured in the resulting image. In each image, at least 100 different points were randomly selected and their diameter was measured by Digimizer software [20].

FTIR

The FT-IR spectra of selected samples were obtained using the FTIR device (Perkin Elmer, Waltham, MA). Samples were prepared by potassium bromide (KBr) and scanned against a blank KBr disk at wave numbers ranging from 4000 to 450 cm^{-1} with resolution of 1.0 cm^{-1}.

Drug Entrapment Determination

Drug loading percent was identified by dissolving a sample piece (2.5×2.5 cm) of electrospun nanofibrous mat in DMF and measuring the absorbance at λ_{max}=284 nm spectrophotometrically (UV-mini 1240, Shimadzu, Kyoto, Japan). The concentration of montelukast in the solution was obtained by using montelukast calibration curve in DMF. The drug loading percent was calculated as follows:

Drug entrapment % = (weight of drug/the weight of the drug loaded web) × 100

***In vitro* Drug Release Studies**

The mat of nanofibers were cut into pices of 2.5×2.5 cm and drug release was measured by immersing a certain weight of the mat in 10 m*l* of PBS (at 7.4 pH) containing 0.5 % Tween 20 under constant stirring at 300 rpm at 37 °C. At particular time intervals, a 50 μ*l* sample of the release media was removed and the concentration of the drug was measured using UV spectrophotometer at λ_{max}=237.5 nm. After each sampling the same amount of fresh buffer was replaced in the release media. Each test was repeated in triplicate and the mean values were reported.

The drug release data were evaluated by various kinetic models including Higuchi ($Q_t=k_H t^{1/2}$), first order ($\ln Q_t= \ln Q_0+k_1 t$) and zero order ($Q_t=Q_0+k_0 t$). Where Q_0 is initial amount of drug in nanofibers, Q_t is amount of drug released at time t, t is sampling time, k is release constant. The best model was selected based on the highest correlation coefficient (R^2).

Cytotoxicity Effects of Nanofibers

For this purpose, mouse fibroblast cells (L929) were used. The cells were cultivated in 6 plates at DMEM medium containing 20 % fetal calf serum (FCS), 200 U/m*l* penicillin and 200 μg/m*l* streptomycin. Before cells seeding, nanofibers were sterilized by UV radiation for 24 h. After the number of cells in each well reached 50,000, the pieces (0.5×0.5 cm^2) of the optimized formulation of nanofibers were placed adjacent to the cells and after 48 h. Negative control groups were cells grown in the culture medium without any nanofibers. After completion the incubation period the cells were counted by trypan blue method [21]. For this purpose 50 μ*l* of the culture medium was mixed with 50 μ*l* of 0.4 % Trypan blue and stirred gently with a pipette, then 20 μ*l* of the mixture was loaded into each chamber of the hemocytometer. Counting was done three times by an analyst according to the standard procedures [22].

Platelet Adhesion Test

This study protocol was approved by the ethics review committee of the Isfahan University of Medical Sciences. Platelet adherence to the nanofibers was quantified by an spectrophotometric measurement based on kinetic determination of lactate dehydrogenase (LDH) activity. For this purpose platelet preparation of human blood (50 m*l*) was collected in a polypropylene tube containing anticoagulant solution (5 units/m*l* of heparin) and centrifuged at 1000 rpm for 15 min to obtain platelet-rich plasma (PRP). The PRP was centrifuged at 3000 rpm for 10 min to yield platelet-poor

plasma (PPP) and a pellet. The pellet was washed in 15 m*l* of Tyrode's buffer and additionally centrifuged at 3000 rpm for 10 min, followed by suspending in 5 m*l* of phosphate buffered saline (PBS). Finally, the concentration of platelets in PBS was adjusted to 5.3×10^7 cells /m*l* by dilution. One m*l* of this platelet suspension was placed on the nanofibers mat of 1×1 cm^2 in a 24 well multidish made of polystyrene (Corning, NY, USA) and kept for 1 h at 37 °C under a static condition. After incubation, the mat was taken out and dip-rinsed twice with PBS in order to remove the platelets which were not attached to the mat surface. At this stage the SEM micrographs were taken from the nanofibers. The surface morphology of the nanofiber mats were analyzed using SEM, after sputter coating with gold for 5 min. The acceleration voltage of 15 kV was used in SEM. After washing, a lysis buffer was added to the mat for subsequent determination of the adhered platelets on the same sample by the LDH method. After thorough washing, adherent platelets were lysed by the addition of 200 μ*l* of 0.2 % Triton X-100 to the well. After 1 h incubation at room temperature, 150 μ*l* of each lysate was collected and mixed with 2.5 m*l* of 0.24 mM NADH, and 0.5 m*l* of 9.76 mM pyruvate. The LDH activity in the lysate was then determined by recording the change in absorbance at 340 nm. A calibration curve was used to relate platelet number (after lysing washed bulk platelets) with LDH activity (A340 nm/min) [23].

Three groups were tested including plasma as the negative control (or blank), montelukast nanofibers, and blank nanofibers.

Results and Discussion

Effect of Formulation Variables on the Properties of PLGA/PMVEMA Nanofibers

Although PLGA has previously been used for electrospinning, but PMVEMA polymer is electrospun for the first time in this study. DMF and other solvents such as hexafloro 2-propanol, have been used as the solvent for electrospinning of PLGA nanofibers [3,24]. DMF is a solvent with high dielectric constant (ε=36.7) and has highly been regarded as a suitable solvent for electrospinning process [25]. In the present study this solvent was used as it could dissolve all three components i.e., PLGA, PMVEMA, and montelukast.

At first, electrospinning was done using only PMVEMA. In our preliminary study different processing conditions were studied and their impacts on the diameter of the nanofibers of this polymer were obtained [19]. The diameter size of nanofibers of just PMVEMA varied between 400-1000 nm and by using the Design Expert Software optimization, the best conditions for electrospinning of PMVEMA nanofibers were peredicted to be at the concentration of 37 %, distance of 19 cm, voltage of 12 kV, and injection rate of 0.2 m*l*/h [19]. After optimization of the processing variables of electrospinning of PMVEMA nanofibers the optimized nanofibers were loaded with 20 % of motelukast and drug release profile was studied. The results showed that the drug was released in less than 5 seconds. Therefore, to control the drug release rate the combination of PLGA:PMVEMA polymers were electrospun with montelukast, and the effects of different ratios of the two polymers and different concentrations of the drug on nanofibers size, drug loading, and drug release efficiency were investigated so that, the best conditions for electrospinning of this combination could be found.

Table 1 shows the physicochemical properties of the nanofibers electrospun from a mixture of PLGA/PMVEMA with different ratio and drug content.

Figure 2(a) shows the effect of the PMVEMA ratio on the viscosity of the polymeric solution. As this figure shows reduction of PMVEMA in the mixture of the polymers reduces the viscosity. Figure 2(b) also shows the effect of increasing the drug concentration on the polymer viscosity. As this figure shows by increasing the drug concentration the viscosity of the polymer solution increased too (Figure 2(b)).

Figure 3(a) shows that by increasing the concentrations of PLGA the diameter of nanofibers reduced. Figures 3(b) and

Table 1. Physicochemical properties of montelukast loaded nanofibers prepared with different concentrations of drug (according to the total polymer weight) and different ratios of PLGA/PMVEMA (total concentration of polymers is 37 %)

Formulation code	PLGA/PMVEMA ratio	Drug concentration (%)	Diameter size (nm)	Drug loading %±SD	RE_{10}% ±SD
$L_{25}M_{75}D_{30}$	25:75	30	262.5	30.0±3.0	63.7±0.7
$L_{50}M_{50}D_{20}$	50:50	20	119.8	6.8±0.3	37.1±1.0
$L_{50}M_{50}D_{30}$	50:50	30	174.3	18.0±1.0	45.0±3.0
$L_{75}M_{25}D_{20}$	75:25	20	101.1	3.4±0.1	74.5±1.5
$L_{25}M_{75}D_{10}$	25:75	10	185.4	9.4±0.1	41.6±2.0
$L_{25}M_{75}D_{20}$	25:75	20	283.0	18.3±0.3	67.5±0.8
$L_{50}M_{50}D_{10}$	50:50	10	137.8	4.2±0.4	47.4±1.2
$L_{75}M_{25}D_{30}$	75:25	30	157.6	43.7±1.0	75.1±2.0
$L_{75}M_{25}D_{10}$	75:25	10	128.3	31.5±1.5	37.8±3.4

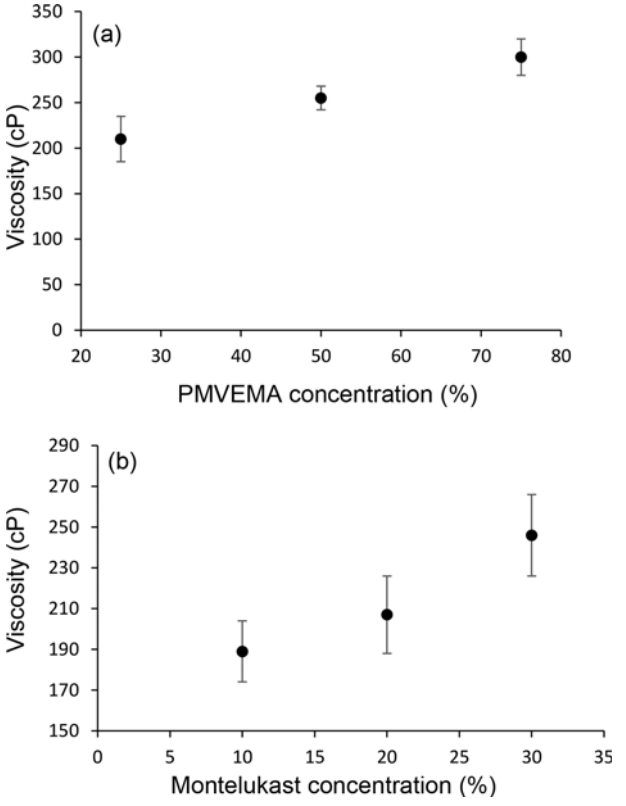

Figure 2. The effect of (a) PMVEMA concentration in the polymer mixture of PMVEMA/PLGA and (b) drug concentration in the polymer mixture of PLGA/PMVEMA (total polymer concentration 37 w/w%) on the viscosity of the electrospinning solution.

3(c) show that when the percentage of PLGA in mixture of polymers increased, at first the drug loading and also the drug release efficiency decreased but after that when the ratio of PLGA increased to more than 50 % of the mixture both these responses increased. This means that in very low or very high concentrations of PLGA, drug-loading percent is higher.

Our previous study showed that when electrospinning was done just by PMVEMA the diameter of the nanofibers was 400 nm [19] while, as the results of Table 1 show the diameter of the nanofibers obtained from the blend of the polymers changed between 101.1-283.0 nm. Also, it is evident that generally by addition of PLGA to PMVEMA, the nanofibers diameter reduced, which may be due to the reduction of the viscosity of the polymeric solution by reduction of the PMVEMA ratio and substitution of some part of it by PLGA (Figure 2(a)). Therefore, by decreasing the viscosity of the electrospinning solution, the fluid jet is stretched and elongated more easily. As a result, the fibers diameter is decreased.

This shows that by incorporation of another polymer in the electospinning solution the changes in viscosity is too important on the diameter of the obtained nanofibers. In the

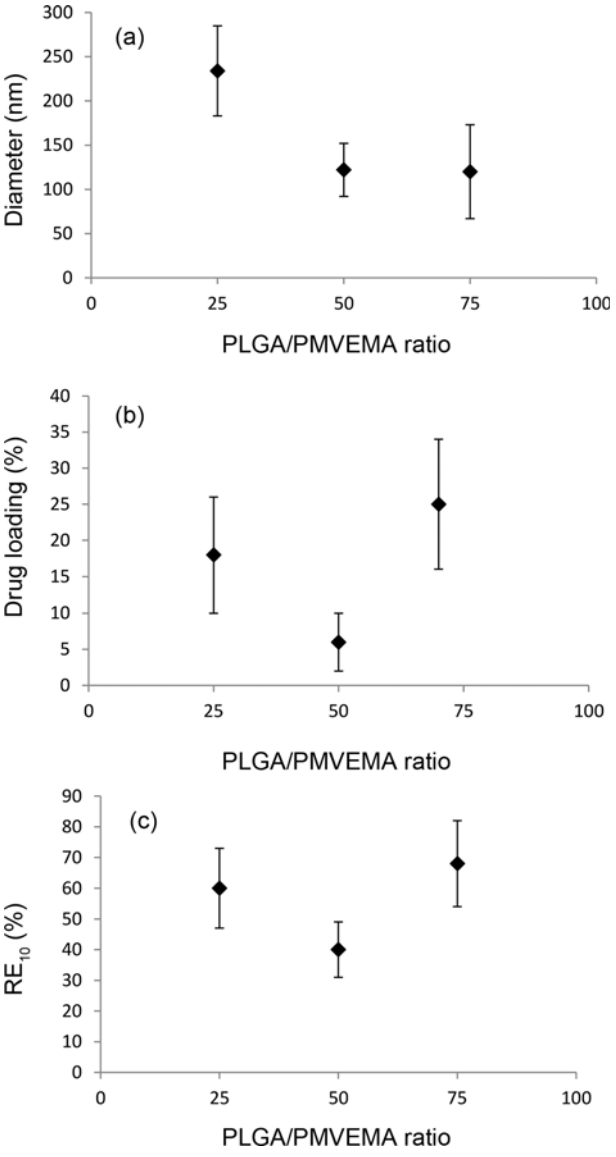

Figure 3. The effect of PLGA/PMVEMA ratio on (a) the average diameter of nanofibers, (b) montelukast loading percent, and (c) release efficiency percent (RE%) of the drug from nanofibers.

present study also by incorporation of the PLGA in the PMVEMA solution the viscosity of the PMVEMA solution decreased (Figure 2(a)) and therefore, the diameter of nanofibers was reduced too (Table 1). This is in accordance with the results of Meng *et al*. [26] who studied the PLGA/ gelatin nanofibers for drug delivery of fenbufen. When they used only PLGA the nanofibers size was 342 nm but when a mixture of PLGA and gelatin with ratio of 9:1 was used the average diameter of nanofibers reduced to 313 nm. In fact, in their study by addition of gelatin, the viscosity of the PLGA solution was reduced and the fibers diameter reduced too. In another study, Meng *et al*. [27] used PLGA in combination to chitosan for electrospinning. They showed

that by increasing chitosan ratio from 0:10 to 0.625:9.375, 1:9 and 3:7, the nanofibers size became 146, 189, 246, and 229 nm, respectively. In fact, as the percentage of PLGA was reduced, the nanofibers size increased, because by increasing the ratio of chitosan the viscosity of the solution increased and therefore, the diameter size increased too. Therefore, the viscosity of the electrospinning solution determines the diameter of the nanofibers respective to the type of the polymers mixture.

The possible reason for low drug loading in some formulations which are mainly those containing 10 or 20 percent of the drug may be the lower viscosity of the electrospinning solution compared to those containing 30 % of the drug (Figure 2(b)). In the fibers containing 30 % of the drug the viscosity was higher and caused electrospinning of better fibers with higher drug loading.

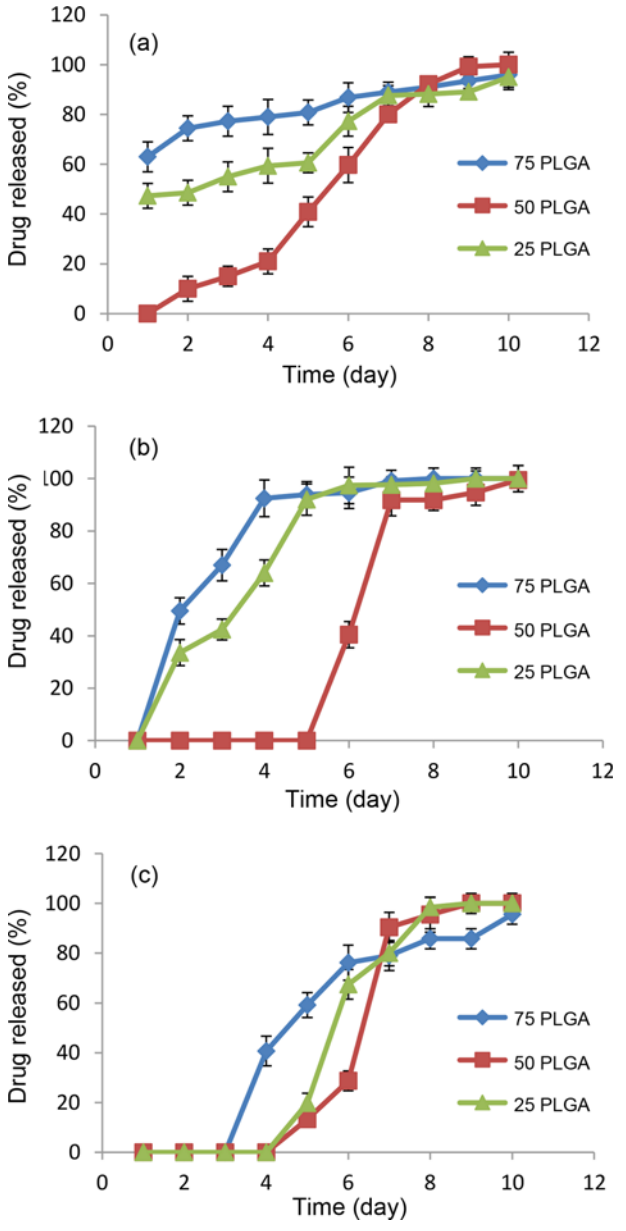

Figure 4. Drug release profiles from nanofibers of PLGA/PMVEMA with different contents of PLGA and (a) 30 %, (b) 20 %, and (c) 10 % of montelukast.

Figure 4 shows the montelukast release profiles from nanofibers of PLGA/PMVEMA with different contents of PLGA and loaded with 10-30 % of montelukast. As this figure indicates the nanofibers electrospun with more drug concentration, show enhanced drug release rate. In the first step a slow release rate which lasted for about 4 hours was seen (Figures 4(b), (c)) (except for the fibers loaded with 30 % of montelukast in which this phase lasted just for 1 hour). Then a fast drug release phase was seen which lasted for about 6 hours and nearly montelukast release reached to 100 %. After this stage drug release reached to a plateau and almost remained constant. The slow stage may be related to the period necessary for wetting of the nanofibers and releasing the drugs which are nearer to the surface but after complete hydration of the fibers the drug diffusion outward got faster until became constant (Figure 4). Overall, the nanofibers loaded with higher percentages of drug (30 %) showed faster drug release (Figure 4(a)) and also the increament of the PLGA ratio to 75 % caused enhanced drug release rate possibly due to the diameter reduction of the nanofibers (Figure 3(a)) which caused greater surface area and more contact with the release medium which in turn caused faster drug release. This difference between nanofibers with different PLGA content was seen in the first 6 hours and after that the drug release rate was similar in all nanofiber and almost reached to 90-100 %. Although the nanofibers with 25 % PLGA were thicker than those with 50 % of PLGA (Figure 3(a)) but had faster drug release rate. To justify the cause of this phenomenon, it can be said that drug release is dependent on two factors: 1) the fiber diameter and 2) the surface hydrophobicity. The balance of these two factors controls the speed of drug release. The fibers containing 25 % of PLGA, had a lower hydrophobicity than those containing 50 % of PLGA, but meanwhile they had a larger diameter (Figure 3(a)). In this case the drug release rate is more affected by the surface hydrophilicity of the fibers with 25 % of PLGA, due to their more pronounced release rate. But in the fibers containing 75 % of PLGA the diameter of the fibers is so diminished (Figure 3(a)) which, masks the effect of surface hydrophobicity and far outstrips than the drug release rate of the fibers with 50 % of PLGA.

The drug release data of the optimized formulation ($L_{75}M_{25}D_{30}$) were fitted with three kinetic models including zero-order (R^2=0.9413), first order (R^2=0.6626) and Higuchi models (R^2=0.97). The best curve fitting with the highest correlation coefficient was obtained for the Higuchi model indicating release of montelukast from nanoparticles is mainly mediated through diffusion mechanism.

Figure 5(a) shows that by increasing in drug concentration,

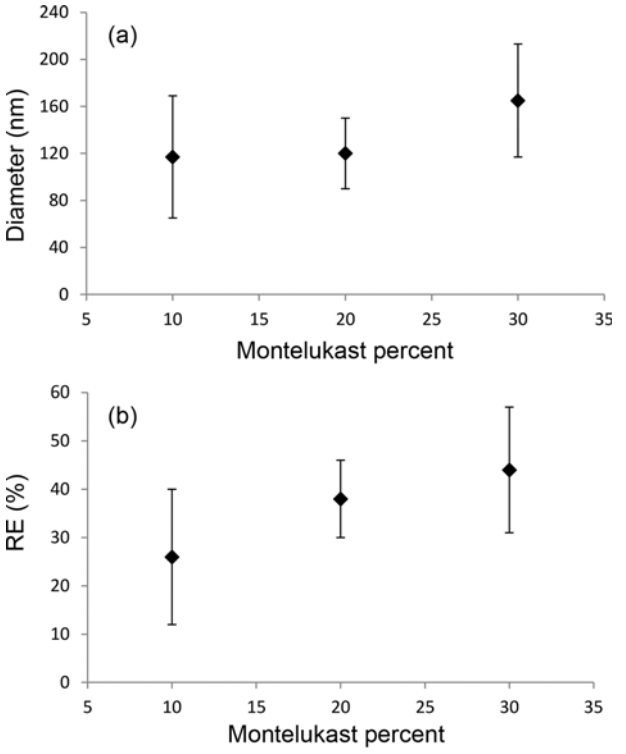

Figure 5. The effect of montelukast concentration on (a) the average diameter of PLGA/PMVEMA nanofibers and (b) release efficiency percent (RE_{10}%) of the drug from nanofibers.

the nanofibers average diameter increased. In fact, increasing the drug concentration led to enhanced viscosity and stickiness of the electrospun solution and consequently thicker nanofibers were achieved (Figure 2(b)). Also Figure 5(b) shows that by increasing the drug concentration, the drug release also increased. This may be due to the presence of more medication in the nanofibers available for release. Zamani *et al.* [20] also electrospun metronidazole nanofibers using polycaprolactone. They showed that by increasing the concentration of metronidazole from zero to 15 %, the nanofibers diameter reduced from 399 nm to 313 nm. In this study they concluded that by increasing the concentration of the payloaded drug the release rate was dramatically increased during the first 4 hours of release test. Jannesari *et al*. [1] electrospun ciprofloxacin nanofibers in polyvinyl alcohol/polyvinyl acetate. Their study also showed that with entry of 10 percent of ciprofloxacin, the average nanofibers diameter reduced from 448 to 405 nm. Moreover, they well showed that by changing the drug concentration in the nanofibers from 5 to 10 percent, the drug release rate almost doubled [2].

The Design Expert Software facilitates the finding of the effect of each variable on the studied response independent from other variables while the other parameters are changing too. It also allows the statistical analysis like many other soft wares and suggests the optimum conditions for electrospinning by predicting the studied responses of the fibers. Computer optimization process by Design Expert Software was carried out and a desirability function determined the effect of the levels of independent variables on the studied responses. The constraints of average diameter of nanofibers, drug loading percent and RE_{10}% were 101.1-283.0 nm, 3.4-43.6 % and 37.1-75.1 %, respectively. Considering the data of Table 1 optimization was carried out by Design Expert Software and the optimized nanofiber formulation suggested by desirability of 80 % was $L_{75}M_{25}D_{30}$ formulation which was prepared by using 37 % total concentration of polymers in a ratio of 75:25 of PLGA: PMVEMA and 30 percent of the drug according to the total polymers weight. This sample had an average diameter of 157.6 nm, loading percent of 43.7 % and release efficiency of 75 % (Table 1).

Figure 6 shows the morphological characteristics of all formulated nanofibers including the optimized nanofibers of $L_{75}M_{25}D_{30}$. As seen in this figure no beaded nanofibers and also no drug crystals were detected by electron microscopy neither on the surface nor outside the fibers loaded with the drug. Therefore, it can be concluded that montelukast was loaded in the nanofibers, demonstrating a good compatibility of drug-polymers-solvent. Figure 7 also shows the frequency distribution of nanofibers diameter in the optimized formulation. This figure indicates the most frequency of diameter in this formulation is between 100-150 nm with average diameter of 157.6 nm.

FT-IR Spectroscopy

FTIR spectra of free montelukast, PLGA, PMVEMA, montelukast loaded nanofibers of $L_{75}M_{25}D_{30}$ and balnk nanofibers of $L_{75}M_{25}$ are shown in Figure 8.

For free montelukast (Figure 8(a)) the tertiaryhydroxyl groups have exhibited a broad peak around 3300 cm^{-1} and a carboxylic acid peak which is in the form of a salt has exhibited a strong peak near 1600 cm^{-1}. Numbers of aromatic C-H peaks are also observed between 2900 cm^{-1} to 3000 cm^{-1}. For PLGA (Figure 8(b)) the absorption band at 3496 cm^{-1} indicates for hydroxyl group of PLGA, the peaks at 2997 cm^{-1} for stretching CH groups, at 1455 cm^{-1} for deformation of CH_3-CH groups and at 1135 cm^{-1} stands for C-O stretching band of ester groups of the polymer. In the spectrum of PMVEMA (Figure 8(c)); a broad absorption band at 3420 cm^{-1} corresponds to the hydroxyl group of this polymer, and the absorption band at 1716 cm^{-1} is assigned to its carbonyl group. In both drug loaded and blank nanofibers (Figures 8(d) and 8(e)) the characteristic peak of C=O which is seen at near 1700 cm^{-1} relates to the carboxylic acid groups of montelukast, PLGA, and PMVEMA but absence of hydroxyl group around 3300-3500 cm^{-1} which was seen in the spectra of these substances, probably indicates a strong hydrogen bonding between them in the nanofibers. Actually this test is done for finding the possible intraction between the drug and the polymers used in the nanofibers. The aformentioned

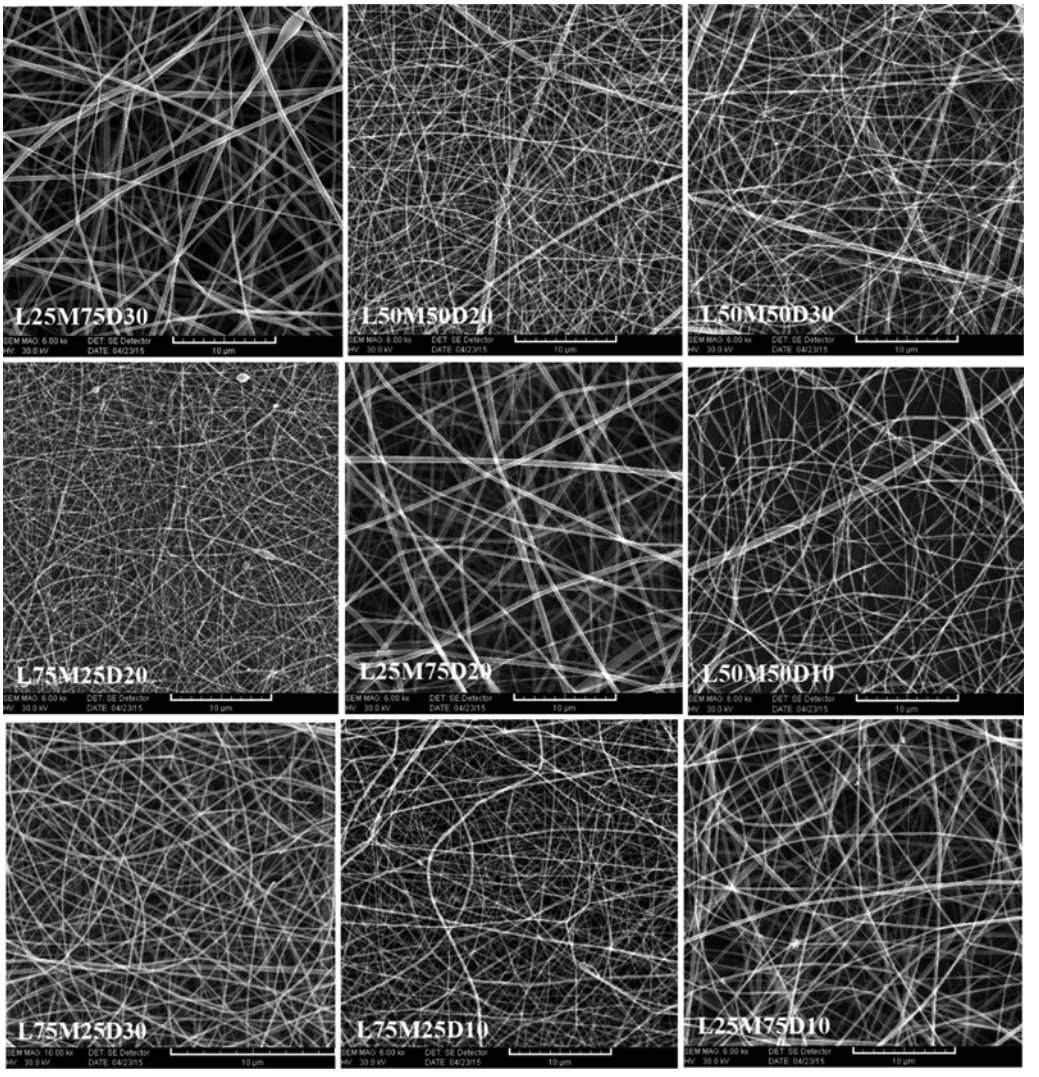

Figure 6. SEM of the nanofibers of PLGA/PMVEMA containing different amounts of motelukast and electrospun at the concentration of 37 % total polymers with different ratios of PLGA/PMVEMA at the distance of 19 cm, voltage of 12 kV, and injection rate of 0.2 ml/h.

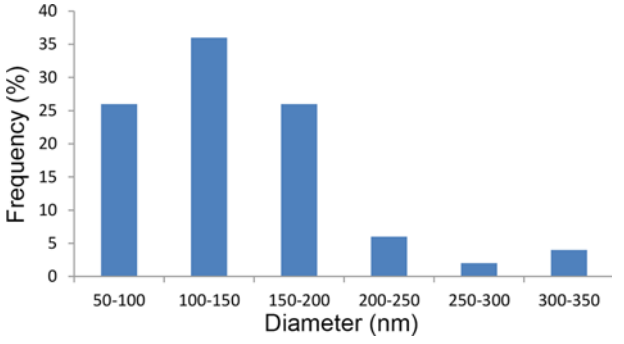

Figure 7. Frequency distribution of diameter in the optimized nanofibers of PLGA/PMVEMA containing 30 % of motelukast and electrospun at the concentration of 37 % total polymers with 75:25 ratio of PLGA/PMVEMA at the distance of 19 cm, voltage of 12 kV, and injection rate of 0.2 m//h.

results obtained indicate no chemical interaction between the drug and the polymers as there was no significant shifting in the functional groups of the drug except for the absence of the hydroxyl vibration peak which indicates establishing the hydrogen bonding between the drug and the polymers and complete incorporation of the drug molecules between the polymers chains [28].

Cytotoxicity Test of Nanofibers

Figure 9 shows the effect of PLGA/PMVEMA nanofibers on the proliferation of mouse fibroblast L929 cells. The results showed that the designed nanofibers had no cytotoxic effect on L929 cells, and the cell survival was not different from the control group ($p>0.05$) and no pronounced changes in cell growth was detected in response to the nanofibers (Figure 9).

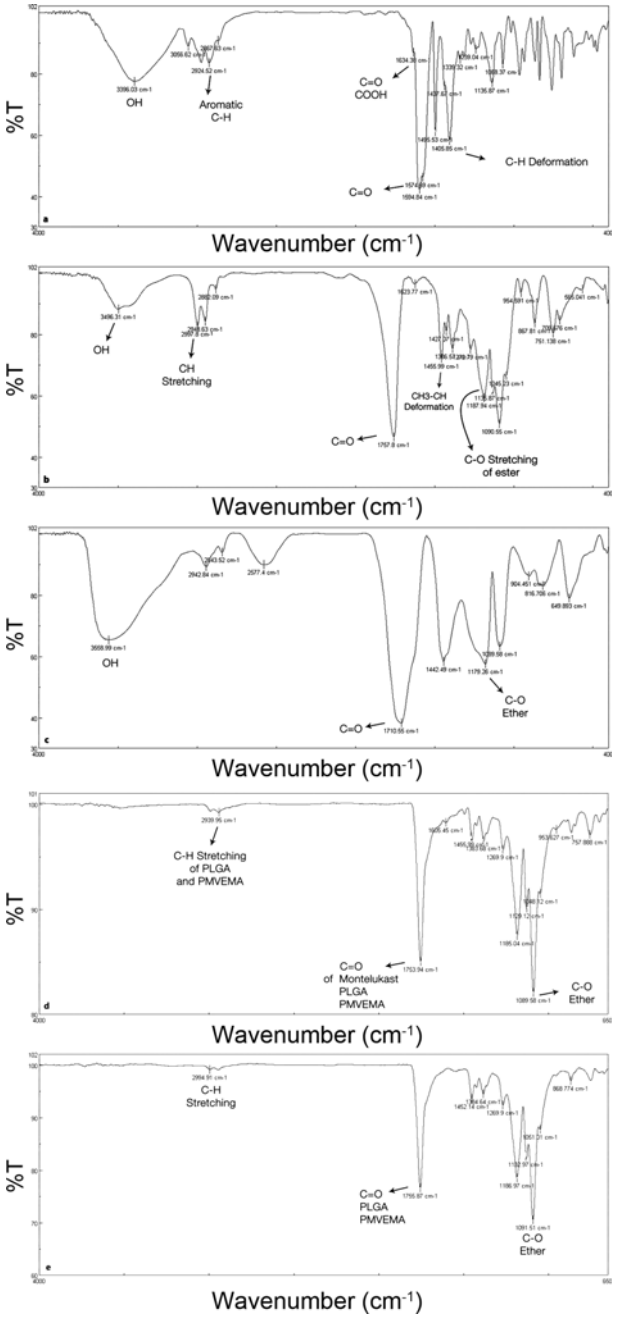

Figure 8. FTIR spectra of (a) free montelukast, (b) PLGA, (c) PMVEMA, (d) montelukast loaded nanofibers of $L_{75}M_{25}D_{30}$, and (e) balnk nanofibers of $L_{75}M_{25}$.

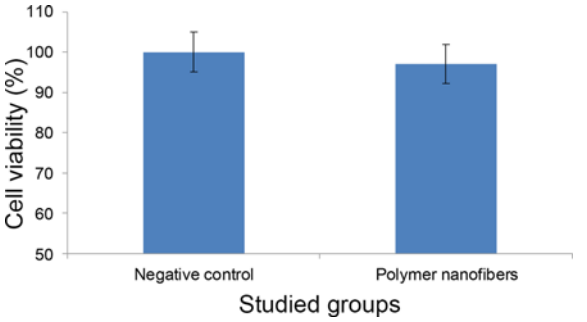

Figure 9. Cell cytotoxicity of PLGA/PMVEMA nanofibers on mouse fibroblast cells of L929 (The stars show significant difference with negative control group $p<0.05$).

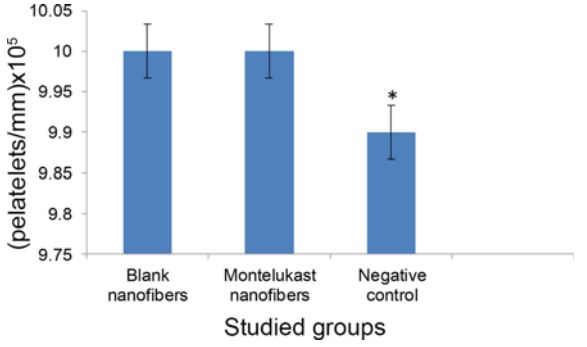

Figure 10. The number of adherent platelets on different sample surfaces obtained from the LDH assay (The stars show significant difference with negative control group $p<0.05$).

Platelet Adhesion Test

Wound healing, as a normal biological process in the human body, is achieved through four precisely and highly programmed phases: hemostasis, inflammation, proliferation, and remodeling. For a wound to heal successfully, all four phases must occur in the proper sequence and time frame. To prevent further bleeding in hemorragic wounds and in surgeries rapid heamostasis is too important. According to lactate dehydrogenase (LDH) activity method, when the cells attach to a surface, they will be lyzed and the LDH enzyme will be released which may be quantified by spectrophotometric method so that the number of lyzed cells are determined. Consequently, the higher the absorption of the sample in the spectrophotometer, the higher the number of attached cells [23,29]. To determine the platelet adhesion properties of the electrospun nanofibers, the LDH activity test was performed. In this test the adhesive properties of the $L_{75}M_{25}D_{30}$ nanofibers loaded with montelukast was compared with plasma as the negative control, and the blank nanofibers. The number of attached platelets for negative control were 9.9×10^5 cells/mm^2 and for blank polymer and montelukast loaded nanofibers it was 1×10^6 cells/mm^2. The results are shown in Figure 10.

As this figure shows the negative control showed significantly less adhesion of cells compared to the montelukast loaded nanofibers and the blank nanofibers ($p<0.05$). The results of the SEM micrographs also confirmed the results obtained by the LDH test. As can be seem in Figure 11 the platelets are attached to the surface of blank and drug loaded nanofibers which confirms the hemostatic effects caused by the PMVEMA polymer.

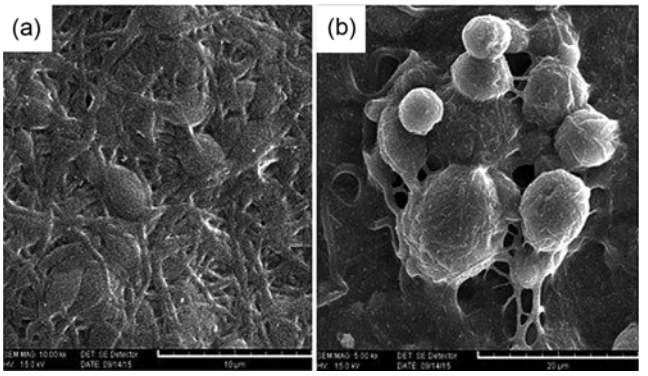

Figure 11. SEM images of platelets adhesion on the (a) blank and (b) montelukast loaded PLGA/PMVEMA nanofibers mat.

Conclusion

Nanofibers of PLGA/PMVEMA containing montelukast were successfully spun. By increase in concentration of PLGA, nanofibers size reduced, montelukast loading and release efficiency were reduced at first and then increased. Adding of montelukast had a similar effect on the above parameters. The effect of PLGA/PMVEMA on the L929 fibroblast cells showed that montelukast nanofibers had a similar effect with the negative control group on the proliferation of the cells and showed no cytotoxicity on these cells. These nanofibers also caused increased platelet adhesion properties compared to the negative control group. The results of this preliminary study show that the PLGA/PMVEMA nanofibers of montelukast can be a good candidate for preparation of nontoxic wound dressings with hemostatic effects. Further studies are going on the possibility of using the designed nanofibers as an effective scaffold for tissue engineering of wounds and the capability of growing the mesanchymal stem cells on this scaffold.

Acknowledgments

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