Original Research Article

Biological properties of Pegylated PLA (PLA-PEG-PLA) and its capability for intracellular delivery of poor soluble peptide drug, gramicidin

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A b s t r a c t

Poly lactic acid-co-poly ethylene glycol-co-Poly lactic acid (PLA-PEG-PLA) co-polymer was synthesized and after determination of its biological properties was used for intracellular delivery of poor soluble peptide drug, gramicidin. Toxicity of this polymer on LNCaP cell line was determined by MTT assay. Treatment of cells with fluorescein isothiocyanate (FITC) loaded nanoparticles and fluorescence microscopy indicates ability of this carrier for intracellular delivery. PLA-PEG-PLA nanoparticles containing poor soluble peptide drug, gramicidin, was prepared by solvent evaporation method. In order to confirm loading of gramicidin on PLA-PEG-PLA nanoparticles, FT-IR, spectrofluoremeteric, circular dichroism (CD), and scanning electron microscopy (SEM) studies were carried out. Our studies revealed treatment of prostate cancer cell line, LNCaP, with gramicidin loaded nanoparticles was more effective than gramicidin alone in killing cancer cells.

Keywords: PLA-PEG-PLA block co-polymer, hemolytic activity, biocompatibility, FITC, gramicidin, nanoparticle

Introduction

Recombinant DNA technology developments led to availability of many protein and peptide drugs and some of these agents can cause cancerous cells death [1] but, physicochemical properties of protein and peptide drugs such as molecular weight, solubility and stability differ from conventional drugs and restrict clinical use of them [2, 3]. Hydrophobic drugs have poor bioavailability and can precipitate in blood vessels which result in undesired side effects [4, 5]. Susceptibility of biomacromolecule drugs to enzymes which are present in the body, recognition of them as antigens and remove of them by reticulo-endothelial system and kidney influence bioavailability of drugs. Cell membrane Impermeability is another obstacle for use of protein and peptide drugs which have intracellular targets. At the other hand many drugs can act on normal and target cells which result in undesired side effects [2, 6].
In order to overcome limitations related to use of biomacromolecule drugs and to maximize therapeutic potential and minimize side effects use of polymeric nanocarriers as drug delivery systems is beneficial. Encapsulation of hydrophobic drugs in the core of micellar nanoparticles formed through the self-assembly of block copolymers in solution not only increase apparent water solubility of drugs [7] but also protect them from premature degradation and contact with biological environment before arriving to disease site. Furthermore, use of nanoparticles cause accumulation of drugs through passive targeting and EPR effect [8, 9].
Polyethylene glycol (PEG) modified nanoparticles are most popular stealth nanoparticles and can evade the recognition by reticuloendothelial system [10]. Nanoparticles prepared from polyethylene glycol-modified poly lactic acid (PEG-PLA) have good biodegradability and biocompatibility and are in phase II of clinical trials as carriers for paclitaxel [11].
In this study, PLA-PEG-PLA was synthesized and after determining biocompatibility of these polymeric nanoparticles on cancerous cell line, LNCaP, they were applied as carrier for a hydrophobic peptide, gramicidin, as drug. Gramicidin loaded nanoparticles were prepared by solvent evaporation method and cytotoxicity of gramicidin in loaded on nanoparticles and free manner was evaluated by MTT assay.

Materials and methods

Materials

L-lactic acid, toluene, ethanol, ethyl acetate, antimony trioxide (Sb_2O_3), dichloromethane (DCM), polyethylene glycol (PEG Mn = 2000), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

(MTT), dimethyl sulfoxide (DMSO), glutaraldehyde, acetone, $CuCL_2$, NaCl, KCl, KH_2PO_4, Na_2HPO_4, HCl, $MgCl_2$, NaOH, $NaHCO_3$ were purchased from Merck. Stannous octoate was purchased from Sigma-Aldrich. anilino naphthalene sulfonate (ANS), fluorescein isothiocyanate (FITC) and gramicidin were obtained from sigma and cell culture media RPMI1640, fetal bovine serum (FBS) and trypsin-EDTA from GIBCO, Invitrogen Corp (USA). Formaldehyde was purchased from Dr.mojallali chemical laboratories,(iran) and prostate cancer cell line, LNCaP, from Pasteur institute and PLA-PEG-PLA synthesized in our lab.

Biological properties

Cell culture and cytotoxicity

In order to polymer toxicity determination MTT assay was performed. Prostate cancer cell line LNCaP was grown under standard cell culture conditions in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. Seeded cells in 24 well plates were maintained in incubator (5% co_2, 37℃ and humidified atmosphere) for 24 h to reach 70-80% confluency. MTT assay was performed as reported [12]. Growth medium was replaced with new ones and nanoparticles were added at different concentrations. Plates were returned to incubator and after desired times of incubation 50μl MTT solution (5mg in 1ml PBS) was added to culture medium and incubated for further 3h. Then growth medium containing MTT was replaced with 1ml DMSO to dissolve formazan crystals and its absorption was determined in 570 nm. All experiments were set up in triplicates and relative growth inhibition compared to control cells was measured.

Fluorescence microscopy and up take by cells

Ability of polymeric nanoparticles in entrance to cells was examined by incorporating fluorescein isothiocyanate (FITC) into polymeric micelles. FITC loaded nanoparticles was prepared by direct dissolution method [13]. Briefly, FITC with appropriate amount of polymer dissolved in PBS and after sonication for 30 min the solution remained for further 30min at room temperature. Unloaded FITC was removed by centrifugation and washing two times in PBS. Cells were treated with FITC loaded nanoparticles for 2h and washed in PBS, fixed for 10min (2% formaldehyde/0.2% glutaraldehyde in PBS) and used for fluorescence microscopy.

Loading studies

FT-IR, Fluorescence, CD and SEM studies for Gramicidin loaded nanoparticle

FT-IR, Fluorescence, CD and SEM studies were performed to confirm loading of gramicidin on nanoparticles. Also loading efficiency and location of gramicidin on nanoparticles were studied by fluorescence spectroscopy. Gramicidin loaded nanoparticles were prepared using solvent evaporation method. Briefly 20μg gramicidin dissolved in 10 μl acetone and different concentrations (1, 2, 3, 4, 5, 6 mg/ml) of polymer (stock: 166.6 mg in 1ml PBS) were added, Sonicated for 30 min (bath sonicator), in order to micelle formation and acetone elimination the prepared solution was kept at room temperature for further 30 min. for FT-IR studies

this loaded nanoparticles (ratio of polymer/peptide: 4mg/20 μg) were dried at 40℃ under vacuum for 24 h and FT-IR spectrum obtained from dried loaded nanoparticles between KBr tablets with PerKin Elmer spectrometer. FT-IR spectrum of free gramicidin was recorded in crystalline form.

Samples for fluorescence studies were prepared as above except that after acetone elimination to decrease turbidity resulted from unloaded poor soluble peptide drug, gramicidin, 30 μl ethanol was added to samples and final volume of 0.5 ml for samples obtained by adding PBS. Also utilized concentrations of polymer were: 3, 5 and 6 mg/ml. The Cu^{2+} ions block the fluorescence of any dye molecules available in the aqueous solution, and hence any dye not encapsulated completely in the core is effectively removed from the fluorescence measurement [7]. To demonstrate gramicidin was contained completely within the core of micelles same amount of $CuCl_2$ (dissolved in PBS) was added for same time to each sample. Measurements were made by exciting tryptophan residues at 292 nm and emitted light detected from 300-500 nm (Cary Eclipse, Australia). Slit number for excitation and emission wavelength was 5 nm.

CD studies also carried out to demonstrate loading of gramicidin on polymeric nanoparticles. 50μg gramicidin dissolved in 25 μl acetone and formulated with 2.5 mg polymer as samples for fluorescence studies (except that no $CuCl_2$ was added). All CD measurements were obtained employing an Aviv 215 (Aviv Instrument, Inc., Lake Wood, NJ, USA) spectropolarimeter.

Size and shape of gramicidin loaded nanoparticles were determined by SEM. For these aim a thin film of gramicidin loaded nanoparticles and gramicidin alone (were prepared as FT-IR sample) on glass (ratio of polymer/peptide: 4mg/20 μg) were applied.Cytotoxicity of free gramicidin and gramicidin loaded nanoparticles

In order to determine the neat effect of gramicidin on cells treatments were classified into two groups. The first one (40ga-pol) was included 40 μg/ml of gramicidin alone and different concentrations of polymer (1, 2, 3, 4 & 5 mg/ml) which were formulated with constant concentration of gramicidin, 40 μg/ml and the second group (pol) consists of different concentrations of polymer alone (1, 2, 3, 4 & 5 mg/ml). Formulation was performed as SEM and FT-IR samples and after 30 min remaining at room temperature were used for cell treatment. Untreated cells considered as negative control.

Results

Biological properties

Cytotoxicity of polymeric nanoparticles on cancerous cell line, LNCaP.

MTT assay was don for five different concentrations of polymeric Nano carriers (1, 2.5, 5, 10 and 15 mg/ml) at different treatment times (16, 24 and 48 hours). Untreated cells considered as negative control and viability calculated against them. As shown in Fig.1 significant cytotoxicity for 16 and 24 h treatments was appeared at concentrations above 5 mg/ml but for 48 h treatment

viability reduced to %91.98 at this concentration (5 mg/ml). Cell treatment with 10 and 15 mg/ml of this nanoparticles show significant reduced viability for all treatment times. Calculated IC50 for treated cells at 16, 24 and 48 hours is 14.71, 9.256 and 7.8 mg/ml respectively. These data indicated that the prepared nanoparticles at concentrations below 5 mg/ml are non-toxic to LNCaP cells and cause cell death in a time and concentration dependent manner.

Fluorescence microscopy and up take by cells

In one of the earlier investigation it was shown that following uptake of FITC-containing nanoparticles by tumor cells if nanoparticles lose their structural integrity and release the FITC into the cytosol fluorescence images of threated cells will exhibit a diffused fluorescence staining which is resulted from presence of FITC in entire intracellular matrix. Fig.2.A. represent fluorescence images of treated cells with FITC loaded nanoparticles. The image obviously reveals a diffused fluorescence staining manner which is related to lose of structural integrity of nanoparticles upon uptake by prostate cancerous cell line, LNCaP. Treated cells with polymer alone was considered as control (Fig.2.B).

Loading studies

FT-IR spectrum of gramicidin and gramicidin loaded nanoparticles

Gramicidin A in crystalline form has $\beta^{5.6}$ conformation. As reported previously FT-IR spectrum of this conformation has six characteristic peaks at 3285, 3078, 1638, 1542, 1285 and 676 cm-1 which are corresponded to type A, B, I, II, III and V amides respectively. Fourier self deconvolution (FSD) analysis was performed for obtained spectra of gramicidin and gramicidin loaded nanoparticles. Gramicidin spectrum show characteristic peaks which had been reported previously [14]. Spectrum of loaded gramicidin on nanoparticles shows a little shift in its peaks which confirm interactions between polymer and peptide (Table 1) (fig.3)

Fluorescence spectra of gramicidin loaded nanoparticles

Gramicidin has intrinsic fluorescence because of the presence of tryptophan residues. Loading of gramicidin in polymeric micelles was investigated by quenching intrinsic fluorescence of gramicidin in the presence of copper ions. As shown in Fig.4 fluorescence of tryptophan residues of gramicidin loaded nanoparticles didn't quenched at the presence of copper ions because tryptophan residues of gramicidin were buried in the core of polymeric micelles so are inaccessible for quenching agent, cu^{2+}. Increasing in polymer to gramicidin ratio result in more fluorescence emission which is related to increasing in gramicidin loading and this continue until gramicidin loaded completely on nanoparticles and there isn't free form of gramicidin.

Far-UVCD spectra of gramicidin and, gramicidin loaded nanoparticles

Far-UVCD studies have been previously used to characterize membrane bounded gramicidin conformations. In order to confirm loading of gramicidin in the core of polymeric micelles CD spectroscopy was utilized. As shown in Fig.5 gramicidin has two different spectrums in the presence and absence of polymer and show that interaction between polymer and peptide has occurred. Spectrum of gramicidin loaded nanoparticle solutions reveals characteristic peaks with negative elliptisity around 194 nm and positive elliptisity around 211 and 193 nm.

Scanning electron microscopy for peptide loaded nanoparticles

As shown in Fig.6.A. sonicated gramicidin forms aggregates with a dimensions about 200 nm and spherical to ovally forms. Micrograph of gramicidin loaded in the core of polymeric micelles exhibit that these drug containing nanoparticles have spherical forms with a diameter smaller than 100 nm (Fig.6.B). Smaller size of gramicidin containing nanoparticles not only confirms the loading of peptide agents also reveals that prepared carriers have a good size for long circulating in blood stream and passive targeting.

Cytotoxicity of free gramicidin and gramicidin loaded nanoparticles

Viability for all treatments was calculated against negative control. These results (Fig.7) indicate that gramicidin alone don't affect viability of cells which is resulted from poor solubility of it but treated cells with gramicidin-polymer complex exhibit reduced viability which is consequence of loading of gramicidin in the core of polymeric micelles and increase in its apparent solubility. As it is evident from results consequence of increase in the ratio of polymer to peptide is improvement in the cytotoxicity effect of gramicidin which results from loading of more amount of gramicidin on nanoparticles. Zero concentration on column chart for the first group is corresponded to the cells which were treated with 40 µg/ml of gramicidin alone and for the second group (pol) related to untreated cells and was considered as negative control.

Discussion

PLA and PEG had been shown good biocompatibility and biodegradability previously and have approved from the food and drug administration of America (FDA) [15, 16]. Previous studies shown that PLA is able to enter the cells and have been applied for intracellular delivery of drugs [17]. At the other hand stealth nanoparticles are very attractive for use in drug delivery. PEG is the most common polymer which is used for making nanoparticles stealth and undetectable for reticulo-endothelial system [5, 7, 18, 19]. Furthermore polymeric micelles which are made from self-assemble amohiphilic block copolymers are of interest for

Table 1. Appeared peaks in FT-IR spectra of gramicidin and gramicidin-polymer nanoparticles

Reported frequencies for crystalline native *ga-A* (cm^{-1})	Assignment	observed frequencies for *ga* (cm^{-1})	observed frequencies for *ga-pol* (cm^{-1})
3415	OH stretch (ethanolamine, tyr)	3416	–
3285	Amide A	3272	–
3078	Amide B	3085	3076
3064	CH stretch (ring)	3066	3062
2964	CH$_3$/CH$_2$ stretch	2960	–
2935	CH$_3$/CH$_2$ stretch	2932	2931
1680 *sh*	amide I	1682	1683
1638	amide I	1634	1635
1542	amide II	1538	1542
1460	CH$_2$ bend (side chain)	1457	1455
1440 *sh*	CH$_2$ bend (side chain)	1436	–
1388	CH$_3$ symmetric bend	1387	1385
1285	amide III	1283	1275
1233	amide III	1234	–
1070	COH (ethanolamine)	1067	1070
676	amide V	674	682
626	amide V	–	628

ga-A = gramicidin A, ga = gramicidin, ga-pol = gramicidin polymer nanoparticles, Sh = shoulder.
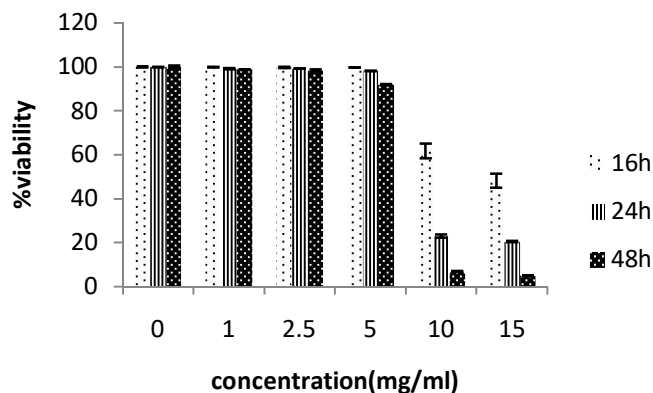

Fig. 1. Calculated viability percent for treated cells (LNCaP cell line) with different concentrations of polymeric nanoparticles (at 16, 24 and 48 hours). (Zero on the chart assigns untreated cells and considered as negative control). viability was calculated against negative control.
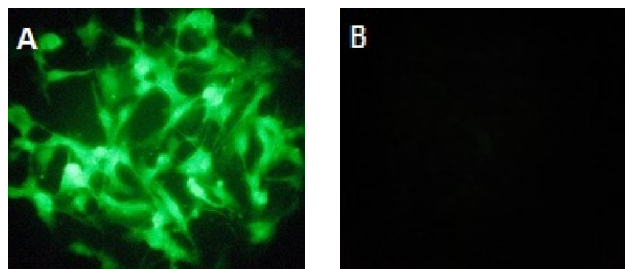

Fig. 2. fluorescence images had been captured from treated cells with FITC loaded nanoparticles (A) and polymer alone (B) (control) for 2 hours.
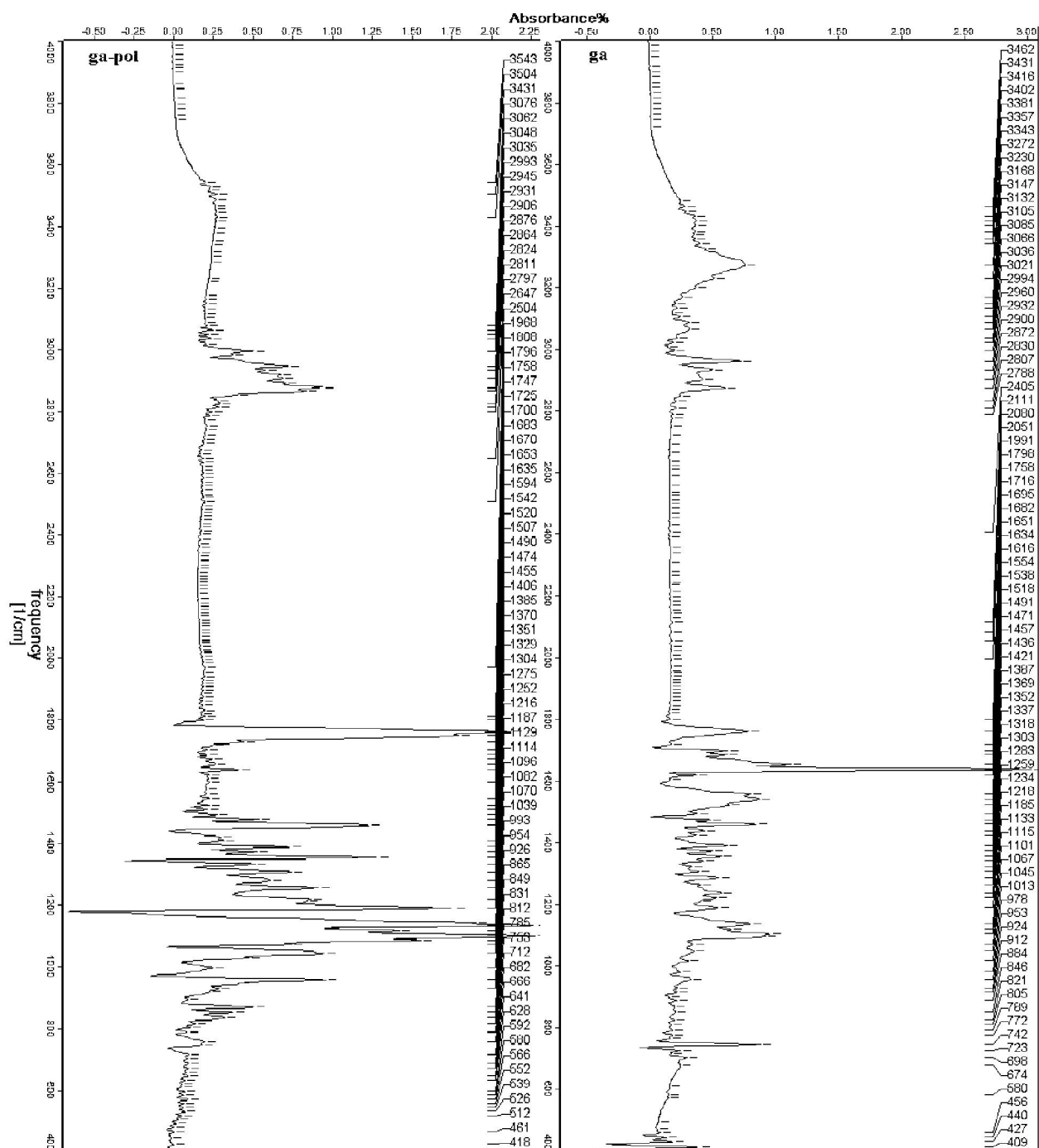
Fig. 3. FT-IR spectra of gramicidin in free form (ga) and when loaded in the core of polymeric micelles (ga-pol)
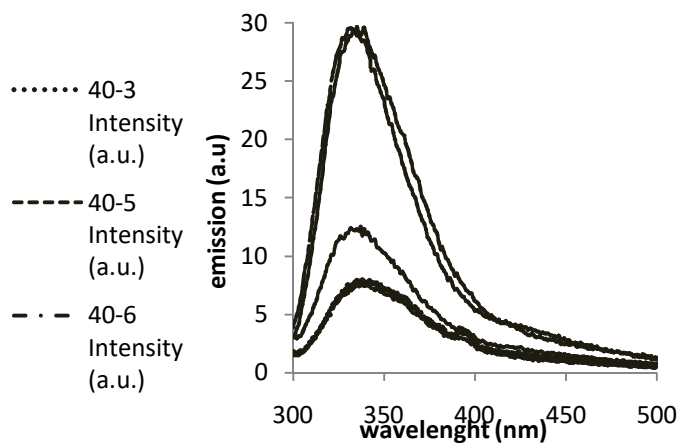
Fig. 4. Fluorescence spectra of 40 µg gramicidin at the presence of copper ions (40ga-cu), absence of copper ions (40ga) and when formulated with 3 (40-3), 5 (40-5) and 6 (40-6) mg/ml of polymer at the presence of copper ions.
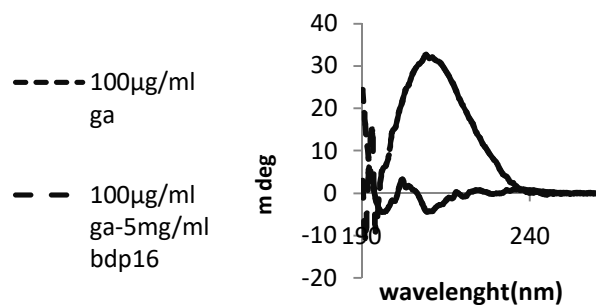

Fig. 5. CD spectra of gramicidin in free form (100 µg/ml ga) and when loaded with 5mg/ml polymer (100 µg/ml ga-5mg/ml copolymer).
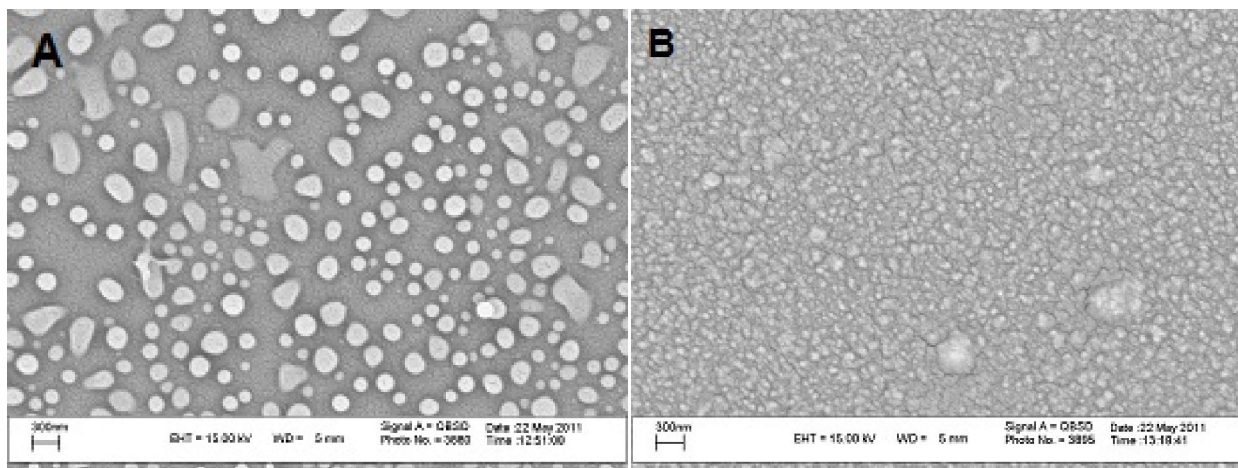

Fig. 6. SEM micrographs of gramicidin aggregates (A) and gramicidin loaded nanoparticles (B) were prepared by solvent evaporation method. (Magnitude: 50000x. scale bar 300 nm).
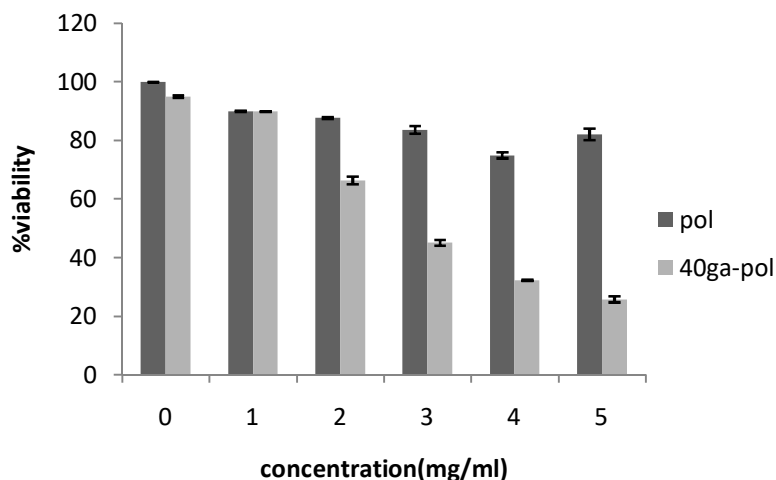
Fig.7. Calculated viability from MTT assay results for treated cells with different concentrations (1, 2, 3, 4 and 5 mg/ml, 0 assign negative control) of polymer (pol) and when constant concentration of gramicidin (40 µg) was formulated with these concentrations of polymer (40ga-pol) (0 assign treated cells with 40 µg gramicidin alone). Viability for all treatments was calculated against negative control.

hydrophobic drugs loading [5, 7, 19]. In this work triblock copolymer of biocompatible segments (PLA-PEG-PLA) was synthesized

MTT assay results reveal low toxicity of prepared nanoparticles on cancerous cell line, LNCaP, and treatment of cells up to 5mg/ml of polymeric nanoparticles don't show significant cytotoxicity. According obtained results from MTT assay (fig.1), we can deduce that these polymeric nanoparticles are applicable for drug delivery at in vivo conditions at concentrations up to 5mg/ml.

Intracellular delivery of drugs through bypassing cell membrane is preferred because it results in resolving chemotherapy problems like multidrug resistances [20-22]. As shown in Fig.2 FITC loaded nanoparticles reveal that these polymeric micelles are able to deliver FITC into the cytosol.

Gramicidin as a hydrophobic drug was loaded in the core of polymeric micelles and its loading was investigated by FT-IR, fluorescence and CD spectroscopies. FT-IR spectra of gramicidin in free form and when loaded in the core of polymeric micelles Show differences in its spectrum (table.1) (fig.3).

The drug within micelles may locate in the corona, the core and the core-corona interface of the micelles. Fluorescence quenching can be used to evaluate amount of drug loaded completely in the micelle core. Bivalent cations like cu^{2+} are able to quench any fluorescent dies and are used for studying loading dye molecules in the core of micelles. Our results clearly show (Fig.4) that loading efficiency greatly affect by polymer concentration which is in agreement with previous studies [7] also gramicidin had been loaded in the core of polymeric micelles and when 40 µg/ml of gramicidin formulated with 6 mg/ml of polymer in the presence of copper ions the detected emission was equal to that detected for 40 µg/ml of gramicidin in the absence of quencher and indicate that gramicidin has been loaded completely. According above statements we can conclude that 6.66 µg of gramicidin can load on 1mg of copolymer. Also we can deduce that gramicidin and its tryptophan residues had been placed in the core of micelles because at this ratio of polymer to peptide in the presence of copper ions the emitted fluorescence is same as free gramicidin in the absence of quencher.Far-UVCD studies (Fig.5) also confirm that interaction between polymer and peptide had been occurred. Gramicidin when placed in the core of micelles has different conformation relative to gramicidin in free form and is a sign of interaction between polymer and peptide.Nanoparticle size has an important effect on efficiency of drug delivery systems. Previous studies report size dependence efficiency of polymeric nanoparticles for intracellular delivery of drugs. Increase in the size of nanoparticles result in decrease in cellular uptake and nanoparticles with a size smaller than 100 nm are more favorable for uptake [24]. At the other hand the optimal nanoparticles size for in vivo applications is generally between 10-100 nm [20, 24]. Also carriers with a size smaller than 100 nm are applicable for brain drug delivery [16, 25]. Particles with smaller than 10 nm are susceptible for remove by renal filtration and particles with a size above 100 nm are more susceptible for elimination by RES [18, 20]. As particles are smaller their bio distributions improve and can

easily distribute through the tumor tissues [11]. Presence of PEG on the surface of nanoparticles result in long circulation time of nanoparticles and cause accumulation of more amount of drug in tumor tissue trough enhanced permeability and retention effect (EPR) [11, 26]. Prepared nanoparticles in this work have a diameter less than 100 nm (Fig.6.B) and because of the presence of PEG on the surface of these nanoparticles these gramicidin loaded nanoparticles appear to be very effective for passive targeting of tumors.

Gramicidin doesn't have significant effect on viability of cells because of its poor solubility. But as it loads in the core of micelles its apparent solubility increase and can affect viability of cells (in agreement with previous studies about paclitaxel [13]. MTT assay for gramicidin loaded nanoparticles (Fig.7) show that by increase in the polymer concentration (as gramicidin concentration is constant) effect of gramicidin on cells increase which is resulted from more loading of gramicidin on nanoparticles (in agreement with fluorescence results (Fig.4)). As previously reported increase in the ratio of polymer/peptide result in loading of more amount of drugs and at a ratio it becomes constant [7]. Reported targets for gramicidin are cell membrane [27], depression of aerobic and anaerobic glycolysis in bovine spermatozoa [28], in the case of gramicidin-S binding to calmodulin [29], DNA, RNA and protein synthesis inhibition in prokaryotic cells [30]. So it's logical that presence of gramicidin in the cytosol results in interaction of it with intracellular targets which their normal function are more necessary for cell viability in compare to extracellular targets and affect gramicidin cytotoxicity.

Conclusion

From above statement it is concluded that these biocompatible and biodegradable polymeric nanoparticles are applicable for intracellular drug delivery at in vivo conditions and have good properties for passive targeting of tumor tissues. Also gramicidin only is used for skin injuries and eye and because of its hemolytic activity and poor solubility is not applicable for systemic administration. Loading of gramicidin on these nanoparticles restrict its hemolytic activity, improve its solubility and accumulation at disease site like tumors by EPR effect hence by this formulation presence of gramicidin in blood vessels is appeared possible and may be useful for cure of many disease like cancer.

Conflict of Interests: The author declare no conflict of interests

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