



Reload Purified Melittin and Lactoferrin on Perfluorooctyl Bromide *nanoparticles* (PFOB-NPs) and Examine the Distribution of Particle Size, Zeta Potential and Confirmation of their Accession on the Nanoparticles via Tryptophan Fluorescence and Circular Dichroism (CD) and its Anti-Cancer Effects on Human Breast Cancer Cell Line MCF7

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ABSTRACT

According to the prevalence of cancer in today's societies, it is too important to find new drugs for that treatment. Cationic antimicrobial peptides that are able to eliminate a wide range of bacteria, fungi, parasites, viruses and unicellular, are natural anti-cancer agents. In the present study, the effect of cationic peptides of bee venom Melittin (ML) and Lactoferrin (LF) of camel milk which was loaded on the Perfluorooctyl Bromide (PFOB) nanoparticles, were examined on MCF7 cells. Nanoparticles using oil in water emulsion was prepared by ultra-thoracic and ultrasonic. Then ML and LF were separately added to the nanoparticles then incorporation of them were confirmed by tryptophan fluorescence and circular dichroism (CD) spectra. The mean diameter of the nanoparticles did not change after addition of ML and LF (~100 nm), but as expected, the zeta potential of the nanoparticles with increasing of ML and LF concentrations to the preformed nanoparticles increased from -18.43(without ML and LF) to +21.61(LF 110.66 μ g/ml) and +20.93(ML 0.1 μ g/ml) and +23.75(LF 110.66 μ g/ml and ML 0.1 μ g/ml). By using of scanning electron microscopy (SEM), the morphology and structure of nanoparticles was investigated, homogeneity and uniformity during processing and loading of ML and LF was observed. Effectiveness of nanoparticles loaded on cancer cells was performed with MTT test. The results showed that ML and LF had a positive effect on cancer cell death. Percent of cell death was higher when ML and LF was loaded on nanoparticles. With increase of ML and LF concentration loading on nanoparticles, increases cancer cell death. Minitab software was used to analyze some data.

Keywords: Bee venom, Camel milk, Melitiin, Lactoferrin, Perfluorooctyl Carbon, PerfluorooctylBromide, MCF7 cell Line.

INTRODUCTION

Despite great advances in cancer treatment, a significant tendency for new anticancer agents due to the increased resistance of cancer cells to anticancer drugs, is currently being expanded.

Cationic antimicrobial peptides can be divided into two categories based on their work: the first group consists of peptides that are toxic to microbes, cancer cells and healthy mammalian cells (non-selective), for example Melittin (ML) of existing peptides the bee venom, tachyplesin isolated from horseshoe crab. The second group consists of peptides that are much potent to microbes and cancer cells, but not healthy cells, for example, cecropin by insects, magainins isolated from the skin of frogs and LF. However, at higher concentrations of more peptides put toxic effect on normal cells because of their fundamental amphipathic property¹⁻³.

ML and other cytolytic peptides are memorable candidate for cancer chemotherapy⁴, for two reasons: (1) Cancer cells due to more negative charge on their surface than normal cells are less likely to develop resistance to pore through the membrane. (2) The combination of a chemotherapy drug (e.g., Doxorubicin, Adriamycin) or selective cationic antimicrobial peptides which is not going to harm healthy cells along (e.g., LF) together with ML can have a synergistic effectiveness, resulting in a therapeutic dose required reduced^{5, 6}

ML is the principal active component of apitoxin (bee venom) and is a powerful stimulator of phospholipase A2. ML is a peptide consisting of 26 amino acids with the sequence GIGAVLKVLTTGLPALISWIKRKRQQ and it constitutes 50% of dry venom. Molecular weight of ML is 2.86 kD and has a net charge of +6 at physiological pH. The amphiphilic property of this peptide makes it water-soluble⁷ ML is a very strong basic peptide and its impact is extreme. ML connected to the hydrophobic membrane and in a matter of milliseconds, either perpendicular or parallel to the surface and cause hemolysis of red blood cells and thereby increase the permeability of their membranes^{8,9}

Lactoferrin (LF) is a globular glycoprotein with a molecular weight about 80 kDa, this protein is composed of 703 amino acid sites. That is one of the constituents of milk and the most important property is that the absorption and transport of iron. LF through chelating the iron ion unavailable to the invading pathogens. And thus inhibit the growth of harmful bacteria indirectly inhibits the spread of infection. Due to the wide distribution of LF in various tissues and the presence of Network of positive charges in it, this protein is a multi-functional protein. In some cases, basic amino acids of LF interactions with anionic molecules at specific levels of bacteria, virus, fungus and parasite and Cause lysis of cells. LF is able to stop the growth of cancer cells through the inhibition of cell proliferation by altering the expression or activity of regulatory proteins and also through the activation of natural killer cells and inhibits tumor growth through activation of apoptosis (programmed cell death).

In order to increase for drug therapeutic effect, must protected until reach to target site in the body and maintained its chemical and biological properties. Some drugs are highly toxic and can cause negative side effects and if they are destroyed during delivery, decreased efficacy. Particles that are not connected easily removed by the liver and spleen^{10, 11} Up to now several transportation system are used for ML and LF, that each with advantages and disadvantages. In this work, used a new drug delivery system for the treatment of cancer is known that the potential utility of cytolytic peptides^{5, 12}

perfluorocarbon nanoparticles used as drug carriers and molecular imaging. Since perfluorocarbon (PFCs) are biologically inert, chemically stable, nonbiodegradable, nontoxic and nonvolatile, that all these features can produce nanoemulsions are biocompatible. Specifically, perfluorooctyl bromide (PFOB) mostly used due to its low vapor pressure, evaporation and may be to reduce the production of pulmonary emphysema¹⁰

MATERIALS AND METHODS

Bioactive Protein

Bee venom was prepared from Shiraz, Iran and stored at -20°C. Camels milk was prepared

from Torkaman Sahra, Iran and stored at -20°C until needed. The bioactive LF (Purity: >85%) from bovine whey and the bioactive ML (Purity: >85%) from BV were purchased from Sigma-Aldrich (USA)

Chemicals and Biochemicals

Perfluorooctyl bromide (PFOB) 99%, carboxymethyl Sephadex C-50 resin, soybean phosphatidylcholine (Lecithin), dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE, assay: >99%), MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) and RPMI-1640 Media were purchased from Sigma-Aldrich (USA).

Other chemicals and biochemicals including protein marker 26612 were purchased from Thermo Scientific (USA). HCl (37%) and NaCl, monosodium phosphate, sodium citrate, acrylamide, bisacrylamide, tris, TEMED, APS, sodium dodecyl sulfate (SDS), ethanol, tetramethylbenzidine, acetic acid, silver nitrate, Sodium thiosulfate, formaldehyde, sodium carbonate, sulfuric acid, hydrogen peroxide, dimethyl sulfoxide, citric acid, bovine serum albumin, C.Blue G250 were purchased from Merck (Germany).

Analytical Equipment

High-performance liquid chromatography (HPLC) system (Agilent, series 1200) and ion exchange chromatography column were used. Cationic exchange chromatography column (10×3 cm) was used. All spectrophotometric readings were carried out using quartz cuvettes in an UV-VIS spectrophotometer, Shimadzu (Kyoto, Japan). Upper Speed Vacuum Centrifuge (Ultracentrifuge) model VS-30000i of VISION SCIENTIFIC CO. LTD was used. Micro-spin/ FUGE/ VOTEX KIAGEN (Molecular Biology Company), magnetic stirrer (yellow MAGHS7), pH meter of Metrohm Swiss (827 pH lab) were used. Eppen dorf centrifuge (5415D), Sodium dodecyl sulfate1 polyacrylamide gel electrophoresis (SDS-PAGE) of Sigma PS 2000-2 (USA) were used. Cationic exchange chromatography column (10×3 cm) was used. Orbital shaker (FINEPCR SH30t), Laminar flow hood (Beasat) and CO2 incubator (N-Biotex) were used. The Zeta potential and particles size of the nanoemulsion particles were determined respectively in a ZetaCompact (CAD Instruments, France) and particle size analyzer (VASCO - 1,

France). SEM analysis of nanoemulsions were carried out in a scanning electron microscope (LEO,model VP 1450, Germany). Also, ultrasound (XL2020-010, USA), ultraturax (IKA, Model T25, Germany), circular dichroism (CD) spectrometer (J-815, CD spectrometer, Japan), fluorescence intensity (model 2500, Hitachi, Japan) were used for analytical assays.

Peptide Synthesis

Bee venom was prepared using Benton electric shock device. 30mg of collected BV was dissolved in 2ml of ddH₂O and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant was centrifuged at the same conditions then was filtered with 0.2µm membrane filter. ML of samples was purified to >95% homogeneity by reverse-phase high-pressure liquid chromatography analysis (RP-HPLC) on a C18 column with semi-polar solution Included water and acetonitrile containing 0.1% TFA as mobile phase under gradient elution at 215 nm¹³.

For the isolation of LF from camel milk, the fat and casein removed from milk by centrifuge at 4500g for 15 min in 4°C (two times) and ultracentrifuge at 30000g for 30 min in 25°C, (two times). What remains was whey protein. LF from camel milk was extracted and purified by ion-exchange chromatography (resin carboxymethyl Sephadex C-50). Since the molecular weight of LF and lactoperoxidase is 80 kDa, to ensure the existence of LF in the output column chromatography, the coloring reaction with tetramethylbenzidin (TMB) was applied. The reaction mixture contains H₂O₂ 30%, mM 88 TMB and sodium phosphate buffer (1 mM 10 at pH=6) with ratio 1: 10: 100. 10 microliters of sample containing of the enzyme with 200 ml of the reaction mixture was incubated for 15 minutes at 37°C. For this purpose, the horseradish peroxidase (HRP) was used as a positive control¹⁴.

The protein solution was desalinated by dialysis where the dialysis bag was surrounded with sucrose or polyethylene glycol (PEG).

Purity of ML and LF was confirmed by SDS - PAGE. ML and LF concentration was measured by the Bradford protein assay and nano-drop spectrophotometry.

ML was identified by comparing the area under the eluting ML peak (retention time 22 minutes) to a standard curve generating using ML sample were purchased from Sigma-Aldrich. Extracted ML lyophilized and stored at -20°C in the dark until needed.

Hemolytic Activity Assay of ML

In general the hemolytic Activity used to evaluate of potential bacteria or drugs and unknown substances in the destruction of red blood cells. In addition, one of the trials evaluating the safety of drug dose is hemolytic activity assay.

The hemolytic activity of bee venom and ML was assayed by a standard procedure¹⁵. In concise, fresh rabbit red blood cells (RBCs) that were collected in the presence of an anti-coagulant (sodium citrate) were washed three times in PBS. ML (in selected amounts dissolved in water 0.1, 0.05, 0.001 µg/ml, respectively) were added to the suspension of red blood cells (2% v/v) in PBS to the final volume of 200 µl and incubated at 37 °C for 35 min. The samples were centrifuged for 10 min at 2000 rpm, and the release of hemoglobin was monitored by measuring the absorbance (A_{sample}) of the supernatant at 540 nm. RBCs in PBS (A_{blank}) and in 0.2% v/v Triton X-100 (A_{Triton}) were used for negative and positive controls respectively. The percentage of hemolysis was calculated according to the equation, Percentage of hemolysis = $[(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{Triton}} - A_{\text{blank}})] \times 100$.

Preparation of Nanoparticles

Perfluorooctyl bromide nanoparticles (PFOB) was prepared by oil in water emulsions. "2% W / V surfactant (98 mol% egg lecithin and 2 mol% dipalmitoylphosphatidylethanolamine, DPPE), 20% V / V (PFOB) and rest of the water to volume 10ml" were stirred on a magnetic stirrer⁵. Then the blend were stirred by Ultra-Turex stirrer for 10 minute at 12000 rpm within water-ice solution. To obtain nano-sized particles, the blend were stirred by Ultrasound stirrer for 10 minutes within water-ice solution¹⁶

Addition of ML and LF to NPs

After the preparation of NPs, LF (in selected amounts in saline phosphate buffered of 110, 55, 27.5 µg/mL, respectively) and ML (in selected amounts in saline phosphate buffered of

0.1, 0.05, 0.001 µg/ml, respectively) were added to the preformed NPs separately and together, and the residual free (unbound) LF and ML washed out by centrifugation at 1000 rpm for 10 min. The amount of unbound LF was measured by measuring the tryptophan fluorescence and Bradford method¹⁷. Control NPs (without LF) were stored for 52 days under the same conditions¹⁸

Entrapment Efficiency (EE)

The concentration of ML and LF added to the nanoparticles was determined by measuring concentration of ML and LF in the supernatant obtained by centrifugation in before step.

Entrapment efficiency (in percentage) was calculated using Eq.1.

$$EE\% = \frac{(C_t - C_f)}{C_t} * 100 \quad \dots(1)$$

Where C_f is the amount of free ML or LF (or mix both of them) and C_t is the total amount of ML or LF (or mix both of them) presented in 500 µL of NPs¹⁹.

Scanning Electron Microscopy Analyses

Scanning electron microscopy (SEM) is a method that uses electrons for high-resolution imaging of surfaces of particles, as a light microscope uses visible light. The advantages of SEM over light microscopy include much higher magnification (>100,000X) and greater depth of field up to 100 times that of light microscopy. Qualitative and quantitative chemical analysis information is also obtained using an energy dispersive x-ray spectrometer (EDS) with the SEM. In this study, the use of SEM, the particle shape and the particle size was checked before and after loading ML and LF.

Determination of Particle Size

Physical dynamic light scattering method is used to determine the distribution of particles in solution and suspension. This nondestructive and rapid method for the determination of particle size in the range of a few nanometers to microns to work. The task of setting a particle size specification for a material requires knowledge of which technique will be used for the analysis and how size affects product performance. All particle size analysis

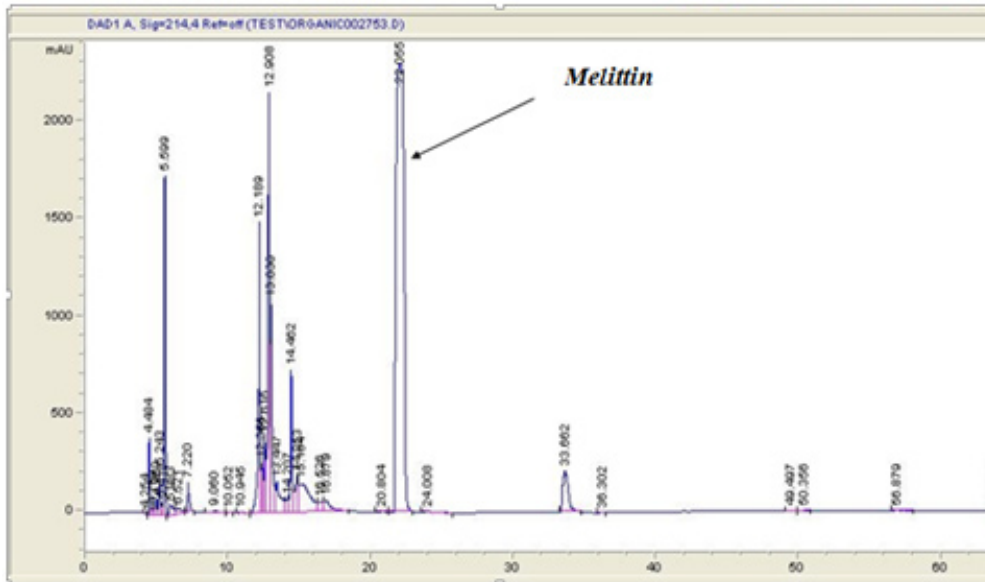


Fig. 1: RP-HPLC chromatograms of bee venom (Column: C18; Mobile phase: Water/ acetonitrile=40/60; Flow rate: 1ml/min; Temperature:25°C; 60 min; Detection: UV 214nm;)

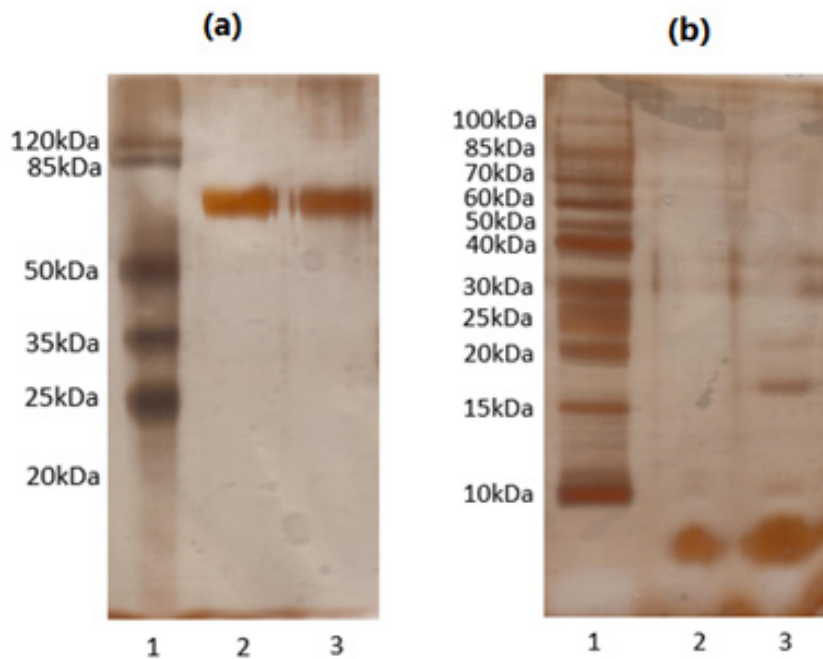


Fig. 2: SDS PAGE analysis on a 15% gel with 120V and 90 minutes (a) Lane 1, 2, and 3 represents molecular weight marker, purified LF and Standard LF, respectively.(b) Lane 1, 2, and 3 represents molecular weight marker, purified ML and Standard ML, respectively

instruments provide the ability to measure and report the particle size distribution of the sample. The size of nanoparticles were obtained before and after loading ML and LF^{20, 21}

Determination of Zeta Potential

Zeta potential is a physical property which is exhibited by any particle in suspension. It can be used to optimize the formulations of suspensions

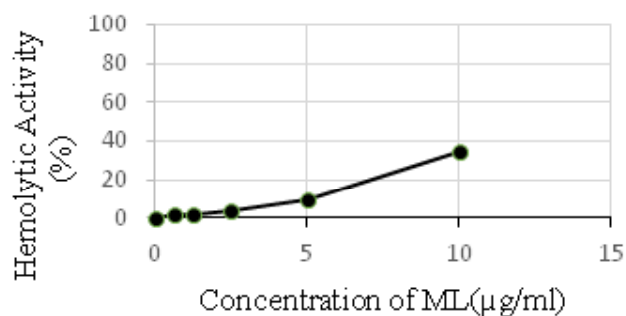


Fig. 3: Hemolytic Activity Assay of ML

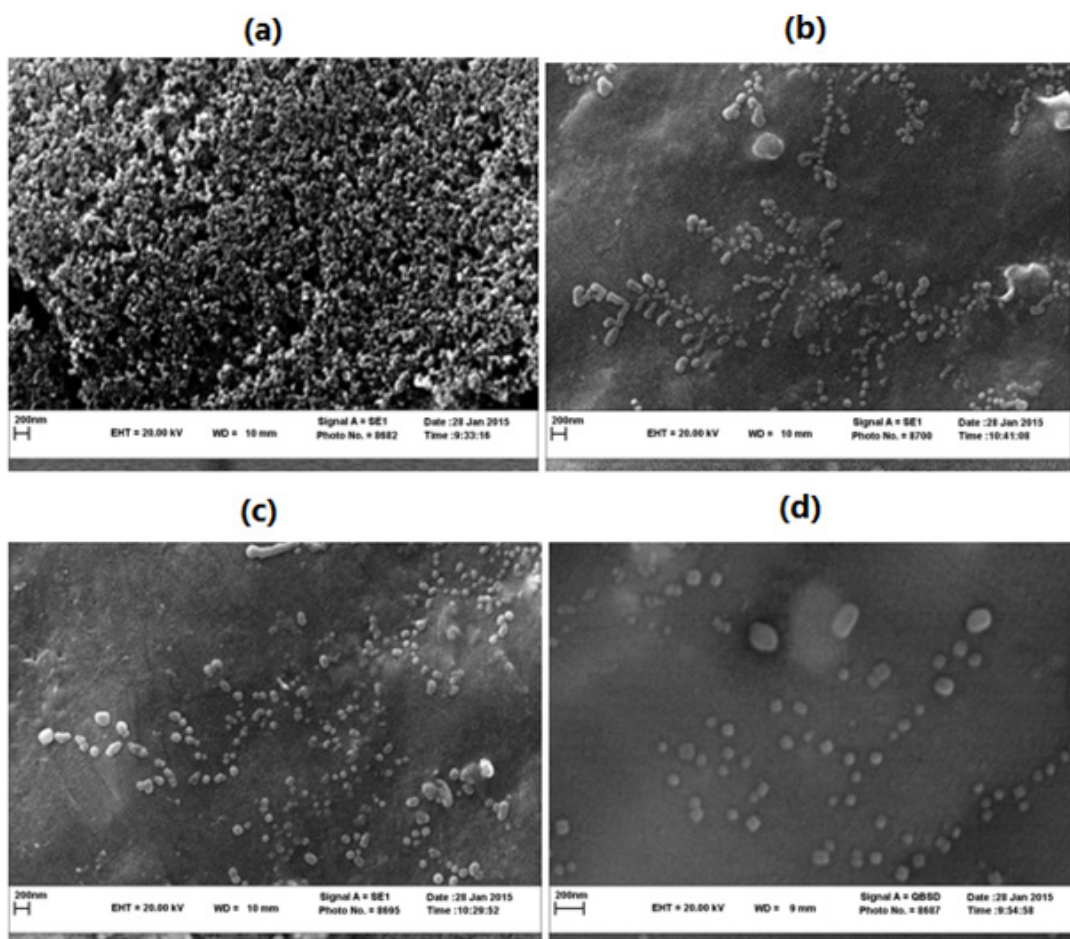


Fig. 4: SEM image (a), (b) PFOB Nanoparticles; (c) PFOB Nanoparticles loaded with ML; (d) PFOB Nanoparticles loaded with LF

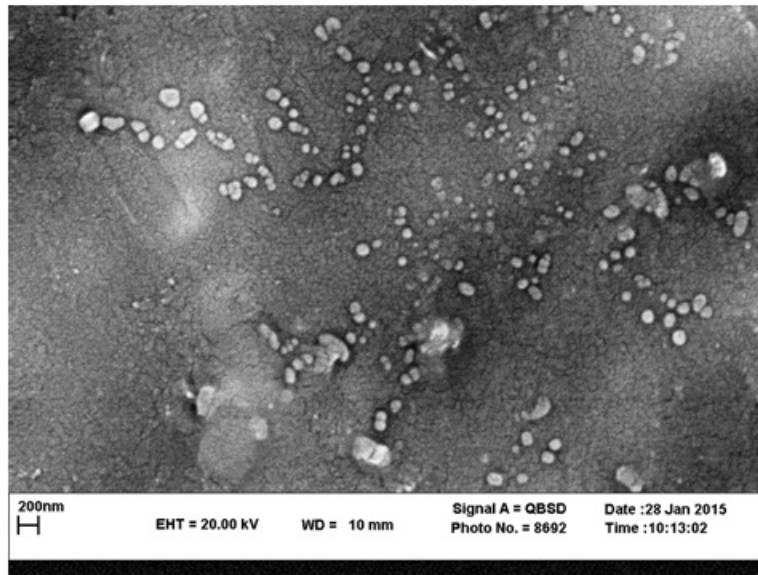


Fig. 5: SEM image of PFOB Nanoparticles loaded with both ML and LF

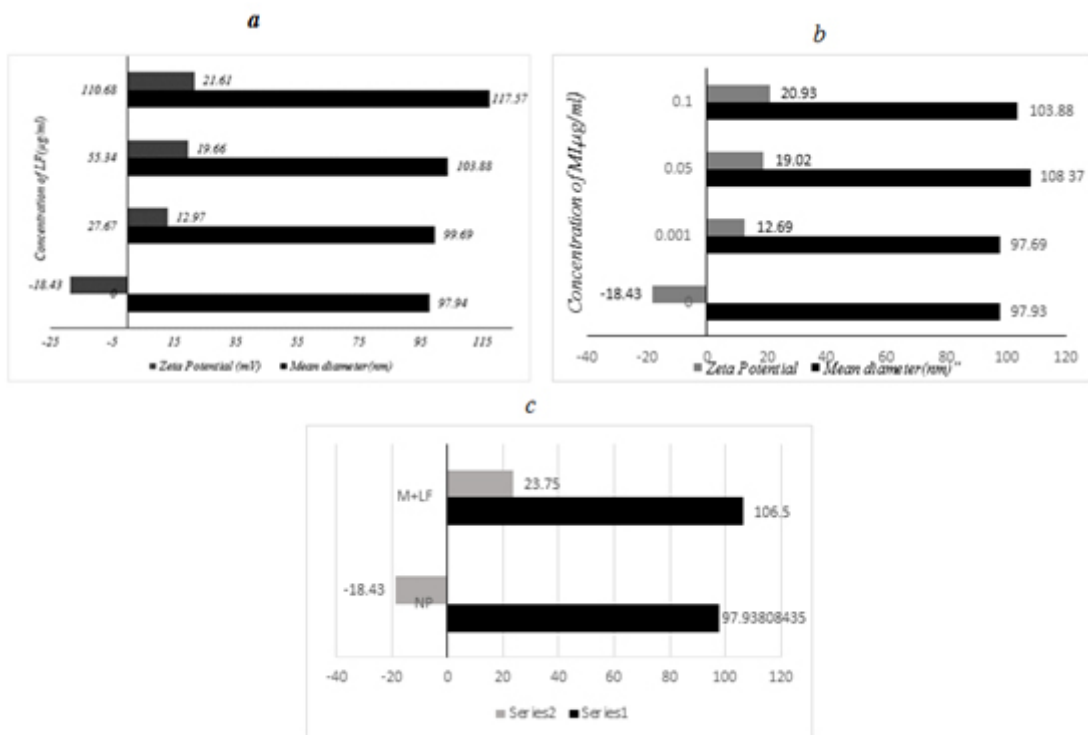


Fig. 6: Mean hydrodynamic diameter and zeta potential of NPs before and after incorporation of a) LF b) ML c) ML and LF

and emulsions. Knowledge of the zeta potential can reduce the time needed to produce trial formulations. It is also an aid in predicting long-term stability.

Always around the level of the particles that have been in fluid, increasing the concentration of charged ions with opposite charge can be observed because the particles have a surface charge. Therefore an additional layer of ions surrounding the particle surface in the form of an electric double layer around the particle is created. The result can be an imaginary distance between the particle and fluid environment are given the impression that this is a double layer that surrounds the particle. These are the so-called hydrodynamic and potential are there in the distance is known as the zeta potential. Here by examining changes of zeta potential of nanoparticles before and after loading ML and LF, their accession was confirmed²²

Circular Dichroism (CD) Spectroscopy

The spectrometer, one of the most important light cognitive techniques to study biological molecules. A powerful way to determine the structure of proteins that an advantage of the method is easy to use, other benefits of this method is the short duration of the trial, and even detectable structural changes at a few milliseconds. Circular dichroism (CD) spectroscopy can be used to observe how secondary structure changes with environmental conditions or on interaction with other molecules. Structural, kinetic and thermodynamic information about macromolecules can be derived from circular dichroism spectroscopy. Herein, CD was used to confirm the incorporation of ML and LF to nanoemulsions.

Fluorescence Spectroscopy

Fluorescence spectroscopy is a method that directly study the interaction of the ligand with

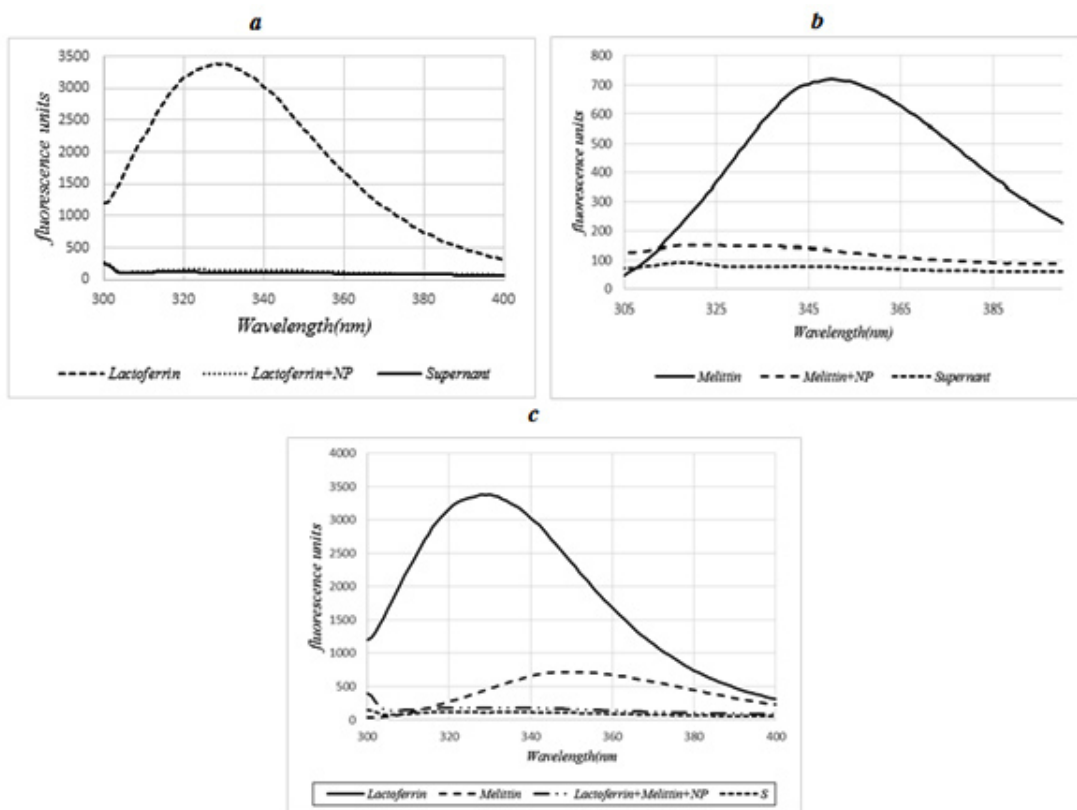


Fig. 7: interaction and the incorporation of ML (a), LF (b) and ML & LF (c) with the lipid monolayer of PFOB nanoparticles intrinsic tryptophan fluorescence (TRP19)

macromolecules, which extensive studies have been done by this method, especially by measuring fluorescence quenching. Fluorescence quenching occurs with static and dynamic quenching that checked in the Results and Discussion. For obtain the information about the incorporation of ML and LF to nanoparticles, fluorescence spectroscopy that is a highly sensitive and useful method were used. Fluorescence ray is the emission ray of UV and visible spectrum with compared to absorption radiation, longer wavelength and less energy. At the Aromatic amino acid in the presence of tryptophan, tyrosine and phenylalanine amino acids, protein absorption at the wavelength of about 280 nm are significant.

Add Free ML and LF and Loaded ML and LF onto the Nanoparticles on Breast Cancer MCF7 Cells Lines

To study the effectiveness of ML and LF and the effectiveness of nanoparticles containing ML and LF and the effectiveness of their combination on the growth of breast cancer MCF7 cells lines, three different concentrations of mentioned compounds were used.

MTT assay is a colorimetric method that is on the basis of breaking and dioxiding of tetrazolium yellow crystals (MTT) with the chemical formula 3 - [4, 5 - dimethylthiazol-2- yl] -2, 5-diphenyl-tetrazolium bromide and it is simpler than other methods of cell proliferation and applicable with the exist facilities in most laboratories.

The MCF-7 breast cancer cell line was prepared from Razi Institute, Ministry of Jihad-e-Keshavarzi, Mashhad, Iran. These cells are thawed to revive and recover. The recovered cells were transferred into culture flasks and were incubated

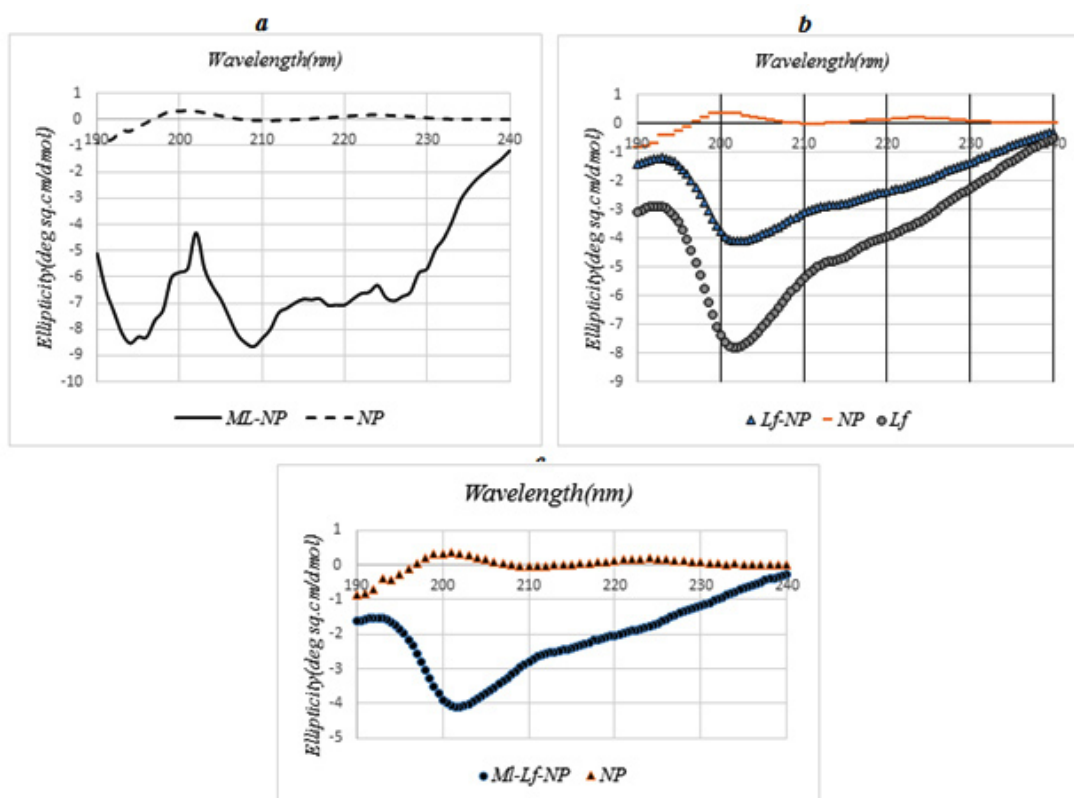


Fig. 8: Far-UV CD spectra of PFOB nanoparticle-bound and free- ML (a) -LF (b) and ML-LF- PFOB NPs (c) at 25°C

with 5% carbon dioxide at 37°C. To investigate the effectiveness of nanoparticles containing of LF in the growth of cancer cells, three different concentrations were used. For this purpose, a day before adding nanoparticles, the cells were transferred in 96-well plates with 200 ml of medium. After adding nanoparticles, plates containing cells were incubated with 5% carbon dioxide at 37°C. After 24 hours the survival rate of the cells was controlled with MTT assay *in vitro*.

RESULTS AND DISCUSSIONS

Identification of ML by HPLC System

As the figure 1 shows, after 22 minutes, HPLC system was created a strong peak that was compared with standard ML as well as SDS-PAGE (figure 2b) was proved that the peak is ML.

The Results of SDS PAGE Analysis

In order to evaluate the pattern of the venom protein extracted and to determine the purity

of the protein (ML and LF) and characterize the molecular weight range, after cationic exchange chromatography and the high performance liquid chromatography (HPLC) respectively sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS- PAGE) was used. The samples were loaded on a 15% gel with 120V and 90 minutes and purified ML and LF were confirmed by comparison with standard ML and standard LF. The fraction in NaCl solution (0.7mol/L) was identified as LF by SDS-PAGE (Figure. 2a, Lane 2). SDS-PAGE results showed that the identified fraction had higher purity. The fraction in 22 minutes was identified as ML by SDS-PAGE (Fig. 2b, Lane 2). SDS-PAGE results showed that the identified fraction in 22 minutes had higher purity.

The Results of Hemolytic Activity Assay of ML

According to Figure 3 the hemolytic activity increases with increasing concentration of ML.

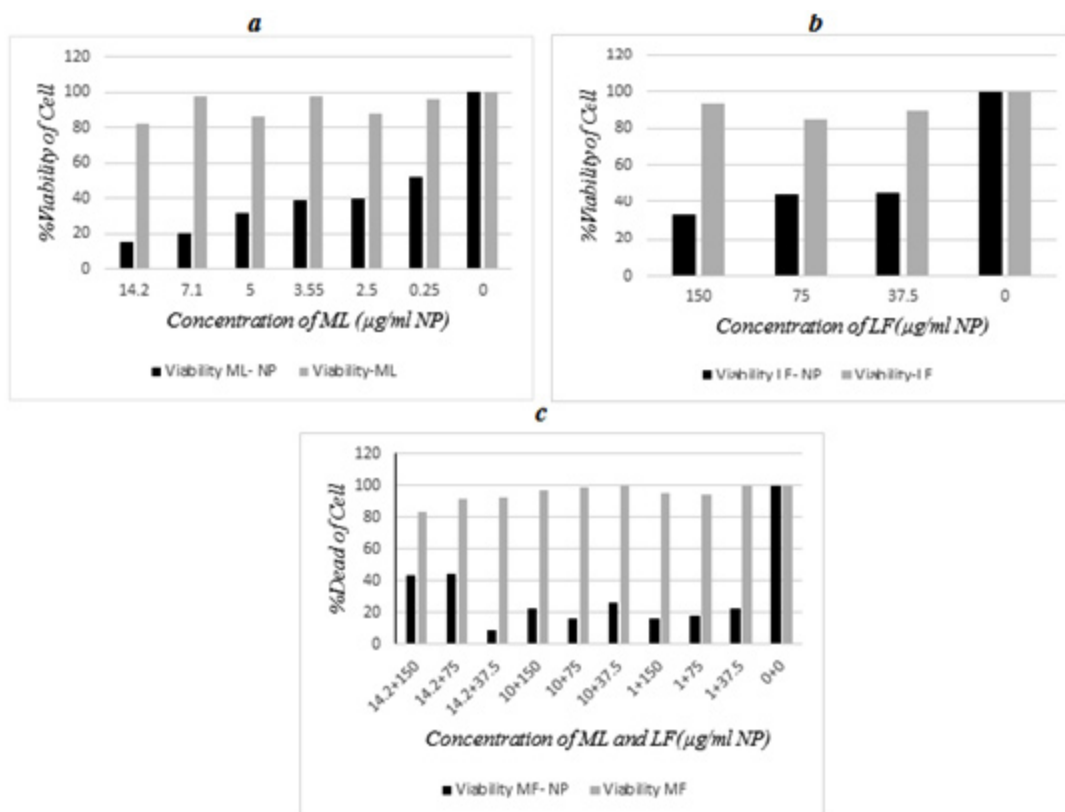


Fig. 9: The Results of the Effectiveness of Nanoparticles Loaded on MCF7 Cells Using MTT Test

Entrapment Efficiency (EE)

According to the description given in section, by measuring the amount of free (unbound) protein (ML and LF) after centrifugation and comparison with ML and LF present in 500 μ L of NPs by tryptophan fluorescence and Bradford method according to equation 1, the *entrapment efficiency* for ML and LF were obtained about 87 and 91 percent, respectively 91% respectively.

The Results of Scanning Electron Microscopy Analyses

SEM images of PFOB nanoparticles and nanoparticles loaded with ML or LF and nanoparticles loaded with both ML and LF, were shown in Figure 4a, 4b, 4c, 4d and figure 5 respectively with a magnification of between 100,000 to 500,000. It clearly that the structure of the nanoparticles during the production process and the incorporation of ML and LF is very homogeneous with uniform distribution diameter and the spherical particle morphology has not changed. Uniform shape of nanoparticles due to their surface charge which stay homogeneously together and do not precipitate. Also, because of the high surface tension, hydrophobicity and lipophobicity of the PFOB nanoparticles core, the general structure was preserved and finally, from the collapse of the nanoparticles was prevented.

The Results of Particle Size Analysis and Determination of Zeta Potential

As Figures 6 (a,b,c) show the surface charge of nanoparticles due to the surfactant lipid monolayer is negative, with the addition of cationic peptide (ML) and cationic protein (LF) the zeta potential increase to positive numbers as a result the incorporation of ML and LF to nanoparticles confirms. The mean diameter (<100 nm) of the nanoparticles after addition of LF and ML has not changed.

The Results of Tryptophan Fluorescence

To study the interaction and the incorporation of ML and LF with the lipid monolayer of PFOB nanoparticles intrinsic tryptophan fluorescence (TRP19) (excitation, 280 nm) were used. The spectra clearly showed a strong fluorescence emission at wavelengths around 350 nm and 332 nm for ML and LF respectively. With the addition of ML and LF to nanoparticles a significant reduction in emission

intensity of fluorescence was observed that the process is called fluorescence quenching.

Quenching the fluorescence emission intensity of the supernatant solution shows that LF added to the nanoparticles completely been connected to the nanoparticles. Results are shown in Figure 7.

The results of Circular Dichroism (CD) spectroscopy

For final confirmation of PFOB nanoparticle-bound ML and LF, CD spectra was used. This is a very sensitive spectroscopy to reveal major structural changes in the secondary structure of ML and LF occurred upon binding to the nanoparticles. When ML is associated with the PFOB nanoparticle lipid monolayer, a double negative peak is observed that characteristic of a R-helical configuration (Figure 8a) and when LF is associated with the PFOB nanoparticle lipid monolayer, a negative peak is observed (Figure 8b) also when ML and LF are associated with the PFOB nanoparticle lipid monolayer, a negative peak is observed (Figure 8c).

The Results of the Effectiveness of Nanoparticles Loaded on MCF7 Cells Using MTT Test

In this study, inhibitory effects of nanoparticles loaded with ML and LF on the viability and proliferation of breast Cancer cell line (MCF7) was investigated. Results of MTT assay indicated that ML and LF and nanoparticles loaded with ML and LF show cytotoxic effect on breast cancer cells. As the figure 9 shows with increase of ML and LF loaded nanoparticles concentration increases cancer cell death. This is despite the fact that with increase of free ML concentration is less effective in cell death. Can be concluded when ML and LF to be loaded on nanoparticles, the most surface of its will be available and therefore a greater effectiveness on cell death. The results show that the higher concentration of ML and LF increase percent of cell death.

CONCLUSION

Due to the unique properties of ML and LF including antimicrobial activity, immune system, and even their anti-cancer properties, appears to be great potential in the use of these natural biological

products to fight diseases. In recent years, much research has been done on them. However, many research experiments in order to gain a better understanding of the activities and their interactions and safe and full use of them is still needed. Due to the sensitive nature of the ML and LF against proteolysis, a lot of research such as the use of nanoparticles to carry has been done. The purpose of this research was to design, formulate and evaluate Perfluorooctyl Bromide (PFOB) nanoparticles for ML and LF deliver in pharmaceutical applications.

The results showed that ML and LF alone effective on cancer cells and also by increasing the concentration of LF from 5.37 to 150 ($\mu\text{g/ml}$) and with increasing the concentrations of ML from 0.005 to 0.1 ($\mu\text{g/ml}$), increase cancer cell death. This effect was intensified when ML and LF was loaded onto the nanoparticles that due to the protein greater availability levels when loaded on nanoparticles.

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