The Novel Method for Delivery miRNA-34a: a New Cationic PEGylated Niosomal Formulation for the Treatment of Breast Cancer

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Abstract

Background: The reactive surface of nanoparticles makes it possible to simply modify with a biocompatible coating and load with therapeutic agents such as siRNA, miRNA, an anti-cancer drug, and antibody. MicroRNAs, like the noncoding RNAs, contribute critical to the regulation of numerous cellular functions via transcriptional silencing. MicroRNAs (miRNAs) have enormous potential in cancer treatment, however, it is difficult to deliver them effectively to most solid tumors. The encapsulation of miRNA-34a in niosome nanoparticles is an attractive strategy for biopharmaceutical resources against cancer. The present study investigated the effectiveness of anticancer activity against MCF-7 and T47D human breast adenocarcinoma cells of a new noiosome system composed of nonionic surfactants.

Methods: We used the optimum formulations to transfer miRNA-34a to breast cancer cells, providing potential benefits, such as exceptionally high entrapment efficiency (almost 100%), spherical shape, suitable positive charge (zeta potential~ + 24 mV) and small diameter (~100 nm).

Results: The miRNA-34a-niosomes represented improved cytotoxic activity against the cancer cells compared to readily dispersed miRNA-34a. The resulting data indicate that delivery of miRNA-34a via niosome can affect tumor suppression, highlighting its promising anticancer effects in breast cancer cells.

Conclusion: In conclusion, the developed a new carrier to improve the delivery of miRNA-34a into the tumor cells. The formulation provided in the present work is stable with a sustained release, high efficiency of miRNA-34a loading having a diameter of 115 nm. miRNA loading is performed without potentially harmful chemical reactions. Niosomes loaded with miRNA-34a in this study represented significant cytotoxic effects against the human breast cancer MCF-7 and T47D cells, which highlights their potential effect on this type of cancer cells.

Background

Breast cancer, the most common cancer diagnosis and is a second most common cause of cancer death in women worldwide. Above 50% women with breast cancer may develop metastases to the bone, liver, lung, or brain (Shah et al., 2018). Although many strategies for breast cancer treatment are currently being followed, this cancer is still considered a major health problem for women. Chemotherapy is one of the most common treatment strategies in breast cancer. However, the use of chemotherapy is restricted to certain circumstances, like before or after surgery or in advanced stages of the disease. On the other hand, chemotherapy with anthracyclines (e.g., doxorubicin), taxanes (e.g., paclitaxel), 5-fluorouracil and/or cyclophosphamide is evident to produce a high toxicity which limits their clinical use. On the other hand, targeted therapy allows to get a selective location of the drug at tumor mass, thereby, decrease in therapy-induced toxic effects in cancer patients. It is also possible to increase of the antitumor efficacy of this process. In this instance, nanocarriers containing anticancer drugs may play an important role to achieve the targeted chemotherapeutic goals (Fraguas-Sánchez et al., 2019).
The MicroRNAs (miRNAs) are eighteen to twenty-eight nucleotides containing single-stranded RNA molecules which are not transcribed into proteins during transcription. It takes part in post-transcriptional regulation by binding to the messenger RNA and inhibiting the expression of specific genes (Treiber et al., 2018). These types of molecules are usually expressed in eukaryotes such as animals and plants, and some viruses (Liu et al., 2017a,b). The first miRNA was reported in Caenorhabditis elegans is Lin-4, then further studies identify more than 18,226 other types of miRNAs in the same organisms such as 22 (nt) lin-4 and 21 (nt) let-7 (Melo et al., 2014).

The p53, a tumor suppressor protein, has lost its function in a large group of human malignancies. Generally, it plays a vital role in cellular reactions to stress, such as activation of oncogenes and DNA damage. After induction, p53 alters the expression of numerous target genes arrested by the regulated cell-cycle, apoptosis, increased DNA repair, in addition to inhibiting angiogenesis. According to independent investigations, the miR-34 class is the most common miRNAs induced by p53. MiR-34s are often silenced in various tumor entities, indicating their importance as the tumor suppressors. MicroRNAs are small, non-coding RNAs that regulate gene expression at the post-transcriptional level and contribute critically to tumorigenesis. They contribute to apoptosis, differentiation and cell proliferation during the development of mammals (Jemal et al., 2011).

Furthermore, miRNAs regulated by p53 mediate numerous p53 tumor suppressor functions. The observations show reduced levels of defined miRNAs that act as suppressors of the tumor that leads to cancer. Mendell and Kent illustrated that miRNAs suppressing tumors include miR-15a, -16-1, -143, let-7, -145, and −34 groups experimentally (Brannon-Peppas and Blanchette, 2004). The miR-34 family members are an important mediator of tumor suppression. Numerous studies indicate that, by the ectopic expression of miR-34s, the epithelial to mesenchymal transition, proliferation, cancer cell metastasis, invasion, and migration are avoided (Bader and Brown, 2010). Furthermore, the delivery of miR-34 must contain the first viral vector. Recently, delivery systems through vesicular carriers have attracted great interest as a result of providing high encapsulation efficiency, enhancing drug solubility, reducing side effects, prolonging blood circulation and the ability to target a specific area. Two useful vesicles that have been used in the miRNA delivery system are liposomes, niosomes. The development of lipid-based therapeutic nanoparticles for delivery of miRNAs (Bader and Brown, 2010; Brannon-Peppas and Blanchette, 2004; Jemal et al., 2011). These researchers addressed the opportunities for therapies based on advanced miRNAs, with a focus on aspects of toxicity and proper cellular uptake, among others. Niosomes are the surfactant tools made from various nonionic surfactants. They are spherical unilateral or multilamellar structures that carry hydrophilic, hydrophobic drugs and genes that can be used to treat many types of cancers (Bartel, 2004).

Breast cancer, one of the most prevalent cancer, is the leading cause of death in women worldwide (Suzuki et al., 2014). The occurrence of breast cancer increases due to the alterations in environmental, lifestyle, and hormonal risk factors. Chemotherapy is one of the three main methods (parallel to surgery and radiotherapy) for the treatment of cancer and has a vital role in the clinical treatment of cancer (Matjaz Rokavec et al., 2014). New therapies are being developed to improve the clinical outcomes of
breast cancer, such as gene therapy, hormone therapy, and combined therapy. In this context, miRNA-based therapies are the promising policy that uses the same principle due to a single miRNA has several targets in the tumor microenvironment (Hermeking, 2010; Li et al., 2014; Okada et al., 2014).

In this study, we loaded miRNA-34a in niosomal formulations to improve efficacy in MCF-7 and T47D human breast adenocarcinoma cells. Along with optimization and formulation design, we assessed the release profile and intracellular delivery to improve the cytotoxic capacity of the miRNA-34a niosomal formulation in the cancer cells.

Results

Physico-chemical characterization of nano complexes

Lipid-based drug delivery systems, or lipid carriers, are being extensively employed to enhance the bioavailability of poorly-soluble drugs as it can incorporate both lipophilic and hydrophilic molecules and protect them against degradation in the system (in vitro and in vivo). Average particle size/diameter and the PDI, among others, are the most important physical attributes of lipid-based nanocarriers that determine their safety, stability, and efficacy in the test systems. The suitability of nanocarrier formulations for a particular route of administration depends on their average diameter, PDI and size stability, among other parameters (Danaei et al., 2018). In this study, after evaluating different niosomal formulations, the optimal formulation was specified to achieve a small vesicle size. Using tween 80 as a surfactant for the preparation of nano complexes, it was observed that the increase in cholesterol content increased the mean diameter of niosomes (F1→F5). The ratio of surfactant cholesterol did not affect PDI and vesicle zeta potential. In all cases, PDI was less than 0.3, implying no aggregations in vesicles (Table 1).
Table 1
The effects of the non-ionic surfactant tween 80 including cholesterol with different molar ratios on Zeta potential (mV) and niosomes ion size.

| Code | Mole tween 80 (%) | Mole cholesterol (%) | PDI       | Size (nm)     | Zeta potential (mV) |
|------|-------------------|----------------------|-----------|---------------|---------------------|
| F1   | 90                | 10                   | 0.281 ± 0.66 | 101 ± 0.12    | -23.00 ± 0.34       |
| F2   | 80                | 20                   | 0.241 ± 0.34 | 103 ± 0.37    | -18.41 ± 0.42       |
| F3   | 70                | 30                   | 0.214 ± 0.13 | 113 ± 0.56    | -1.86 ± 0.86        |
| F4   | 60                | 40                   | 0.235 ± 0.26 | 119 ± 0.45    | -0.97 ± 0.65        |
| F5   | 50                | 50                   | 0.264 ± 0.12 | 125 ± 0.65    | -21.78 ± 0.71       |

Values are mean ± SD

The specimens were assessed at 100 kV. When depositing a drop of the specimen on a carbon-covered copper screen, it was covered with uranyl acetate for 2 min after drying. Then, it was rinsed with distilled water. To determine the physical stability of F8, the vesicle size, zeta potential, and the PDI were examined at 4°C after 60 days of storage. No considerable changes were found in the vesicle size, zeta potential and PDI of the enhanced formulation (F8) compared to specimens taken recently (p-value < 0.05). The optimal stability of the formula was confirmed by these findings.

To obtain less aggregation, smaller niosomes, and enhanced stability, 5% of PEG was introduced in F2. The niosomal formula F6, comprising 5% of PEG, represented a smaller diameter and lower PDI than the formula F2. The negative charge of the polymer can be limited by the use of PLGA nanoparticles in gene delivery. A fine interaction with the negative charge of the nucleic acid phosphate group may be obtained (Ramezani et al., 2017). In our study, the number of positive charged particles was increased by adding 10 and 15% of DOTAP to F6. A marked sharp increase in the positive charge is observed in F8. The findings indicated that the niosomal formulations comprising tween 80; cholesterol: PEG DOTAP with a 4:1:0.9:0.3125 molar ratio (F8) as the optimal formula (Table 2).
Table 2
The effects of DSPE-mPEG (2000) and cationic phospholipid DOTAP on Zeta potential (mV), size ion Niosomes.

| Code | Mole tween 80 (%) | Mole cholesterol (%) | Mole DOTAP (%) | Mole PEG (%) | PDI | Size (nm) | Zeta potential (mV) |
|------|-------------------|----------------------|----------------|--------------|-----|-----------|-------------------|
| F6   | 76                | 19                   | -              | 5            | 0.166 ± 0.22 | 93 ± 0.19 | -19.24 ± 0.2      |
| F7   | 68                | 17                   | 10             | 5            | 0.153 ± 0.01 | 89 ± 0.23 | +14.23 ± 0.54     |
| F8   | 64                | 16                   | 15             | 5            | 0.154 ± 0.17 | 82 ± 0.25 | +23.56 ± 0.23     |

Values are mean ± SD

In this study, the nanoparticles were synthesized by using tween 80 as a safe surfactant and were compared based on the PI, size, and zeta potential of several formulations. Cholesterol is a stabilizing factor that increases the mean diameter of niosomes by incrementing the cholesterol content (Emi et al., 2005). The improvement in stability and the decrease in the mean size diameter are caused by the presence of PEGylation, such as the formula of noisome (Misso et al., 2014). Hence, 5% of noisome PEG was inserted in the F5 formula. The results showed that the F6 has a smaller diameter and a smaller PDI than the formula F5. Moreover, the addition of DOTAP (cationic lipids) affected the vesicle size, transfection efficiency, and polydispersity index. As noted, the decrease in the polydispersity index, vesicle size and increase in zeta potential happened with the addition of 10–15% of DOTAP to the formula F6 and F7. In order to interrupt the nanovesicles, aggregation is vital for the introduction of a charge on its surface. Zeta potential is a good indicator for this due to particles with large zeta potential presumably repelled by each other, hence, they will have no tendency to aggregate (Saito et al., 2015). Following storage for 60 days, the existence of PEG and DOTAP in vesicles was stable and other physical characterization was similar. The internal structure of niosomes was evaluated by AFM (atomic force microscopic) and SEM (scanning electron microscopy). As shown in Fig. 1a, F8 was spherical in shape. SEM photographs indicated that the niosomal vesicles were round with smooth surfaces (Fig. 1b).

**In vitro drug release study**

A pH-sensitive outline was revealed by the release of miRNA from the displayed targeted niosome (Fig. 2). Complexes of niosome–miRNA were stored in a medium (PBS/RPMI) at two pH values (5.4 and 7.4). The release of miRNA was dependent on time and the pH value. For the non-targeted formulation, a similar trend was found. According to Fig. 2, the decrease in pH enhanced the release of the gene (pH 5.4 mimics the pH in cancer tissues). The cumulative miRNA-34a release from the niosome reached 40% after 48 h, at a pH of 5.3, and only 23.56% at a pH of 7.4, respectively. The pH impact consists of our previous work and controlled release modeling as previously published. The localized and efficient delivery to the tumor
region and intracellularly within tumor cells is ensured by the low release at neutral pH and improved release at a lower pH that normally occurs through hypoxic circumstances in the tumor tissue and within the intracellular lysosomes (Hämälistö and Jäättelä, 2016).

**Encapsulation of miRNA-34a**

To encapsulate the miRNA-34a, a new representation of the assessed nanoplexes is required to evaluate the impact of this macromolecule on the physicochemical properties of the nanocarrier. DLS analysis was performed to evaluate the mean size, zeta potential, and PDI of empty and miRNA-34a-loaded nanoparticles. In the incubation time miRNA-34a was introduced in a different volume of nanoplexes and incubated for one hour at 37°C. Gel electrophoresis was performed on 1% (w/v) of agarose gel at 120 V for 40 min using TAE buffer (most effective ratio of miRNA (ng) niosome (mg). The figure was arranged from left to right as lane A for free miRNA, lane B for nanoplex (20 mg), lane C for miRNA (4 ng), lane D for miRNA (2 ng), lane E for miRNA (1 ng) and lane F for DNA ladder (non-migrated niosome) (Fig. 3).

**Leakage stability**

Complexes were kept for 4 months at 4 °C to analyze the impacts of long-term storage on the leakage of mi-RNA from the nanoplexes. For all prepared formulations, the stability was determined spectrophotometrically (at 260 nm) (Fig. 4(a)). The complexes showed stability for all formulations, after 4 months with the lowest leakage (1%) for code E and highest for code B (10%).

**Zeta potential stability and size**

Zeta potential is the electrostatic potential at the electrical double layer surrounding a nanoparticle in a solution. Nanoparticles having zeta potential value between −10 and +10 mV are considered approximately neutral, while more than +30 mV or less than −30 mV are considered strongly cationic and strongly anionic, respectively. Since most cell membranes are negatively charged, zeta potential can affect a nanoparticle's tendency to permeate membranes, with cationic particles generally displaying more toxicity associated with cell wall disruption (Clogston and Patri, 2011). In this study, the zeta potential and size of all formulations of miRNA nanoplexes were monitored over a month (Fig. 4(b) and (c)). It is observed that the biggest change in the diameter was in the formulation of group A and the most stable formulation was E, respectively. This result was repeated in the zeta potential approximately, in contrast, the zeta potential increased, while reducing the size trend. More stability was revealed by the counterpart, reaching an increase of only 2.8%.

**Cytotoxicity assays**

The *in vitro* antitumor impact of miRNA-34a-loaded nanoplexes was assessed using the MTT test on MCF-7 and T47D cells as a cancer cell, and MCF-10a as a normal cell. First, the toxicity of the empty nanoplexes to MCF-7 cells was confirmed. The result showed that the empty nanoplexes did not impose any toxicity, indicating that any anti-proliferative activity is associated with the entrapped miRNA-34a. A considerable reduction was found in cell viability after adding miRNA-34a/nanoplex to cells, followed by
incubation for 24 h, which was confirmed after 48 h (Fig. 2(b)). The miRNA-34a/noisome significantly decreased the growth of cancer cells in a concentration-dependent manner. We found a similar cycle for MCF-10a cells, although resistance to normal cells is greater than the cancer cells (Jabr-Milane et al., 2008; Youle and Strasser, 2008; Zhang et al., 2011; Lv et al., 2014; Misso et al., 2014; Saito et al., 2015; Wang et al., 2016; Baek et al., 2017; Emi et al., 2019). A recent study reveals that the miRNA-34a gene coloaded formulation presented a potent antitumor efficacy in colorectal cancer cells, especially when cotreated with irinotecan (a potent antitumor chemotherapeutic agent in clinical practice which is used for treating various malignant tumors) with good biocompatibility (Li et al., 2020).

**miRNA-34a niosomal cellular uptake tests**

Cellular uptake tests were carried out to assess the cell uptake performance of miRNA-34a niosomal formulations within MCF-10a cells, as a model for normal human mammary epithelial cells and MCF-7 and T47D cells as a model for cancer cells. To monitor the intracellular delivery of miRNA-34a, stimulations of miRNA-34a were labeled with FAM fluorescent dye to visualize the miRNA-34a cell uptake. According to Figure 5, followed by incubation for 4 h, FAM-labeled miRNA-34a (red in the web version) was found in the perinuclear areas of the cytoplasm. Yellow stains (in the web version) were created on the merged image by a blue and red fluorescence overlay (DAPI) of the FAM-miRNA-34a NPs-treated cells (Figure 5b). The nucleus was marked with DAPI in blue (in the web version) in all cells. It was broadly reported that, after 4 h, data indicated the possibility of effective delivery of miRNA-34a to breast cancer cells through nanoparticles. Cellular uptake experiments were demonstrated by adding empty noisome, free miRNA-34a, and miRNA-34a/noisome to the cells. The entry of miRNA-34a into the cytoplasm of cells was clearer with more noisome than the free miRNA-34a in both cell lines (Kent and Mendell, 2006; Bommer et al., 2007; Ibrahim et al., 2011; Wang et al., 2018)

**Discussion**

The use of miRNAs for cancer therapy is based on the finding that the expression of miRNA is deregulated in cancer tissues (Chang et al., 2007). The highly effective therapies of miRNA are based on their ability to express oncotic genes, regulating the pathways that are involved in the development and progression of tumor growth. Moreover, distinctive miRNA expression profiles have been associated with specific types of cancer. On the other hand, replacement therapy with miRNA mimics is used to restore miRNA levels and their tumor suppression properties when miRNAs are down-regulated. Because the objective of this replacement mimics miRNA should be loaded into RISC to silence its target mRNAs. This double-stranded miRNA mimics are preferred over single-stranded mimics because the duplex structure has been found to facilitate RISC loading and thereby enhance the gene silencing efficacy (He et al., 2007). A major challenge in this therapy is the delivery of miRNAs to cancer cells because miRNAs are quickly degraded by nucleases and are cleared. Moreover, the entering mimic of miRNA into cells is prevented by negative charge and high molecular weight (Raver-Shapira et al., 2007). To overcome this problem, nanocarriers can be used. It also enhances the delivery of anticancer therapies to tumor cells. In this technology different materials can be used, including polymeric and many colloidal formulations.
The new biopharmaceutical modeling of the encapsulation of old drugs, whose delivery systems make them more effective at lower dosages and results in decreased side effects.

MiRNA-34a belongs to a signaling network involving p53 and Sirt-1, results in DNA damage with further downstream signals that induce senescence or apoptosis in cancer cells (Smit-McBride et al., 2014). It is evident that transactivation of miRNA-34a by p53 influences the gene expression, thereby promotes apoptosis in cancer cells (Tarasov et al., 2007). In this study, we also found a significant cytotoxic effect of the niosomal-miRNA-34a. It may be due to the nanocarrier incorporated with miRNA-34 significantly increased the cellular uptake of cationic PEGylated niosomal formulation which may increase the transfer of miRNA-34a effectively in the cancer cells.

Improving stability while reducing the mean size diameter due to the presence of PEGylation in the formula of noisome can impart an improved cytotoxic effect on the cancer cells (Chang et al., 2007). We added 5% noisome PEG to the F5 formula. However, the findings suggest that F6 has a smaller diameter with a smaller PDI than the F5 formula. An addition of DOTAP (cationic lipids) affected on the vesicle size, transfection efficiency and PDI values of the niosomes. As can be seen, a decrease in PDI, the vesicle size and the increase in zeta potential occurred when 10–15% DOTAP was added to the F6 and F7 formula. For stopping the vesicular aggregation, it is essential to introduce a charge on their surface. A good indicator of the size of this barrier is the zeta potential. If all particles have sufficiently large zeta potential, they presumably repel each other to protect from the aggregation of the niosomal particles (Chiche et al., 2010). We have seen that the presence of DOTAP and PEG in the vesicles was established and other physical characterization was similar in the nanoplexes after storage for 60 days,

In this study, we observed a clear difference of the cellular uptake capacities of the cell lines for empty noisome, free miRNA-34a and miRNA-34a/niosome. The entry of miRNA-34a into the cytoplasm of cells was improved with noisome than free miRNA-34a into both cell lines. p53 expression of miRNA-34a/nanoniosome formulation is compared to the free miRNA-34a in MCF-7 and T47D cells in which the expression of this protein was significantly (p < 0.05) increased in the miRNA-34a/nanoniosomal group.

**Conclusion**

Considering the deregulation of miRNA expression in cancer tissues, miRNAs are used for cancer therapy. Highly effective miRNA therapies are based on their ability to express oncotic genes, regulating the pathways included in the development and progression of the tumor growth. Furthermore, specific types of cancer have distinctive miRNA expression profiles. By down-regulating miRNAs, miRNA levels and their tumors suppressing features are restored by replacement therapy with miRNA simulators. miRNA simulators need to be loaded on RISC to silence their target mRNAs. These double-stranded miRNA simulations are favored over single-stranded simulations, since the duplex structure facilitates the loading of RISC and enhances the efficacy of gene silencing. A major challenge in this therapy is to deliver miRNAs to cancer cells, since miRNAs are degraded and cleared quickly by the nucleases. Besides, the entry of miRNA mimics into cells is prevented by high molecular weight and negative charge. To
overcome these problems, nanocarriers can be used. We have developed a new carrier to improve the delivery of miRNA-34a into the tumor cells. The formulation provided in the present work is stable with a sustained release, high efficiency of miRNA-34a loading having a diameter of 115 nm. miRNA loading is performed without potentially harmful chemical reactions. Niosomes loaded with miRNA-34a in this study represented significant cytotoxic effects against the human breast cancer MCF-7 and T47D cells, which highlights their potential effect on this type of cancer cells.

Materials And Methods

Materials

Ovarian cancer cells (MCF-7 and T47D human breast adenocarcinoma cells) were prepared from the Pasteur Institute (Tehran, Iran). Tween 80 was bought from DaeJung Chemicals & Metals (Seoul, Korea). DOTAP and cholesterol (1, 2-dioleoyl-3-trimethylammonium-propane) were obtained by Sigma-Aldrich (MO, USA), respectively. PBS tablets, DMSO (dimethyl sulfoxide), dialysis bag (MW=12 kDa), MTT (5-diphenyl tetrazolium bromide; 3-(4, 5-dimethylthiazol-2-yl)-2) and paraformaldehyde solution were supplied by Sigma-Aldrich (MO, USA). DAPI (40, 6- diamidino-2-phenylindole) was obtained from Thermo Fisher Scientific (MA, USA). No additional purification was considered for other chemicals, salts and solvents with analytical grades, unless specified. The sequences of the miRNA-34a Primers were synthesized as follows: forward, CTTGAACTCCTGGGGCCTGAAG; reverse, GCCAAAGAAACACTCACAGCT. Eurofins Genomics Ebersberg (Ebersberg bei München, Germany) was used to synthesize the sequences of the miRNAs. Fluorescence microscopy was used to label the 50th end with FAM to allow for tracking.

Niosome preparation

The thin-film hydration technique was used to make niosomes (Matsumura and Maeda, 1986). Tween 80 (DaeJung Metals and Chemicals, South Korea) cholesterol (Sigma-Aldrich, MO, USA) was accurately calculated and dissolved in 100 μL of choloform C. A thin lipid layer was formed under reduced pressure on a rotary flash evaporator (Ultrasonics GmbH, Heidolph, Germany). Then, the film was hydrated using 3 mL of phosphate buffered saline (PBS) at 60 °C and at a pH of 7.4. A microtip probe sonicator (Ultrasonics GmbH, Hielscher Germany) was used to sonicate the hydrated thin lipid for 30 min to reduce the mean size of vesicles. Niosomal formulations were screened for physical characterization.

Then, the polyethylene glycol (Lipoid GmbH, DSPE-mPEG 2000, Lipoid PE 18:0/18:0-PEG2000, Darmstadt, Germany) and cationic lipid DOTAP (1,2-dioleoyl- 3-trimethylammonium-propane, Sigma-Aldrich, MO, USA) were inserted to improve the stability of niosomal formulations. We used a rotary evaporator (Ultrasonics GmbH, Heidolph, Germany) to remove the organic solvent at 45 °C. Then, the layers were hydrated by the addition of PBS (pH 7.4) for 45 min at 60 °C to achieve the niosomal suspensions. A microtip probe sonicator (Ultrasonics GmbH, Hielscher, Germany) was used to sonicate the niosome suspensions for 15 min to decrease the mean size of the vesicles (Bader et al., 2011; Whitehead et al., 2010; Fernandez-Piñeiro, 2017; Babaei et al., 2020).
Physical characterization of niosomal vesicles

The PDI (Poly-Dispersity Index), zeta potential, and the size distribution of the noisome particles were calculated by the dynamic light scattering method, using a ZetaPALS particle size and zeta potential analyzer (Holtsville, Brookhaven Instruments, NY, USA). The dispersed light was found at an angle of 90° and at room temperature, and specimens in 1700 µL of deionized water (0.1 mg/mL) were made and calculated after preparation. All measurements were triplicated to calculate the mean value. Photomicrography representing the surface morphology of niosomes were taken using a scanning electron microscope (SEM) (model sm-5510, JELO Company, Tokyo, Japan). A drop of the Nanoniosome solution dissolved in water was placed on a mesh copper grid 400 and then placed in a vacuum desiccator to evaporate the solvent. Finally, specimens were covered with a gold coating to make them conductive, after assessing the surface morphology using SEM with a 100 W power instrument (model KYKY-EM3200-30 kV, Peking, China). To use AFM (Atomic Force Microscopy), similar samples were also prepared (Chiche et al., 2010).

Physical stability examination

After 60 days of storage, the physical stability of the niosomal was determined. Changes in zeta potential, particle size, and PDI were evaluated at 14, 28, and 60 days (Seo et al., 2019).

Cell lines and culture conditions

T47D cells (the Iranian Biological Resource Center, Tehran, Iran) and human breast cancer MCF-7 were cultured in the combination of Ham RPMI1640 (InoClon, Tehran, Iran) supplemented with 1 mg/mL penicillin/ streptomycin (Gibco, MA, USA), 2 mM GlutaMAX™-I (100X, Gibco, MA, USA), and 15% of FBS (Fetal Bovine Serum, Gibco, MA, USA). The non-tumorigenic human breast epithelial cell lines MCF-10a (Iranian Biological Resource Center, Tehran, Iran) were developed in a mixture of Ham DMEM/F12, supplemented with 5% horse serum (Gibco, MA, USA), 2 mM GlutaMAX™-I, and EGF (Epithelial growth factor, Sigma-Aldrich, MO, USA) hydrocortisone 0.5 μg/mL (Sigma-Aldrich, MO, USA), 20 ng/mL, 1 mg/mL penicillin/streptomycin, and insulin 10 μg/mL (Sigma-Aldrich, MO, USA), 100 ng/mL cholera toxin (Sigma-Aldrich, MO, USA).

Cytotoxicity examines

The cytotoxicity of different concentrations of miRNA34-a was determined through MTT analysis (Sigma, USA) (Mohammady et al., 2019; Uchegbu and Vyas, 1998). MCF-7, MCF-10a, and T47D cells were cultured in 96-wells plates at 10,000 cells per well. After attaching for 24 h, 200 μL of fresh medium comprising serial dilutions of the various miRNA-34a/niosome formulations were used to treat the cells, including free- miRNA-34a solution miRNA-34a/noisome, and empty noisome. The addition of 20 μL MTT or 5 mg/mL in PBS in each 96 well plate was performed, followed by incubation for 24 and 48 h, and incubation at 37 °C for 3 h. The medium was carefully eliminated during the introduction of 180 μL of DMSO into each well to dissolve the formed formazan crystals. The EPOCH Microplate
Spectrophotometer (synergy HTX, BioTek, VT, USA) was used to record the absorption of each well at 570 nm.

**Nanoniosomal cellular uptake**

For each well, 5×10⁴ cells of MCF-7, MCF-10a and T47D were planted in a 6-well plate and incubation was performed for 24 h for attaching them. Then, the empty noisome, miRNA-34a/noisome, and free-miRNA-34a solutions were used to treat the cells. Rinsing the cells with cold PBS was performed 3 times after 4 and 8 h of incubation and fixed with a mixture of citric acid and methanol (Sigma, USA). To stain the cells, DAPI (0.125 μg/mL, Thermo Fisher Scientific, MA, USA) was used, moreover, a fluorescence microscope was used to photograph (BX61, Olympus, Japan) (Uchegbu and Vyas, 1998; Ertekin et al., 2015; Mohammady et al., 2019).

**Statistical analysis**

All data were analyzed by using the GraphPad Prism (version 6.00 for Windows GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) and the data were expressed as mean ± standard deviation (SD). To compare two independent groups, a student t-test was used, and multiple samples were compared using an ANOVA test. A p value <0.05 was considered significant.

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

**Competing interests**

The authors declare that they have no competing interests.

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**Author Contributions**
AS, MLP, VN and PW conceptualized, designed and supervised the study. NAA, FH, FY and JZR conducted and validated the formal analysis including data analysis. NAA, FH, FY conducted the experiment and wrote original draft and MLP, TM, VN, AS, RH, MTI helped to review the final draft. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1
The physicochemical characteristics of empty nanoplexe nanospheres. Panel (a): AFM micrographs of empty nanosystems; panel (b) SEM micrographs of empty nanosystems.

Figure 2

(a) Investigating the pH-sensitive release of miRNA at pH 5.3 and 7.4 (b) In vitro cytotoxicity of miRNA-34a- on Mcf-7, T47-d and MCF1-a cells as a function of exposure time and miRNA concentration.
Figure 3

Agarose gel electrophoresis of free miRNA, free niosome and miRNA loaded in niosome vesicles to determine the most effective ratio of niosome miRNA.
Figure 4

(a) Monitoring the stability of miRNA loading during storage for 4 months at 4 °C, (b) Monitoring the size stability of nanoplex during storage for 4 weeks at 4 °C, (c) Monitoring the zeta-potential stability of nanoplex during storage for 4 weeks at 4 °C
Figure 5

Cellular uptake of empty noisome and niosomal mi-RNA34-a in (a) MCF-7 cells and (b) T47D cells