Drug Development and Industrial Pharmacy

ISSN: 0363-9045 (Print) 1520-5762 (Online) Journal homepage: https://www.tandfonline.com/loi/iddi20

New approaches to tumor therapy with siRNA-decorated and chitosan-modified PLGA nanoparticles

Behiye Şenel & A. Alper Öztürk

To cite this article: Behiye Şenel & A. Alper Öztürk (2019) New approaches to tumor therapy with siRNA-decorated and chitosan-modified PLGA nanoparticles, Drug Development and Industrial Pharmacy, 45:11, 1835-1848, DOI: 10.1080/03639045.2019.1665061

To link to this article: https://doi.org/10.1080/03639045.2019.1665061

Accepted author version posted online: 06 Sep 2019.
Published online: 19 Sep 2019.

DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY
2019, VOL. 45, NO. 11, 1835–1848
https://doi.org/10.1080/03639045.2019.1665061

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

New approaches to tumor therapy with siRNA-decorated and chitosan-modified PLGA nanoparticles

Behiye Şenel[a] and A. Alper Öztürk[b]

[a]Department of Pharmaceutical Biotechnology, Anadolu University, Eskisehir, Turkey; [b]Department of Pharmaceutical Technology, Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey

ABSTRACT

Objective: In this study, we aimed to develop a candidate modifited polymeric nanoparticle (NP) system that will kill cancer cells by facilitated to apoptosis and also reduce pain.

Significance: The primary goal of treatment, especially for metastatic cancers, is to control the growth of the cancer and to alleviate the symptoms. Pain is one of the commonest symptoms of cancer. In cancer treatment, directing cancer cells to death while simultaneously relieving pain will be a new approach.

Methods: Chitosan-modified PLGA NPs were prepared using an nanoprecipitation technique. The NPs were loaded with flurbiprofen and decorated with folic acid. STAT3-siRNA was adsorbed to these polymeric NPs using antisense technology.

Results: The NPs were small in size (176.9–220.3 nm) with positive zeta potential (+14.1 mV to +27.2 mV). They had high loading capacity and prolonged release properties over 144 hours. Cytotoxicity studies performed with siRNA showed effective electrostatic interaction due to the positively charged NPs. Folic acid facilitated entry into cancer cells and helped to kill them.

Conclusion: The formulation we developed is a potential carrier system for both treatment of cancer and prevention of pain, especially for metastatic cancers.

ARTICLE HISTORY
Received 12 July 2019
Accepted 31 August 2019

KEYWORDS
STAT3 siRNA; flurbiprofen; PLGA nanoparticles; folic acid; chitosan

Introduction

Cancer is characterized by the uncontrolled and irregular division of cells in tissue or an organ. These cells multiply to form the tumor. Over time, cancer cells can spread to other parts of the body through blood and lymph circulation, and continue to grow. This phenomenon is called metastasis, and it is a process that changes the clinical course and treatment methods [1].

Pain is one of the commonest symptoms in cancer patients, especially in bone metastases. Cancer pain presents a challenge for the patient during the disease, and its treatment requires a comprehensive strategy [2].

Studies show that 50% of early-stage and 75% of advanced-stage cancer patients suffer from moderate to severe pain [3]. Although the methods and medications used in the treatment of pain can be seen as adequate from the point of view of physicians, unfortunately one of the two cancer patients has not yet benefited from this treatment. The cause of pain is a verifiable lesion or disorder that is likely to sustain the pain through a related process, such as direct tissue injury or inflammation [4].

Flurbiprofen, derived from phenyl alkanoic acid, is a powerful nonsteroidal anti-inflammatory drug, with analgesic, antipyretic, and anti-inflammatory effects, used to treat acute and chronic pain [5]. The analgesic effect is due to inhibition of prostaglandin synthesis by blocking the cyclooxygenase enzyme [6].

RNA interference (RNAi) is a newly discovered biological process in which RNA molecules inhibit gene expression. It has recently attracted attention in biomedical research. RNAi molecules transported to cells initiate the disruption of complementary messenger RNA (mRNA) molecules through intracellular mechanisms. This halts the production of proteins encoded by mRNAs and decreases gene expression. RNAi molecules include microRNA (miRNA), small interfering RNA (siRNA), and short hairpin RNA (shRNA) [7].

Expression and functionality of siRNAs and miRNAs vary in cancer cells. Since these molecules have been found to significantly inhibit cancer cells, RNAi is an attractive target for the development of innovative therapeutics. However, in clinical practice, there are many obstacles, such as access to appropriate tissue and cell types at safe and effective dosages, long-term stability during circulation and delivery, increased cellular uptake, and monitoring of therapeutic efficacy [8–10].

Therefore, in the development of these therapies, nanoparticles (NPs) that can mediate the transport of effective RNAi molecules with targeting potential are of great interest. These NPs have different dimensions and strategies for surface modification and functionalization. They can be categorized mainly into polymeric, lipid-based or inorganic NPs, dendrimers, metal ions, or exosome mimetics [11,12].

Although many NPs have been designed for RNAi treatments to date, there are no products available on the market, except for Patisiran which received US Food and Drug Administration (FDA) approval in 2018 [13].

In light of this, the aim of our study is to develop a formulation to reduce the most frequently complained types of cancer pain. The formulation contains flurbiprofen, a painkiller, and an siRNA molecule that can cause cancer cells to die. Flurbiprofen, has proven efficacy for pain relief, has been studied previously and was selected as a model drug. The STAT3 gene was selected as the genetic material for regulation of genes related to cell growth and

CONTACT Behiye Şenel behiyek@anadolu.edu.tr Department of Pharmaceutical Biotechnology, Anadolu University, Eskisehir, Turkey

division, cell movement, and cell self-destruction (apoptosis). Folic acid was added to the polymeric NPs in the formulation to facilitate targeting of the cancer cells.

Materials and methods

Materials

Flurbiprofen was provided by Sanovel (Istanbul, Turkey). Resomer®RG504 H (poly(D,L-lactide-*co*-glycolide), acid terminated, lactide:glycolide 50:50, M_W: 38,000–54,000) and Span®60 were purchased from Sigma-Aldrich (St. Louis, MO). Low-M_W chitosan (deacetylated chitin/poly(D-glucosamine), M_W: 50,000–190,000 Da, viscosity 20–300 cP) was purchased from Sigma-Aldrich (Darmstadt, Germany). Pluronic®F-68 (poloxamer-188) was purchased from Alfa Aesar (Kandel, Germany). All other chemicals used were of analytical grade. Folic acid, spectrophotometry-grade dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), and MTT dye were purchased from Sigma-Aldrich (Darmstadt, Germany). STAT3-siRNA (sc-29493) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Methods

Preparation of polymeric nanoparticles

Chitosan-modified PLGA (poly-(lactic-co-glycolic acid)) NPs were prepared by following the nanoprecipitation technique with some modification [14,15]. Formulation ingredients are shown in Table 1. In brief, PLGA (45 mg) was dissolved in 3 ml of acetone together with Span®60 (15 mg), and 3 ml of this solution was added dropwise at a rate of 5 ml/h into 10 ml of aqueous-phase solution under magnetic stirring. The aqueous-phase solution was a 1% (*v/v*) acetic acid solution containing chitosan (0.25% *w/v*) and/or Pluronic®F-68 (0.5% *w/v*) and/or folic acid (0.5% *w/v*). In preparing the folic acid formulations, folic acid was first dissolved in 2 M NaOH and added dropwise to the chitosan solution under magnetic stirring (750 rpm, 5 min). Some turbidity was seen at first. When the pH was adjusted to 5.5 with 2 M NaOH, a clear yellowish solution was formed. In the preparation of formulations containing Pluronic®F-68 and folic acid, Pluronic®F-68 and chitosan solution were mixed first, and then folic acid dissolved in NaOH was added dropwise to this solution. A transparent solution was obtained by the addition of NaOH. Acetone was then allowed to evaporate at room temperature under magnetic stirring for 4 h. The resulting aqueous dispersion was centrifuged to collect the NPs (10,000 rpm, 45 min, 4 °C). The pellet of NPs was washed three times with distilled water. For the preparation of flurbiprofen-loaded chitosan-modified PLGA NPs, in brief, the procedure began with adding 4.5 mg of flurbiprofen to the organic phase. Then, the same procedure above was performed.

Binding of STAT3 siRNA to polymeric nanoparticles

To prepare the siRNA-loaded polymeric formulation, STAT3-siRNA was mixed with all the polymeric dispersions at ratios of 1:1, 2.5:1, 5:1, and 10:1 (N/P, *w/v*). The dispersions were then incubated at 37 °C for 20 min to form electrostatic interactions between the genetic material and NPs [16].

Characterization studies

Particle size, polydispersity index, and zeta potential. Average particle size, size distribution, and zeta potential values of the formulations were characterized by a Zetasizer NanoZS Instrument (Malvern Instruments, Worcestershire, UK). For each parameter, the average of three repeated measurements was taken [16].

Entrapment efficiency. The flurbiprofen content of NPs was assessed directly by extracting flurbiprofen from the nanoparticles (NPs) [17]. Lyophilized NPs (about 5 mg) were weighed and 2 ml of ethyl acetate was added, and the mixture was vortexed to dissolve the particles in the organic phase. All solutions were filtered through a 0.22 μm polyamide filter prior to analysis with a UV-visible spectrophotometer (Shimadzu UV-vis 160, Kyoto, Japan) at 246 nm [18,19]. The entrapment efficiency (EE%) of NPs was calculated by Equation (1) [17,20].

$$EE\% = \left[\frac{\text{actual amount of flurbiprofen in NPs}}{\text{theoretical amount of flurbiprofen in NPs}}\right] \times 100 \quad (1)$$

Dissolution. *In vitro* release of flurbiprofen from NPs was investigated over 144 h, using a dialysis membrane. The release study was carried out in phosphate buffered saline (pH 7.4) in order to simulate physiological conditions. Nanoparticles containing 1 mg of flurbiprofen were placed in a cellulose acetate dialysis bag (M_W cutoff 14,000). After the addition of 1 ml of phosphate buffered saline (dissolution medium), the bag was sealed at both ends. It was then placed in a glass beaker containing 100 ml of phosphate buffered saline at 37 ± 1 °C under continuous stirring at 100 rpm (IKA®Labortechnik RT 15 S000, Staufen, Germany). The receptor compartment was closed to prevent evaporation of the dissolution medium. A sample (2.5 ml) was withdrawn periodically, diluted with an appropriate volume of phosphate buffered saline, and measured at 246 nm [17,20].

Thermal, Fourier-transform infrared spectroscopy, and ^{1}H NMR analyses. The physical state of NPs was characterized by DSC (differential scanning calorimetry; DSC-60 differential scanning calorimeter, Shimadzu Scientific Instruments, Columbia, MI). Samples weighing 3 mg each were analyzed in aluminum crucibles under nitrogen gas (flow rate 50 ml/min), with a heating rate of 10 °C/min and a temperature range between 50 and 350 °C.

Table 1. Formulation ingredients.

Code	PLGA	Span 60	Acetone	Flurbiprofen	CS solution-1	CS solution-2	CS solution-3	CS solution-4
F1-Blank	45 mg	15 mg	3 ml	–	10 ml	–	–	–
F2-Blank	45 mg	15 mg	3 ml	–	–	10 ml	–	–
F3-Blank	45 mg	15 mg	3 ml	–	–	–	10 ml	–
F4-Blank	45 mg	15 mg	3 ml	–	–	–	–	10 ml
F1	45 mg	15 mg	3 ml	4.5 mg	10 ml	–	–	–
F2	45 mg	15 mg	3 ml	4.5 mg	–	10 ml	–	–
F3	45 mg	15 mg	3 ml	4.5 mg	–	–	10 ml	–
F4	45 mg	15 mg	3 ml	4.5 mg	–	–	–	10 ml

CS: chitosan; CS solution-1: 0.25% *w/v* chitosan solutions; CS solution-2: 0.25% *w/v* chitosan solutions including Pluronic®F-68 (0.5%); CS solution-3: 0.25% *w/v* chitosan solutions including folic acid (0.5% *w/v*); CS solution-4: 0.25% *w/v* chitosan solutions including Pluronic®F-68 (0.5%) and folic acid (0.5% *w/v*).

Fourier-transform infrared (FTIR) spectra of NPs were recorded using a spectrometer (Perkin Elmer BX II, Waltham, MA) with KBr discs, and were reported at a spectral range from 4000 to 500 cm^{-1}.

^1H NMR analyses were performed using a JEOL NMR-400 spectrometer (Peabody, MA). Samples were prepared by dissolving formulations in deuterated chloroform ($CDCl_3$).

Pure flurbiprofen, pure folic acid, and blank formulations were also analyzed for use as references for all analyses.

Cell culture studies

Cell viability. The colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to examine the effects of the prepared formulations on the cancer cell lines 3T3, A549, MDA-MB-231, and MCF-7. The tests were performed according to our previous study [16], and involved a 24–48 h incubation period and 2.5, 5, 10, and 20 µl volumes of the formulations. The experiment was repeated three times, and the results were plotted with standard deviations.

Gel retardation studies and serum degradation protection. Gel retardation studies were performed to determine whether there is electrostatic interaction between siRNA and the prepared dispersions or whether polymeric NPs protect siRNA against enzymatic degradation in the presence of serum. For this purpose, dispersions prepared for electrostatic interaction at NPs(µl)/:siRNA (µg) ratios of 1:0.5, 2.5:0.5, 5:0.5, and 10:0.5 were loaded on to gel. In the presence of serum, the 5:1 ratio of dispersion was kept in a DMEM medium containing 20% fetal bovine serum for 30 min, 1, 4, 6, 12, 24, 48, and 72 h. Before being loaded on to a TBE-buffered 1.5% agarose gel, the samples were incubated in a bath incubator at 60 °C for 3 min to terminate serum activity. The analysis was run for 45 min at 50 mV. The images were analyzed by a gel documentation system (Uvitec Alliance 4.7, Cambridge, UK).

Transfection studies. To analyze the internalizing properties of genetic material and NPs to cells, transfection assays were performed that were similar to those previously carried out in our laboratory [16]. FITC-labeled siRNA (Santa Cruz Biotechnology, Heidelberg, Germany, cat. no.: sc-36869) was used as the genetic material. After 24 h, images were evaluated under a fluorescence microscope with 20× magnification.

Statistical analysis. The MTT and transfection assay data are presented as mean ± standard deviation. In the MTT assay, the standard deviation was given on the graphic. The differences between treatment and control groups were analyzed using linear regression for concentration–effect–time curves in Minitab 18. The differences between treatment and control groups for other parameters were analyzed using analysis of variance or Student's *t*-test. A p value less than .05 was considered to be statistically significant.

Results and discussion

Preparation of nanoparticles

Nanoparticles have recently attracted great attention as vectors for gene delivery [21]. One of the major advantages of using NPs for transport of genetic material is the enhanced permeability and retention (EPR) effect, which allows NPs to accumulate in tumors at much higher concentrations than in normal tissue [22]. If carrier size and surface coating are appropriate, they can prevent secretion in the bloodstream before reaching the target cell [23,24].

There are specific formulations based on PLGA and its related homopolymers, e.g. poly(lactic acid) and poly(glycolic acid), which have been approved by the FDA for medical applications. PLGA NPs have proven potential as drug delivery systems for many therapeutic agents (e.g. chemotherapeutic or anti-inflammatory agents) and can be favorable for tumor- and/or DNA-targeting [25,26]. Due to these good properties of PLGA, we preferred to use Resomer®RG 504 H in our study.

Cell surfaces, especially cancer cell surfaces, are usually charged negatively due to the translocation of negatively charged constituents of the inner layer of the cell membrane (e.g. anionic phospholipids, phosphatidylserine, proteoglycans, and glycoproteins) to the cell surface [27]. PLGA in neutral medium has negative surface potential, attributed to the terminal carboxyl groups. It can often be difficult for PLGA NPs to target and interact with cells [28]. For this reason, we performed surface modification with chitosan to enable the NPs to interact with negatively charged cancer cells and to convert the negative zeta potential of PLGA to positive.

Specific ligands can be added to the NP surface to actively target the target site. Ligands functionalize NPs to increase specificity and cell uptake, and bind to receptors or antigens in tumor cells, tumor microenvironment or tumor vasculature [29]. Folic acid, biotin, aptamers, antibodies, and peptides are ligands frequently used in NP preparation [26]. For this reason, we prepared the NPs with a chitosan solution without folic acid (formulations coded F1-Blank/F1 and F2-Blank/F2) and a chitosan solution containing folic acid (formulations coded F3-Blank/F3 and F4-Blank/F4). We used cytotoxicity studies to assess whether or not targeting was achieved.

In this study, we aimed to treat cancer cells with active targeting, while effectively relieving pain and inflammation in the tumor-forming regions. The World Health Organization has published a treatment template aimed at controlling cancer pain. The template uses adjuvant drugs and non-opioids or weak or strong opioids as analgesics in three steps, according to the severity of the pain. In general, non-opioid drugs, such as paracetamol and nonsteroidal anti-inflammatory drugs, are used alone or in combination, and if the pain becomes more severe, weak or powerful opioids are used [30,31].

In this study, we have successfully prepared flurbiprofen-loaded chitosan-modified PLGA NPs by the nanoprecipitation technique for effective relief of pain and inflammation in tumor-induced regions. The nanoprecipitation technique is a straightforward single-step process with high reproducibility, and was initially applied for hydrophobic drugs, such as flurbiprofen [26]. When preparing the NP, the surfactant present in the aqueous phase can have a direct effect on NP properties, such as particle size, distribution, and release rates of the prepared NP [32]. In order to determine the effect of the surfactant on the prepared NPs, the formulations were prepared with and without Pluronic®F-68.

Particle size, polydispersity index, and zeta potential

Characterizations of NPs are primarily evaluated with particle size, particle size distribution (PDI), and zeta potential. The relevant information is presented in Table 2. Particle size obtained in the blank formulation was 176.9–217.3 nm while particle size in the flurbiprofen-loaded NPs was 194.2–220.3 nm. In particle size analysis, it is important to note that particle size increases with active substance loading.

Table 2. Particle size, polydispersity index, and zeta potential.

Code	PS	PDI	ZP
F1-Blank	197.3 ± 2.5	0.278 ± 0.169	26.2 ± 2.2
F2-Blank	176.9 ± 3.7	0.218 ± 0.011	21.33 ± 0.8
F3-Blank	217.3 ± 9.0	0.369 ± 0.014	14.11 ± 0.7
F4-Blank	201.3 ± 3.5	0.264 ± 0.012	18.26 ± 0.2
F1	204.8 ± 3.2	0.302 ± 0.032	27.2 ± 1.1
F2	194.2 ± 4.6	0.261 ± 0.008	26.6 ± 0.2
F3	220.3 ± 9.3	0.363 ± 0.002	16.0 ± 0.5
F4	213.3 ± 3.5	0.284 ± 0.019	17.4 ± 0.6

PS: particle size (nm); PDI: polydispersity index; ZP: zeta potential (mV).

This effect has also been reported for NPs – their particle size increased with the loading of the active substance [14,20,33,34]. Our results are also consistent with this finding. In the comparisons between formulations coded F1, F2 also, F3, and F4 (also F1-Blank, F2-Blank, F3-Blank, and F4-Blank), F2 and F4, prepared with a surfactant (Pluronic®F-68) added to the chitosan solution, were found to have smaller particle sizes. The presence of a surfactant in the aqueous phase during preparation reduces surface tension and stabilizes newly developed surfaces during nanoprecipitation and the production of smaller particles [35–37]. An increase in particle size was also detected in formulations F3 and F4 prepared with a chitosan solution containing folic acid. In this case, due to the strong electrostatic interaction between the cationic amino group of chitosan and the anionic carboxyl group of folic acid, natural conjugation was achieved, and, therefore, an increase in particle size was observed [38]. The NPs loaded with the active agent must efficiently release the drug at the targeted site *in vivo* without aggregation. Therefore, it is important that the NPs have an optimum particle size that prevents them from being excreted by the reticuloendothelial system. It is known that a particle size around 200 nm is optimum to prevent the removal of NPs by body fluids, the kidneys, and phagocytes in the immune system [39]. Although particle size is affected by the presence of surfactant and folic acid, results of previous studies confirm that we produced NPs with appropriate particle size in our study.

The average PDI of blank NPs varied between 0.218 and 0.369, whereas the average PDI of flurbiprofen-loaded NPs was between 0.261 and 0.363. The PDI, which is a ratio that gives information about the homogeneity of the PDI in a given system, reflects the quality of the NP dispersion within the range 0.0–1.0. PDI values $\leq$0.1 indicate the highest quality of dispersion. Most authors recognize PDI values $\leq$0.3 as optimum; however, values $\leq$0.5 are also acceptable [40]. In this study, the PDI values of the F2 and F4 formulations were lower than those of F1 and F3. It is thought that the presence of a surfactant in the aqueous phase during NP preparation reduces PDI values of NPs [41,42]. PDI values obtained in our study support this hypothesis.

Physicochemical properties of NPs, such as particle size and zeta potential, play a key role in the cellular uptake of the NP system. The uptake of NPs by cells is a two-step process-binding to the cell membrane followed by internalization. Binding of NPs to the cell membrane is affected mostly by the zeta potential of the NPs [27]. Cancer cell surfaces are usually negatively charged due to the translocation of negatively charged components of the inner layer of the cell membrane to the cell surface [43]. In our study, positive values for zeta potential are observed in all NPs (Table 2). Zeta potential was in the range from +14.1 to +26.2 mV and from +16.0 to +27.2 mV in blank NPs and flurbiprofen-loaded NPs, respectively. The positive values for zeta potential are a result of the amino groups present in the chitosan and suggest that the NPs are sufficiently modified by the chitosan [28]. A decrease in zeta potential was observed in the formulations F3-Blank and F3 and F4-Blank and F4 prepared with chitosan solution containing folic acid. This may be because folic acid molecules have modified the surface of the chitosan-modified particles and the number of protonated amino groups in chitosan decreased, leading to the drop in zeta potential [38]. Therefore, NPs with positive zeta potential may be effective for cancer treatment.

Entrapment efficiency

Drug EE of NPs is an important factor in formulations because higher loading leads to a lower number of NPs for a given dose of treatment [44,45]. EE% for flurbiprofen were 74.26 ± 3.24%, 72.60 ± 4.80%, 77.24 ± 1.76%, and 76.30 ± 1.84% for F1, F2, F3, and F4 NP formulations, respectively. These results suggest that the range of EE% values obtained is acceptable. Due to poor solubility of flurbiprofen in the external aqueous phase, transition of flurbiprofen to the aqueous phase is relatively small; therefore, a high EE% is obtained in this study [46]. The common ingredients in all formulations are chitosan and PLGA, but folic acid was added to the chitosan solution when preparing the F3 and F4 formulations. EE% of the folic acid-containing chitosan-formulated NPs does not differ from the folic acid-free NP formulations. A similar finding has been reported by previous studies [47].

Dissolution study

A dissolution profile over 24-h is also presented in Figure 1(b). The release of flurbiprofen from NPs continued over 144-h while pure flurbiprofen exhibited a rapid release of 97.28 ± 1.8% (mean ± SD) in 3-h. The dissolution rates for F1, F2, F3, and F4 NP formulations after 144 h were 53.4 ± 6.2%, 40.7 ± 1.2%, 63.2 ± 3.8%, and 59.6 ± 3.9%, respectively, demonstrating extended release from all formulations relative to pure flurbiprofen.

The two main release mechanisms associated with drug release from PLGA NP systems are diffusion and degradation/erosion. The drug release rate is initially controlled by diffusion followed by degradation/erosion [48,49]. There seems to be an inverse relationship between the amount of drug released and particle size. Large microspheres degrade faster than small microspheres [48,50]. This is probably due to the increased accumulation of acidic products during polymer hydrolysis in large microspheres where hydrolysis starts immediately in PLGA systems. Further catalysis of hydrolysis by the acids produced during initial hydrolysis, i.e. the autocatalytic process, leads to more rapid degradation at the center than at the surface of the PLGA matrix. This effect becomes more pronounced as the size of the NP system increases [46]. The F1 formulation with a larger particle size probably degraded relatively faster than F2, F3, and F4, leading to higher release. This hypothesis is further supported by the fact that the F2 formulation had the smallest particle size and the lowest release rate. In addition, slow release rates may be due to both the natural structure of PLGA and the modification of NPs with chitosan [51]. Figure 1(a,b) shows rapid release up to 24 h. Cumulative release rates with burst release at the 24th hour for formulations F1, F2, F3, and F4 were 29.3 ± 2.4%, 26.0 ± 2.8%, 37.5 ± 1.6%, and 30.2 ± 2.6%, respectively. The rapid release of these formulations stopped at the end of the 24th hour and passed to the second phase-slow release. Figure 1(a) clearly shows biphasic release in the NP formulations [52].

As a result, they agree with previous studies, and show that release rates change in relation to particle size. Formulations containing folic acid have not been observed to change release rates, confirming results of previous studies [47].

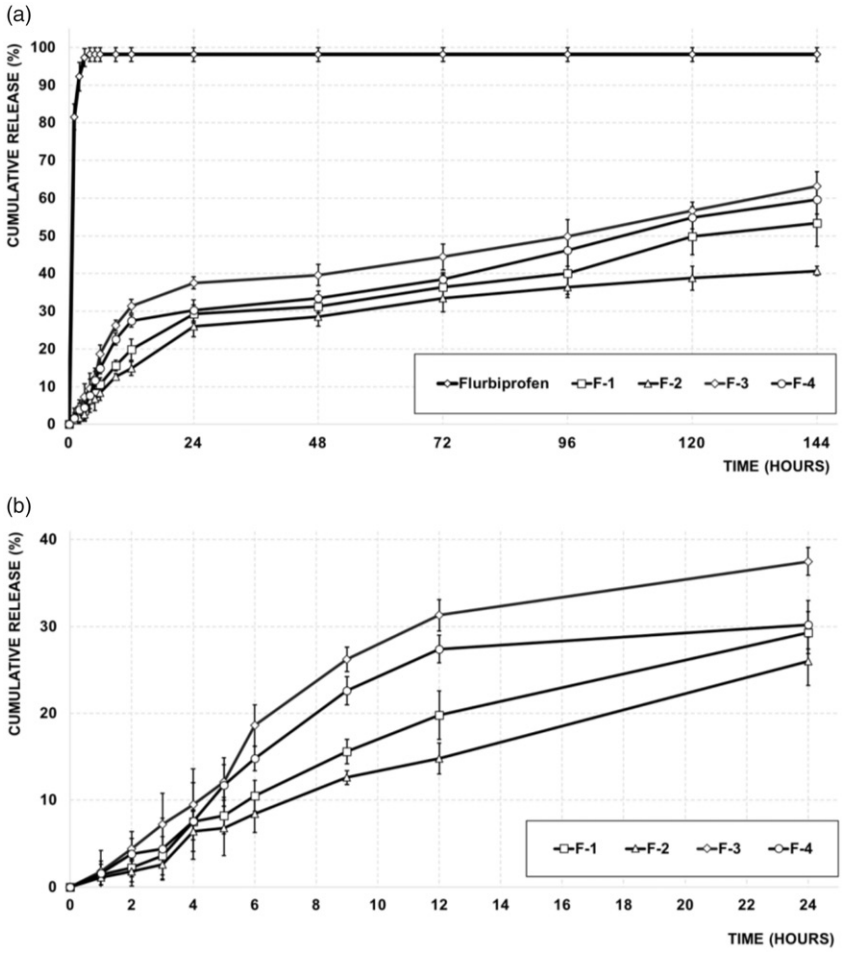

Figure 1. Dissolution profile of pure flurbiprofen and prepared nanoparticles. (a) 144 hours' profile and (b) 24 hours' profile.

Thermal (DSC) analysis

The results are given in Figure 2. The DSC curve for flurbiprofen showed a sharp endothermic peak at 124.82 °C, consistent with previous findings [17,53,54].

When folic acid is heated, the Glu moiety breaks away first at ~180 °C, after which degradation of pterin and aminobenzoic acid takes place at the same time. Moreover, on further heating, amide and acid functionalities of folic acid are lost at ~195 °C, and heating above 200 °C degrades the crystalline form of folic acid to an amorphous form [55]. The DSC curve for NP formulations showed that the sharp endothermic peak for flurbiprofen and folic acid disappeared at 124.82 °C and 196.05 °C, respectively. Complete disappearance of the peaks for flurbiprofen and folic acid may be due either to the formation of a homogeneous polymeric matrix or to the dilution effect of the polymer [14]. The disappearance of the endothermic peaks of flurbiprofen and folic acid NP formulations indicate incorporation of flurbiprofen and folic acid, homogenous matrix formation, and formation of an amorphous structure due to the loss of crystallinity of the drug [17].

Fourier-transform infrared analysis

The FTIR spectra for flurbiprofen and folic acid are similar to those in other studies [56]. PLGA consists of two monomers, lactide and glycolide. Intense bands observed in the region between 1770 and 1750 cm^{-1} are attributed to the stretching vibration of the carbonyl groups present in the two monomers. Medium intensity bands between 1300 and 1150 cm^{-1} are attributed to asymmetric and symmetric C–C(=O)–O stretches. Bands at around 3500 cm^{-1} and 3450 cm^{-1} in the FTIR spectra for lactide and glycolide are attributed to stretching vibrations of the OH group [57]. The absorption bands for chitosan at around 2900 cm^{-1} can be attributed to symmetric and asymmetric stretching of C–H (Figure 3).

Chitosan, a polysaccharide derivative, shows bands typical for these compounds. The presence of residual N-acetyl groups was confirmed by the bands at around 1600 cm^{-1} (C=O stretching of amide) and 1385 cm^{-1} (C–N stretching of amide I) [58]. Figure 3 shows FTIR peaks specific to PLGA and chitosan in all formulations. However, the intensity of the peaks specific to flurbiprofen and folic acid is reduced in the FTIR spectra. Distinctive peaks for flurbiprofen and folic acid were not seen in the spectra, indicating molecular dispersion of flurbiprofen and folic acid in the polymeric matrix, a finding also supported by the DSC analysis. The absence of distinctive peaks for flurbiprofen and folic acid confirmed encapsulation of the drug within the polymeric structure

^{1}H NMR analysis

The results are shown in Figure 4. The spectrum for flurbiprofen exhibits dense bands at $\delta = 1$–2 ppm, at approximately $\delta = 3.5$ ppm, and at $\delta = 7$–8 ppm (Figure 4(a)). Peaks in the $\delta = 7$–8 ppm interval indicate aromatic C–H and peaks at $\delta = 3$–4 ppm represent CH_3 in the spectrum for flurbiprofen.

The appearance of the characteristic resonance peak signals at $\delta = 6.5$ ppm indicates a phenyl ring, which corresponds to the

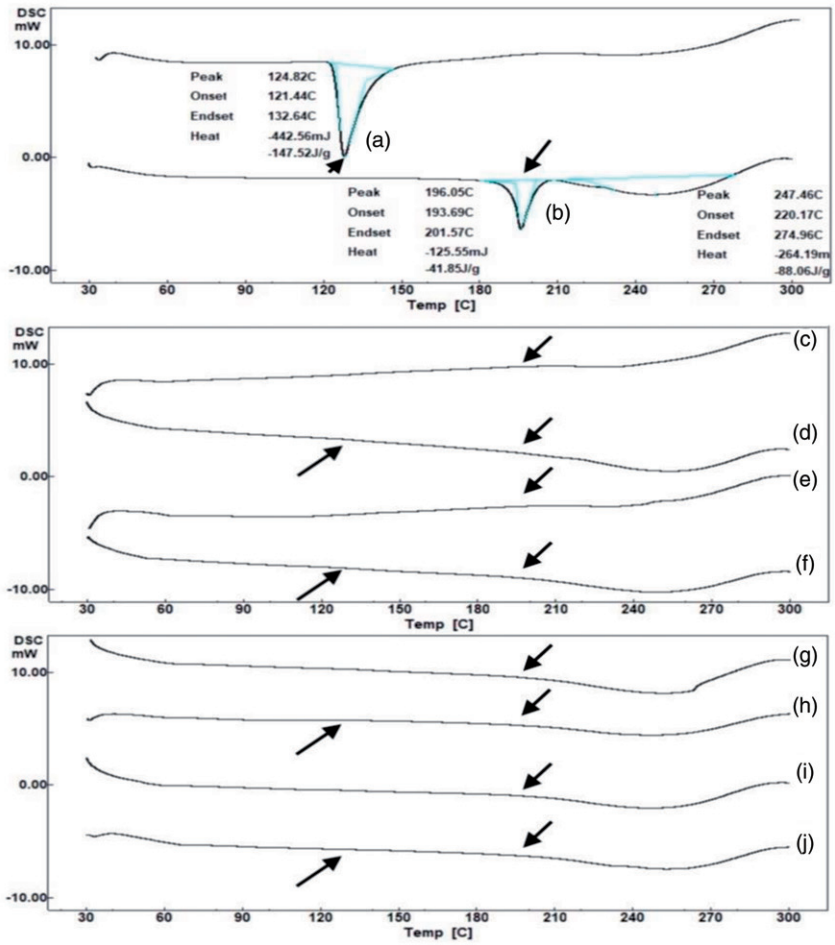

Figure 2. DCS curves of flurbiprofen, folic acid, and prepared nanoparticles. (a) Flurbiprofen, (b) folic acid, (c) F1-Blank, (d) F1, (e) F2-Blank, (f) F2, (g) F3-Blank, (h) F3, (i) F4-Blank, and (j) F4.

aromatic protons of folic acid (Figure 4(b)). The peak signal at $\delta = 4.5$ ppm also assigns a C–H functional group to folic acid. In addition, the peak signal at $\delta = 2.3$ ppm, which corresponds to the CH functional group, is close to the γ-carboxylic group in folic acid [59]. PLGA contains two types of structural unit, the most intense signals for CH ($\delta = 5.1$ ppm) and CH_3 ($\delta = 1.4$ ppm) from lactic acid, and CH_2 ($\delta = 4.8$ ppm) from glycolic acid. Higher CH_2, CH_3, and CH signals in the copolymer caused the corresponding peaks to expand. The ^{1}H NMR spectra for both blank and flurbiprofen-loaded NPs show specific peaks for the PLGA polymer (Figure 4(c–j)). The peak at around 3.5 ppm was assigned as H_2 glucosamine and the peak at 2.5 ppm corresponded to the hydrogens of the methyl group of N-acetyl-glucosamine in chitosan [58]. The spectra for both blank and flurbiprofen-loaded NPs also show specific peaks for chitosan (Figure 4(c–j)). Figure 4(d,f,h,j) shows specific ^{1}H NMR signals for flurbiprofen in NPs, indicating that flurbiprofen has been successfully loaded into the NP formulations. Specific ^{1}H NMR signals for folic acid were also observed in folic acid-containing formulations (Figure 4(g–j)), confirming that folic acid is physically entrapped within NP formulations [59].

Cell viability test

Folic acid-conjugated formulations are most probably internalized through receptor-mediated endocytosis. Uptake of NPs into cells can increase in the presence of the folic acid receptor in cells, and, thus, NPs may show cytotoxic effects [60,61].

To date, NP systems containing folic acid have been developed in different studies; these NPs have been loaded with anticancer agents and biological molecules (DNA, RNA, etc.) and show to be effective *in vitro* [62–64].

In this study, the presence of the folic acid receptor was investigated with the western blot method before analyzing cytotoxicity (data not shown). The results showed that the cell lines MCF-7 and MDA-MB-231 contained high levels of folic acid, but A549 and NIH-3T3 were not adequately expressed. We then completed a cell viability test. As shown in Figures 5 and 6, the cytotoxicity profiles of the folic acid-free and folic acid-conjugated formulations are relatively similar, and the difference between them is not statistically significant ($p > .05$). Moreover, cell viability did not decrease below 80%, even at the highest dose used. However, folic acid-conjugated formulations began to show more cytotoxic effects when STAT3-siRNA (200 ng/well) was added to the formulations. When all cells were evaluated, folic acid-free STAT3-siRNA-loaded formulations caused 25% cell death only at the highest dose applied, while in folic acid-conjugated and STAT3-siRNA-loaded formulations, the highest dose caused cell deaths at a rate of 25%, 16%, 20%, and 38% for A549, NIH-3T3, MDA-MB-231, and MCF-7, respectively. MCF-7 cell lines carrying the folic acid receptor were observed to be affected more because they internalized the NPs better. These results support the

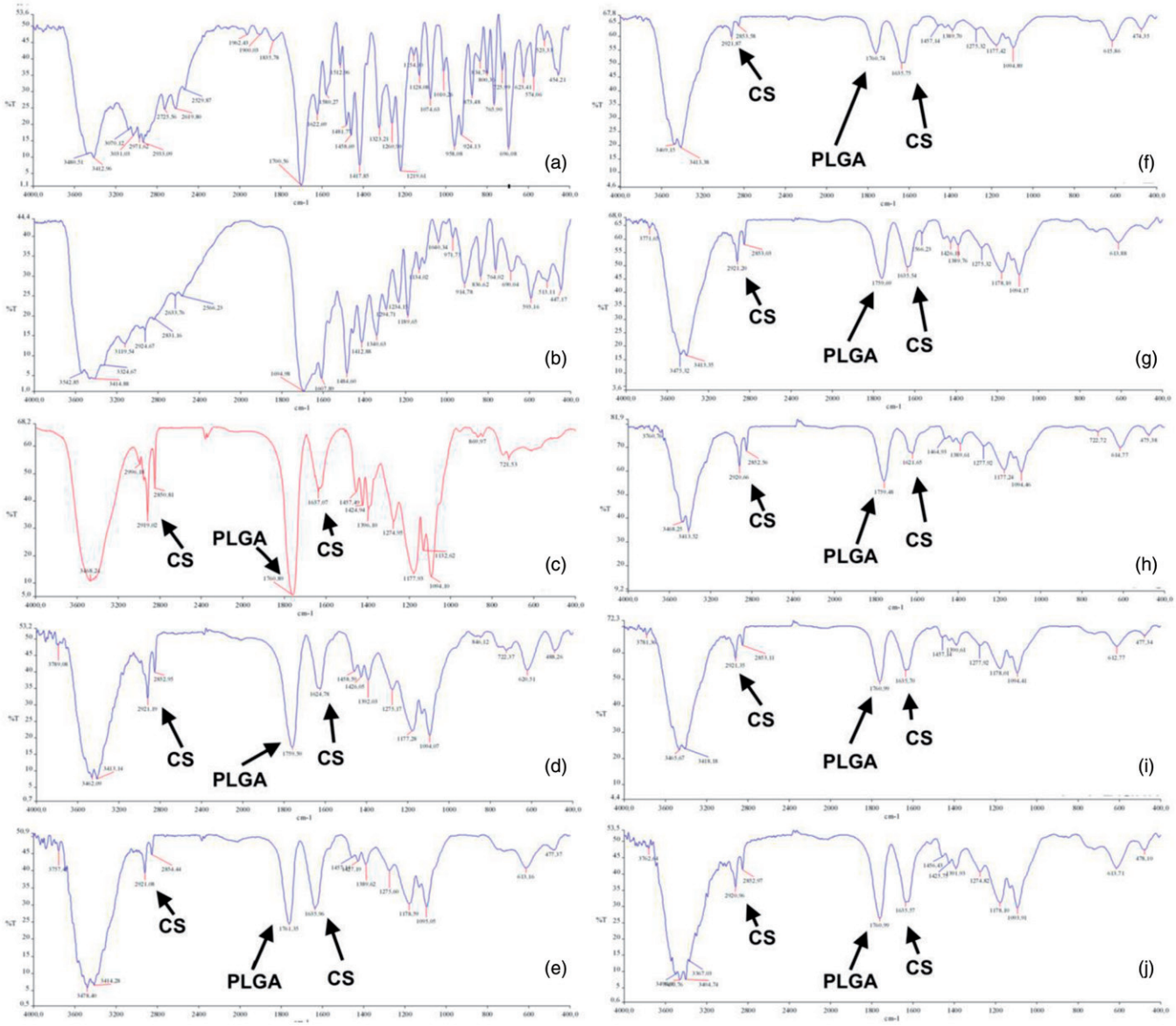

Figure 3. FT-IR spectrum of flurbiprofen, folic acid, and prepared nanoparticles. (a) Pure flurbiprofen, (b) pure folic acid, (c) F1-Blank, (d) F1, (e) F2-Blank, (f) F2, (g) F3-Blank, (h) F3, (i) F4-Blank, and (j) F4. CS: chitosan.

statistically significant cytotoxic increase in the folic acid receptor-containing MCF-7 cells compared to folic acid receptor-free A549 and NIH-3T3 cells ($p \leq .001$) [65–68].

On the other hand, although MDA-MB-231 cells contained the folic acid receptor, STAT3 inhibition did not have a sufficient effect on cell death. Previous studies have shown that STAT3 inhibition of MDA-MB-231 did not cause any apoptotic cell death *in vitro*; this finding supports our results [69].

Since cell viability was more than 90% with flurbiprofen even at the highest dose, no effect of the drug on cell viability was found.

Naked-STAT3 was applied to cells at 100, 200, and 300 ng concentrations, and based on previous studies [40], it was found to be effective on cells at a concentration >125 ng; 200 ng per well was the preferred genetic material concentration in this study (data not shown). When naked-STAT3 (200 ng) was applied to cells, cell viability (%) after 48/72 h was 94/90, 93/90, 90/85, and 85/80% for A549, NIH-3T3, MDA-MB-231, and MCF-7, respectively. This is also due to the difficulty of effective internalization of naked siRNA on its own into the cell, as confirmed by other studies [70,71].

Gel retardation assay

A major obstacle for siRNA reaching the target site is the sensitivity of genetic material to circulation and intracellular compartment nucleases. One of the most important strategies for developing a successful genetic material delivery system is to ensure its ability to bind to genetic material and protect it against serum nuclease [72–74].

Therefore, in order to evaluate the siRNA binding capacities of the formulations, we prepared 1:1, 1:2.5, 1:5, and 1:10 ratio of STAT3-siRNA:formulation mixtures. Before loading onto the gel, the mixtures were incubated for 20 min at 37 °C and electrostatic interaction was performed. While siRNA binding studies were performed in all formulations (blank and flurbiprofen loaded formulations), serum studies were performed only in flurbiprofen loaded formulations. The analysis results are shown in Figures 7 and 8.

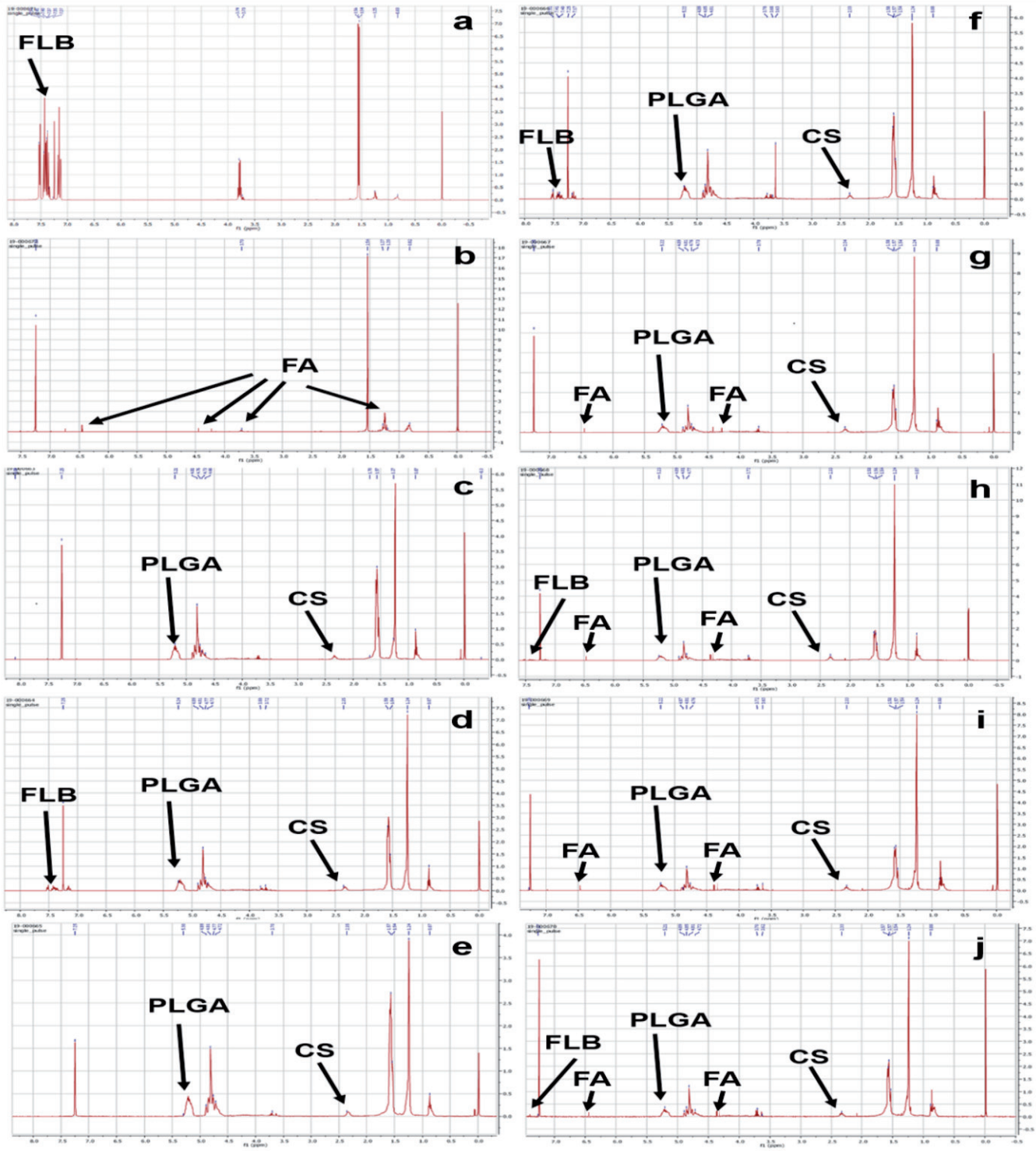

Figure 4. [1]H NMR analysis results of flurbiprofen, folic acid, and nanoparticles. (a) Flurbiprofen, (b) folic acid, (c) F1-Blank, (d) F1, (e) F2-Blank, (f) F2, (g) F3-Blank, (h) F3, (i) F4-Blank, and (j) F4. FLB: flurbiprofen; FA: folic acid; CS: chitosan.

Particle size, zeta and polydispersity index of siRNA loaded formulations prepared before gene retardation studies were measured and the results are given in Table 3.

As shown in Figure 7(a,b), F1-Blank, F2-Blank, and F3-Blank are observed to release the STAT3-siRNA in 1/1 ratio over time. However, F1, F2, F3, F4-Blank, and F4 formulations appear to strongly bind to siRNA at all ratios used.

To evaluate the siRNA protection capacities of the formulations we prepared, 1:5 STAT3-siRNA: formulation mixtures were added to a cell culture medium containing 20% fetal bovine serum and incubated for specific time intervals. At the end of each time interval, samples were taken and loaded on to the gel. Results of this analysis are shown in Figure 8(a,b). While the F1 and F2 formulations released genetic material after 24-h, and the released genetic material started to degrade after 48-h, the F3 and F4 formulations started to release genetic material only after 72-h.

The addition of Pluronic®F-68 to the formula had no effect on protection or release of genetic material because there was no

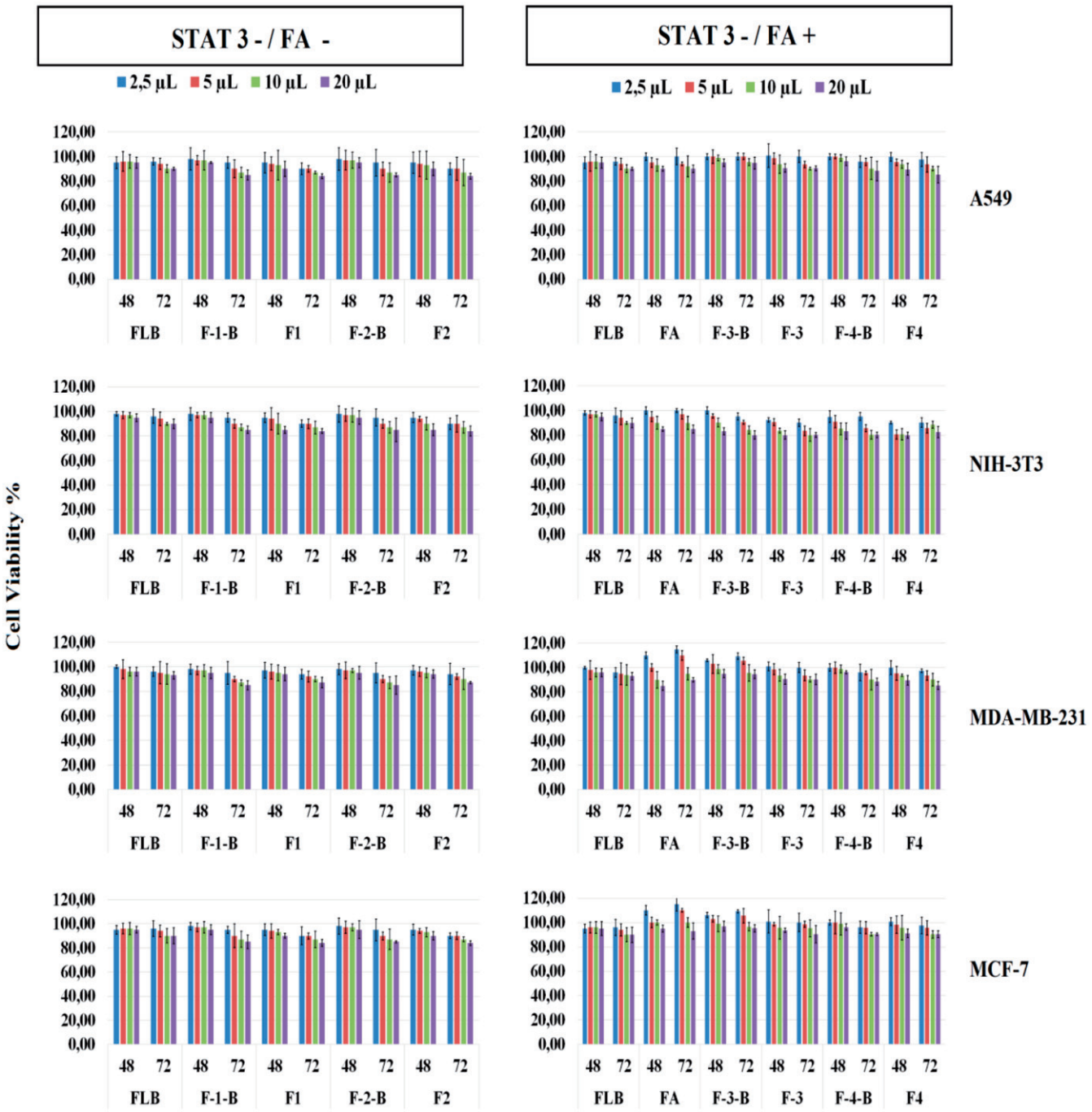

Figure 5. The cytotoxicity results of non-STAT3-siRNA loaded formulations.

difference between F1 and F2 and between F3 and F4. However, no aggregation of polymeric NPs in serum was observed during 48 h of the analysis.

Transfection assay

Transfection efficiency is one of the most important parameters used to determine the efficiency of NPs in carrying genetic material because it is clearly the case that prepared NPs will not be effective unless they pass into the cell. Although minimally distributed in normal tissues, overexpression of the folate receptor in a wide range of human tumors is a subject of considerable attention in targeting NPs to the tumor [75].

Therefore, folic acid, a high-affinity ligand of this receptor, exhibits receptor binding and endocytosis properties very well when covalently or non-covalently bound to nanoparticulate carrier systems [65,66,70,76,77].

Transfection assays were used for all cells undergoing cytotoxicity analysis. In this study, folic acid-free F2 and folic acid-conjugated F4 formulations containing flurbiprofen were used in the transfection study because Pluronic®F-68 used as surfactant only reduced particle size but had no effect on siRNA binding or serum degradation. A formulation sample of 5 µl and 200 ng of FITC siRNA were used for transfection. At the end of the 24-h incubation period, cells with and without transfection in 10 sites were visually counted at fluorescence + brightfield microscope under 20× magnification (Figure 9). A transfection index (TI) was

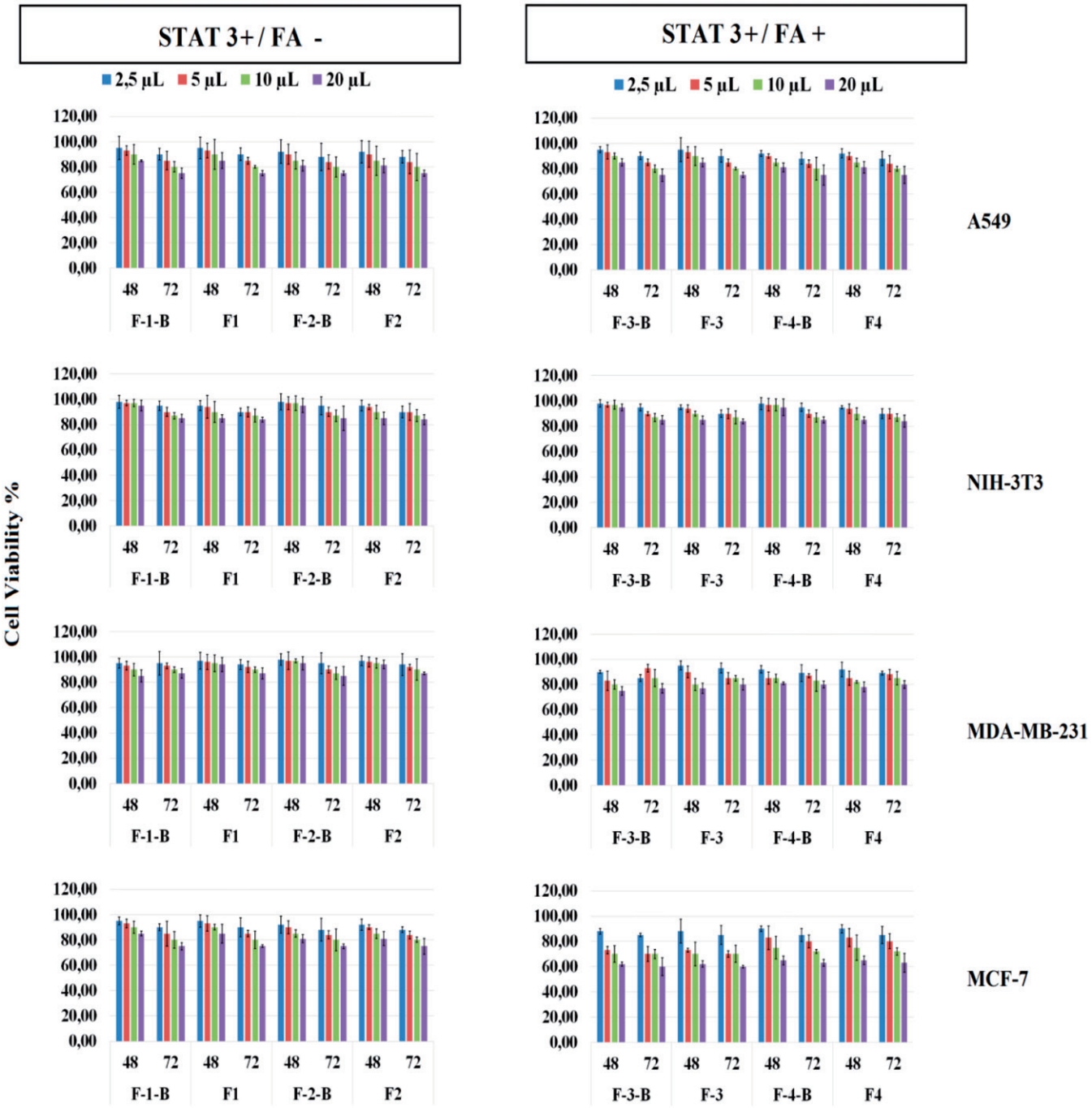

Figure 6. The cytotoxicity results of STAT3-siRNA loaded formulations.

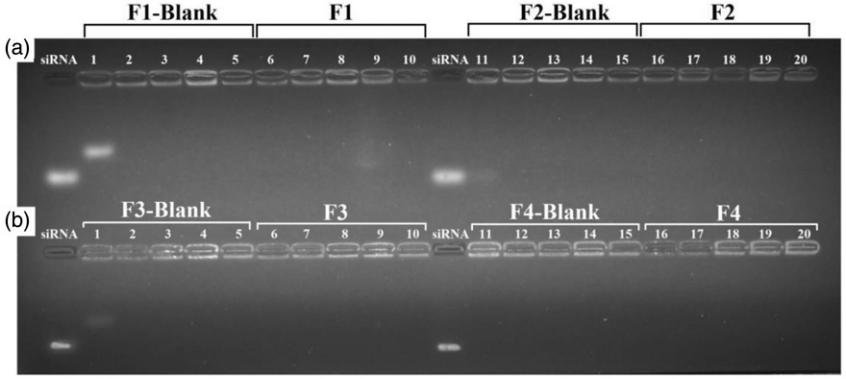

Figure 7. The gel retardation results of the formulations. Lanes 1–4, 6–9, 11–14, and 16–19; 0.5:1, 0.5:2.5, 0.5:5, and 0.5:10 siRNA (µg)/formulations (µl) ratios, respectively. Lane 5-(a): only 5 µl F1-Blank formulations, lane 10-(a): only 5 µl F1 formulations; lane 15-(a): only 5 µl F2-Blank formulations, lane 20-(a): only 5 µl F2 formulations. F1 formulations, lane 5-(b): only F3-Blank formulations, lane 10(b): only F3 formulations; lane 15-(b): only F4-Blank formulations; lane 20-(b): only F4 formulations.

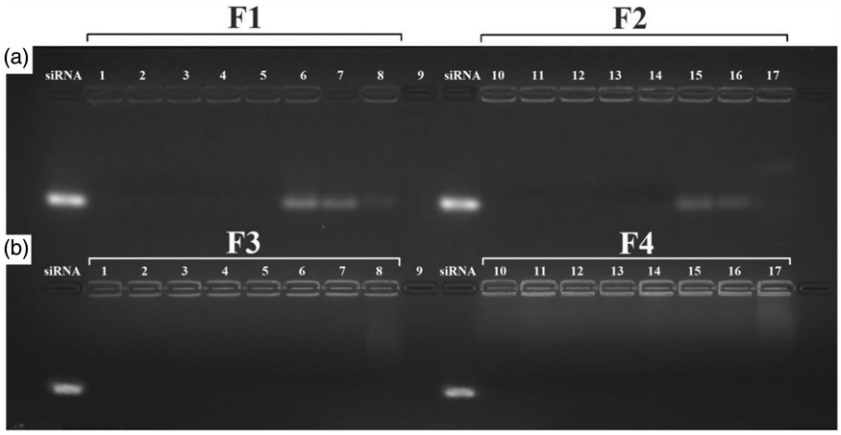

Figure 8. The results of serum protection properties of formulations at eight time intervals (lanes 1–8 and 10–17; 30 min, 1, 4, 6, 12, 24, 48, and 72 h, respectively) for 05.5/5 (μg/μl) (ratios of siRNA/formulation).

Table 3. Particle size, polydispersity index, and zeta potential results of STAT3-siRNA loaded formulations.

Code	PS	PDI	ZP
F1-Blank/STAT3	220.5 ± 1.7	0.245 ± 0.123	18.14 ± 1.6
F2-Blank/STAT3	182.5 ± 2.2	0.256 ± 0.01	17.22 ± 0.6
F3-Blank/STAT3	222.2 ± 3.2	0.333 ± 0.012	10.71 ± 0.5
F4-Blank/STAT3	235.5 ± 3.7	0.278 ± 0.001	14.95 ± 0.5
F1/STAT3	221.5 ± 4.1	0.365 ± 0.012	22.5 ± 2.3
F2/STAT3	205.3 ± 2.7	0.277 ± 0.005	22.5 ± 1.4
F3/STAT3	232.8 ± 4.3	0.388 ± 0.23	12.9 ± 2.9
F4/STAT3	231.6 ± 2.6	0.301 ± 0.024	13.6 ± 4.2

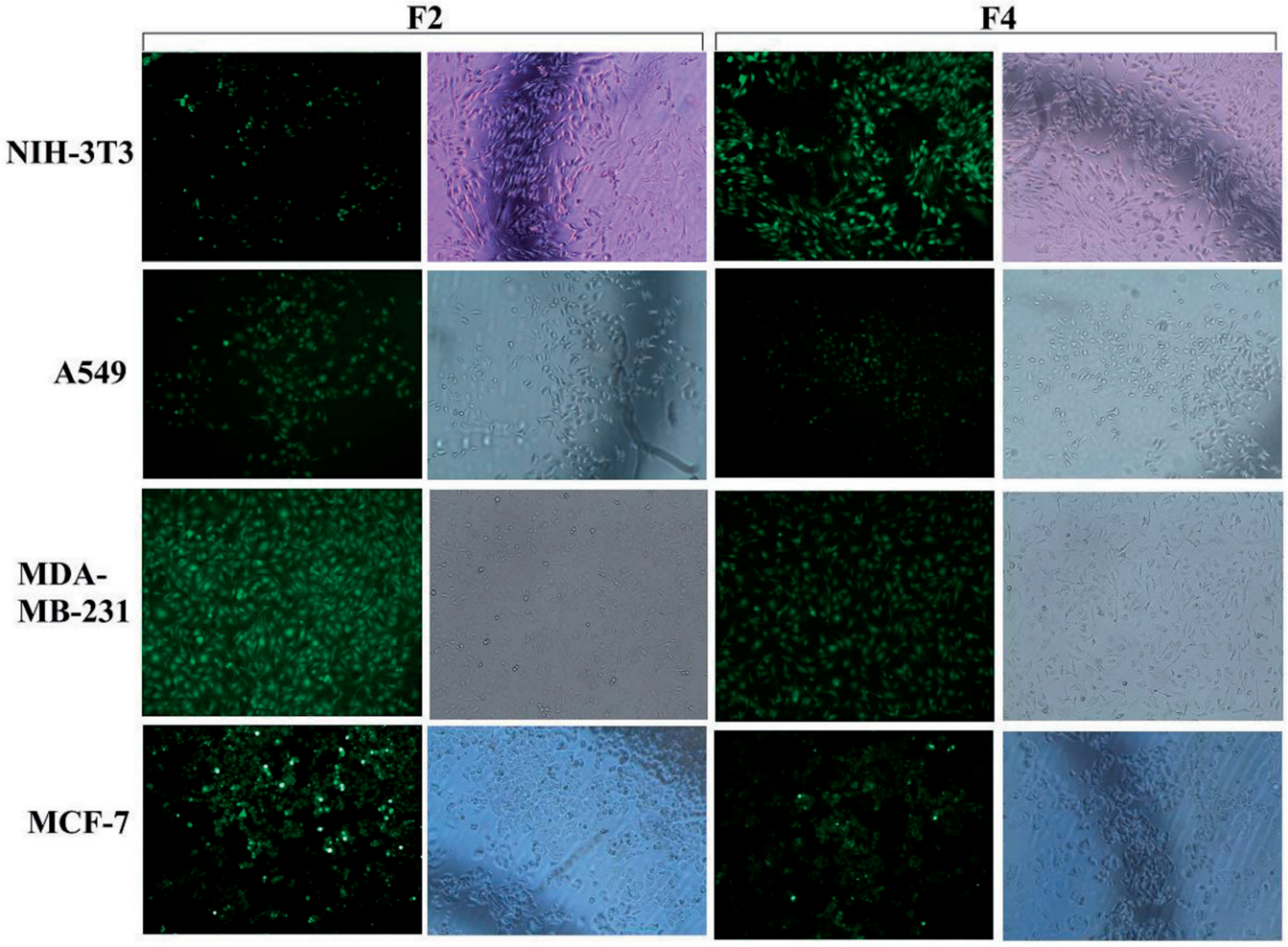

Figure 9. Transfection images at 20× magnification. On the left are the fluorescence images of the formulation and on the right are the brightfield images.

calculated using Equation (2).

$$TI = \frac{\text{number of transfected cells}}{\text{number of transfected cells} + \text{number of non} - \text{transfected cells}} \times 100 \quad (2)$$

While the transfection rate did not differ significantly between A549 and 3t3 cells ($p > .05$) in the F2 (65%) and F4 (67%) formulations, the MDA-MB231 and MCF-7 cells showed significantly greater rates of transfection ($p \leq .001$) in the F4 (88%) formulations than in the F2 (78%) formulations.

Conclusion

The number of people who will develop cancer will increase exponentially in the near future if precautions are not taken. However, despite all the advances in medicine and related fields promising treatment methods have not been developed yet for some types of cancer. To achieve successful results in these areas, research efforts need to be interdisciplinary and sustainable to create a long-term roadmap. Our study shows that folic acid receptor-containing MCF-7 cells was affected more by STAT3 siRNA. This indicates that we have developed an innovative chitosan/PLGA folate nano-carrier system which can carry or distribute genetic material and selectively target cancer cells. The addition of a drug with analgesic and anti-inflammatory properties, such as flurbiprofen, to NPs suggests that there may be a different, more efficient, approach to cancer treatment. Thus, the potential for reducing the number of doses of drugs administered to the patient, or reducing the number of drugs to be taken, would increase the patient's compliance and reduce side effects. The prolonged release of drugs from drug-loaded prepared NPs and the long-term preservation of genetic material in the presence of serum may be sufficient for both the drugs and genetic material to remain in circulation until reaching the target region. Therefore, prepared NPs are a promising system for gene and drug transport.

Acknowledgements

We greatly appreciate to Associate Professor Mustafa YILDIZ for the 1H-NMR and FTIR analyses from Çanakkale Onsekiz Mart University. We would like to thank to Associate Professor Gülay Büyükköroğlu for her valuable scientific contribution.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Behiye Şenel http://orcid.org/0000-0001-9747-8307
A. Alper Öztürk http://orcid.org/0000-0001-9596-0538

References

[1] Sarkar S, Horn G, Moulton K. Cancer development, progression, and therapy: an epigenetic overview. Int J Mol Sci. 2013;14(10):21087–21113.
[2] Portenoy RK. Treatment of cancer pain. Lancet. 2011; 377(9784):2236–2247.
[3] Uyar M. Inceleme yazıları meme kanserlerinde agrı tedavısı. SSK Tepecık Hast Derg. 2002;12(1):1–12.
[4] Schneider G, Voltz R, Gaertner J. Cancer pain management and bone metastases: an update for the clinician. Breast Care (Basel). 2012;7(2):113–120.
[5] Sultan A, McQuay HJ, Moore RA, et al. Single dose oral flurbiprofen for acute postoperative pain in adults. Cochrane Database Syst Rev. 2009;2009(3):1–39.
[6] Yin Y, Yi Y, Yu J, et al. Effects of flurbiprofen on serum level of interleukin-6, prostacyclin and corticosteroid A2 in patients with bone metastases of cancer. Oncol Lett. 2018; 15(2):1545–1548.
[7] Xin Y, Huang M, Guo WW, et al. Nano-based delivery of RNAi in cancer therapy. Mol Cancer. 2017;16(1):134–139.
[8] Soutschek J, Akinc A, Bramlage B, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature. 2004;432(7014):173–178.
[9] Devi GR. siRNA-based approaches in cancer therapy. Cancer Gene Ther. 2006;13(9):819–829.
[10] Wang J, Lu Z, Wientjes MG, et al. Delivery of siRNA therapeutics: barriers and carriers. AAPS J. 2010;12(4):492–503.
[11] Lee JM, Yoon TJ, Cho YS. Recent developments in nanoparticle-based siRNA delivery for cancer therapy. BioMed Res Int. 2013;2013:1–10.
[12] Lunavat TR, Jang SC, Nilsson L, et al. RNAi delivery by exosome-mimetic nanovesicles – implications for targeting c-Myc in cancer. Biomaterials. 2016;102:231–238.
[13] Setten RL, Rossi JJ, Han S. The current state and future directions of RNAi-based therapeutics. Nat Rev Drug Discov. 2019;18(6):421–446.
[14] Öztürk AA, Martin-Banderas L, Cayero-Otero MD, et al. New approach to hypertension treatment: carvediol-loaded PLGA nanoparticles, preparation, *in vitro* characterization and gastrointestinal stability. Trop J Pharm Res. 2018;37(9): 1730–1741.
[15] Khan N, Ameeduzzafar Khanna K, Bhatnagar A, et al. Chitosan coated PLGA nanoparticles amplify the ocular hypotensive effect of forskolin: statistical design, characterization and *in-vivo* studies. Int J Biol Macromol. 2018;116: 648–663.
[16] Şenel B, Büyükköroğlu G, Yazan Y. Solid lipid and chitosan particulate systems for delivery of siRNA. Die Pharm. 2015; 70(11):698–705.
[17] Öztürk AA, Güven UM, Yenilmez E, et al. Effects of different derivatives of eudragit polymer on entrapment efficiency, in vitro dissolution, release kinetics and cell viability results on extended release flurbiprofen loaded nanomedicines. Lat Am J Pharm. 2018;37(10):1981–1992.
[18] Yılmaz-Usta D, Demirtaş Ö, Ökçelik C, et al. Evaluation of in vitro dissolution characteristics of flurbiprofen, a BCS class IIa drug. FABAD J Pharm Sci. 2018;43(2):117–124.
[19] Matias R, Ribeiro PRS, Sarraguca MC, et al. A UV spectrophotometric method for the determination of folic acid in pharmaceutical tablets and dissolution tests. Anal Methods. 2014;6(9):3065–3071.
[20] Öztürk AA, Martin-Banderas L, Cayero-Otero MD, et al. Dexketoprofen trometamol-loaded poly-lactic-co-glycolic acid (PLGA) nanoparticles: preparation, in vitro characterization and cytotoxicity. Trop J Pharm Res. 2019;18(1):1–11.
[21] Dizaj SM, Jafari S, Khosroushahi AY. A sight on the current nanoparticle-based gene delivery vectors. Nanoscale Res Lett. 2014;9(1):252.
[22] Drummond DC, Meyer O, Hong K, et al. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol Rev. 1999;51(4):691–743.

[23] Ragelle H, Vandermeulen G, Préat V. Chitosan-based siRNA delivery systems. J Control Release. 2013;172(1):207–218.
[24] Haussecker D. The business of RNAi therapeutics. Hum Gene Ther. 2008;19(5):451–462.
[25] Kang F, Singh J. Preparation, in vitro release, in vivo absorption and biocompatibility studies of insulin-loaded microspheres in rabbits. AAPS Pharm Sci Tech. 2005;6: 487–494.
[26] Rezvantalab S, Drude NI, Moraveji MK, et al. PLGA-based nanoparticles in cancer treatment. Front Pharmacol. 2018;9: 1–19.
[27] Honary S, Zahir F. Effect of zeta potential on the properties of nano-drug delivery systems—a review (part 1). Trop J Pharm Res. 2013;12(2):255–264.
[28] de Lima IA, Khalil NM, Tominaga TT, et al. Mucoadhesive chitosan- coated PLGA nanoparticles for oral delivery of ferulic acid. Artif Cells Nanomed Biotechnol. 2018;46(Suppl. 2):993–1002.
[29] Perez-Herrero E, Fernandez-Medarde A. Advanced targeted therapies in cancer: drug nanocarriers, the future of chemotherapy. Eur J Pharm Biopharm. 2015;93:52–79.
[30] Ripamonti CI, Bandieri E, Roila F. ESMO Guidelines Working Group. Management of cancer pain: ESMO Clinical Practice Guidelines. Ann Oncol. 2011;22(6):69–77.
[31] Arslan D, Tatlı AM, Üyetürk Ü. Kansere bağlı ağrı ve tedavisi. Turk J Bioch. 2013;2(3):256–260.
[32] Heinz H, Pramanik C, Heinz O, et al. Nanoparticle decoration with surfactants: molecular interactions, assembly, and applications. Surf Sci Rep. 2017;72(1):1–58.
[33] Ilkan MGY, Özdemir N. Investigation of the parameters affecting the release of flurbiprofen from chitosan microspheres. Braz J Pharm Sci. 2017;53(4):1–12.
[34] Dora CP, Singh SK, Kumar S, et al. Development and characterization of nanoparticles of glibenclamide by solvent displacement method. Acta Pol Pharm. 2010;67(3):283–290.
[35] Zirak MB, Pezeshi A. Effect of surfactant concentration on the particle size, stability and potential zeta of beta carotene nano lipid carrier. Intl J Curr Microbiol App Sci. 2015; 4(9):924–932.
[36] Mainardes R, Evangelista RC. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution. Int J Pharm. 2005;290(1–2):137–144.
[37] Koppolu B, Rahimi M, Nattama S, et al. Development of multiple-layer polymeric particles for targeted and controlled drug delivery. Nanomedicine. 2010;6(2):355–361.
[38] Song H, Su C, Cui W, et al. Folic acid-chitosan conjugated nanoparticles for improving tumor-targeted drug delivery. BioMed Res Int. 2013;2013:1–6.
[39] Menon JU, Kona S, Wadajkar AS, et al. Effects of surfactants on the properties of PLGA nanoparticles. J Biomed Mater Res A. 2012;100(8):1998–2005.
[40] Büyükköroğlu G, Şenel B, Başaran E, et al. Preparation and in vitro evaluation of vaginal formulations including siRNA and paclitaxel loaded SLNs for cervical cancer. Eur J Pharm Biopharm. 2016;109:174–183.
[41] Tripathi A, Gupta R, Saraf SA. PLGA nanoparticles of anti tubercular drug: drug loading and release studies of a water in-soluble drug. Int J Pharm Tech Res. 2010;2: 2116–2123.
[42] Song KC, Lee HS, Choung IY, et al. The effect of type of organic phase solvents on the particle size of poly(D,L-lactide-co-glycolide) nanoparticles. Colloids Surf A. 2006; 276(1–3):162–167.
[43] Ran S, Downes A, Thorpe PE. Increased exposure of anionic phospholipids on the surface of tumor blood vessels. Cancer Res. 2002;62(21):6132–6140.
[44] Shen S, Wu Y, Liu Y, et al. High drug-loading nanomedicines: progress, current status, and prospects. Int J Nanomedicine. 2017;12:4085–4109.
[45] Senapati S, Mahanta AK, Kumar S, et al. Controlled drug delivery vehicles for cancer treatment and their performance. Signal Transduct Targeted Ther. 2018;3(7):1–19.
[46] Martín-Banderas L, Alvarez-Fuentes J, Durán-Lobato M, et al. Cannabinoid derivate-loaded PLGA nanocarriers for oral administration: formulation, characterization, and cytotoxicity studies. Int J Nanomedicine. 2012;7:5793–5806.
[47] Ji J, Zuo P, Wang Y-L. Enhanced antiproliferative effect of carboplatin in cervical cancer cells utilizing folate-grafted polymeric nanoparticles. Nanoscale Res Lett. 2015;10(1):1–8.
[48] Fredenberg S, Wahlgren M, Reslow M, et al. The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems—a review. Int J Pharm. 2011; 415(1–2):34–52.
[49] Ansary RH, Awang MB, Rahman MM. Biodegradable poly(D,L-lactic-co-glycolic acid)-based micro/nanoparticles for sustained release of protein drugs-A review. Trop J Pharm Res. 2014;13(7):1179–1190.
[50] Berkland C, Kim K, Pack DW. PLGA microsphere size controls drug release rate through several competing factors. Pharm Res. 2003;20(7):1055–1062.
[51] Singh PK, Sah P, Meher JG, et al. Macrophage-targeted chitosan anchored PLGA nanoparticles bearing doxorubicin and amphotericin B against visceral leishmaniasis. RSC Adv. 2016;6(75):71705–71718.
[52] Huang WZC. Tuning the size of poly(lactic-co-glycolic acid) (PLGA) nanoparticles fabricated by nanoprecipitation. Biotechnol J. 2018;13(1):1–19.
[53] Sohail MF, Shah PA, Tariq I, et al. Development and *in vitro* evaluation of flurbiprofen microcapsules prepared by modified solvent evaporation technique. Trop J Pharm Res. 2014;13(7):1031–1038.
[54] Paradkar A, Maheshwari M, Tyagi AM, et al. Preparation and characterization of flurbiprofen beads by melt solidification technique. AAPS Pharm Sci Tech. 2003;4(4):1–9.
[55] Nayak G, Trivedi MK, Branton A, et al. Consciousness energy healing treatment: impact on the physicochemical and thermal characteristics of folic acid. Int J Nutr. 2018;3(1): 30–42.
[56] Varshosaz J, Hassanzadeh F, Aliabadi HS, et al. Synthesis and characterization of folate-targeted dextran/retinoic acid micelles for doxorubicin delivery in acute leukemia. BioMed Res Int. 2014;2014:1–14.
[57] Singh G, Kaur T, Kaur R, et al. Recent biomedical applications and patents on biodegradable polymer-PLGA. Int J Pharmacol Pharm Sci. 2014;1(2):30–42.
[58] Queiroz MF, Melo KRT, Sabry DA, et al. Does the use of chitosan contribute to oxalate kidney stone formation? Mar Drugs. 2015;13(1):141–158.
[59] Chuacharoen T, Sabliov CM. Zein nanoparticles as delivery systems for covalently linked and physically entrapped folic acid. J Nanopart Res. 2017;19(2):1–12.
[60] Jin H, Pi J, Yang F, et al. Folate-chitosan nanoparticles loaded with ursolic acid confer anti-breast cancer activities in vitro and in vivo. Sci Rep. 2016;6(30782):1–11.

[61] Zhao F, Zhao Y, Liu Y, et al. Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. Small. 2011; 7(10):1322–1337.
[62] Lee KD, Choi SH, Kim DH, et al. Self-organized nanoparticles based on chitosan-folic acid and dextran succinate-doxorubicin conjugates for drug targeting. Arch Pharm Res. 2014;37(12):1546–1553.
[63] Li HL, He YX, Gao QH, et al. Folate-polyethylene glycol conjugated carboxymethyl chitosan for tumor-targeted delivery of 5-fluorouracil. Mol Med Rep. 2014;9(3):786–792.
[64] Mansouri S, Cuie Y, Winnik F, et al. Characterization of folate-chitosan-DNA nanoparticles for gene therapy. Biomaterials. 2006;27(9):2060–2065.
[65] Li K, Liu Y, Zhang S, et al. Folate receptor-targeted ultrasonic PFOB nanoparticles: synthesis, characterization and application in tumor-targeted imaging. Int J Mol Med. 2017;39(6):1505–1515.
[66] Fernández M, Javaid F, Chudasama V. Advances in targeting the folate receptor in the treatment/imaging of cancers. Chem Sci. 2018;9(4):790–810.
[67] Kim YK, Jiang HL, Choi YJ, et al. Polymeric nanoparticles of chitosan derivatives as DNA and siRNA carriers. Vol. 243. In: Jayakumar R, Prabaharan M, Muzzarelli R, editors. Chitosan for biomaterials. I. Advances in polymer science. Heidelberg: Springer; 2011. p. 1–22.
[68] Muhamad N, Plengsuriyakarn T, Na-Bangchang K. Application of active targeting nanoparticle delivery system for chemotherapeutic drugs and traditional/herbal medicines in cancer therapy: a systematic review. Int J Nanomedicine. 2018;4(13):3921–3935.
[69] Banerjee K, Pru C, Pru JK, et al. STAT3 Knockdown induces tumor formation by MDA-MB-231 cells. Clin Oncol Res. 2018;1(1):2–8.
[70] Huh MS, Lee EJ, Koo H, et al. Polysaccharide-based nanoparticles for gene delivery. In: Cheng Y, editor. Polymeric gene delivery systems. Switzerland: Springer; 2017. p. 65–84.
[71] Marquez AR, Madu CO, Lu Y. An overview of various carriers for siRNA delivery. Oncomedicine. 2018;3:48–58.
[72] Riley MK, Vermerris W. Recent advances in nanomaterials for gene delivery—a review. Nanomaterials (Basel). 2017; 7(5):94–19.
[73] Raja MAG, Katas H, Jing Wen T. Stability, intracellular delivery, and release of siRNA from chitosan nanoparticles using different cross-linkers. PLOS One. 2015; 10(6):1–19.
[74] Kim TK, Eberwine JH. Mammalian cell transfection: the present and the future. Anal Bioanal Chem. 2010;397(8): 3173–3178.
[75] Hofland HE, Masson C, Iginla S, et al. Folate-targeted gene transfer *in vivo*. Mol Ther. 2002;5(6):739–744.
[76] Zheng B, Yang S, Wang M, et al. Non-covalent nanocomplexes of folic acid and reducible polyethylenimine for survivin siRNA delivery. Anticancer Res. 2015; 35(10): 5433–5441.
[77] Du YZ, Cai LL, Li J, et al. Receptor-mediated gene delivery by folic acid-modified stearic acid-grafted chitosan micelles. Int J Nanomedicine. 2011;6:1559–1568.