**AS1411 aptamer-decorated biodegradable PEG-PLGA nanopolymersomes for
the targeted delivery of gemcitabine to non-small cell lung cancer *in vitro***

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Abstract

Molecularly targeted drug delivery systems represent a novel therapeutic strategy in the treatment of different cancers. In the current study, we have developed gemcitabine (GEM)-loaded AS1411 aptamer surface-decorated PEG-PLGA nanopolymersome (Apt-GEM-NP) to target nucleolin-overexpressing non-small cell lung cancer (A549). The prepared Apt-GEM-NP showed average particle size of 128 ± 5.23 nm and spherical morphology with encapsulation efficiency and loading content of $95.32 \pm 2.37\%$ and $8.61 \pm 0.27\%$, respectively.

Apt-GEM-NP exhibited a controlled release pattern. A sustained release of drug in physiological conditions will greatly improve the chemotherapeutic efficiency of a system. Enhanced cellular uptake and the cytotoxicity of aptamer-conjugated nanoparticles in A549 cancer cells obviously verified nucleolin-mediated receptor-based active targeting.

Nucleolin-mediated internalization of the targeted polymeric nanoparticle (NP) was further confirmed by flow cytometry and fluorescence microscopy. MTT assay clearly showed the enhanced cell proliferation inhibitory effect of AS1411-conjugated NP due to the selective delivery of GEM to the nucleolin-overexpressing cancer cells. Our results showed that AS1411 aptamer conjugation on the surface of NP could be a potential treatment strategy for A549 as a nucleolin-overexpressing cell line. This suggests that AS1411-GEM-NPs could be potentially used for the treatment of non-small cell lung cancer (NSCLC).

Keywords: Cancer chemotherapy; Drug delivery system; Encapsulation; Nanoparticle; PLGA; Polymeric drug delivery systems; Targeted drug delivery

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1. Introduction

Lung cancer is the most common cause of cancer-related death, with a low survival rate.¹

In spite of the various strategies to treat it, such as tumor resection, radiation, and/or chemotherapy, resistance to chemotherapy, cancer relapses, and metastasis occur.²The reasons for chemo and/or radio therapy resistance in non-small cell lung cancer (NSCLC), one of the main types of lung cancer are not completely understood.

Serious limitations in delivering adequate anti-cancer drugs to tumor cells due to systematic toxicity and the extravasation of drugs from the tumor tissue through the fenestrated vessels are a likely cause of this resistance.^{3, 4}Therefore, versatile approaches for transmitting highly cytotoxic anti-cancer agents are extremely desirable in order to improve drug delivery and accumulations in the tumor site while minimizing systematic toxicity.

Gemzar (GEM) is a powerful FDA-approved anticancer drug for the treatment of pancreatic and non-small-cell lung cancers.⁵A serious limitation of GEM is its fast metabolic degradation and narrow therapeutic index.⁶ Therefore, the improvement of the GEM therapeutic window is a high-priority requirement in treating non-small cell lung cancer (NSCLC).

One of the ideal approaches is to employ nanoscale vehicles to modify the pharmacokinetic properties of GEM and to increase its half-life and anticancer capability. The nanoscale dimension of these therapeutic systems confers the advantage of prolonging the circulation time of the drug in the blood stream by preventing its enzymatic inactivation. Also advantageous is the enhancement of GEM accumulation in tumor tissue by passive tumor-site targeting through enhanced permeation and retention (EPR) effects, which increase an encapsulated drug's therapeutic potency.^{7, 8}

It has been shown that liposomes have showed capability in encapsulating GEM, greatly prolonging its circulation time, decreasing its inactivation *in vivo*, and consequently

exhibiting a higher tumor growth inhibitory effect in mice than free GEM.^{9, 10} Meanwhile, the liposomal formulation of GEM modifies the pharmacokinetics and prolongs the circulation time of the drug, also causing excessive drug accumulation in the liver and spleen between 12-24h post injections, which likely stimulates chronic toxicities in these vital organs.¹¹

Over the past few decades, there has been an increasing interest in the potential use of polymeric nanoparticles (NPs) as delivery vehicles for GEM, and studies have demonstrated that these nanocarriers can significantly enhance the anti-tumor efficiency of GEM and also reduce its systematic toxicities.¹²⁻¹⁵ In this regard, Zhao et al.¹⁶ employed biocompatible lipid-polymer hybrid nanoparticles to co-deliver HIF1 α siRNA and GEM for pancreatic cancer treatment in subcutaneous and orthotopic tumor models. The obtained results demonstrated the prolonged lifetime of GEM in the bloodstream and enhanced tumor tissue accumulation. Moreover, the prepared formulation showed a significant tumor metastasis inhibitory effect in an orthotopic tumor model. Other researchers have compared liposomal and poly(lactic-co-glycolic acid) (PLGA)-based formulations of GEM *in vitro*.¹⁷ They proved the preferential biological effects of PLGA formulation versus liposomal formulation of GEM and further indicated that when GEM is delivered in PLGA, it can target endoplasmic reticulum and mitochondria organelles more potently.

Among the various kinds of polymer-based nanoparticles, which are self-assembled structures from biodegradable amphiphilic block polymers, polymeric vesicles (polymersomes) are considered promising as drug delivery vehicles for both hydrophilic and hydrophobic drugs.¹⁸

The polymersomes hold many advantages, including high stability, a tuneable size and release pattern, high entrapment efficiency for hydrophilic and hydrophobic drugs, and desirable bio-distribution due to accumulation at the tumor site through the EPR effect.^{19, 20}

In this regard, polymersomes based on amphiphilic copolymers of PEG (as a hydrophilic block) and biodegradable polyesters are the most commonly used in an FDA-approved, non-toxic, biodegradable and non-immunogenic system for the encapsulation of molecules without the inclusion of any organic toxic solvent.²¹

We have recently shown that PEG-*b*-PLGA provides a protective shield for the entrapped doxorubicin and increases the doxorubicin therapeutic index *in vivo*.^{22, 23}

Furthermore, through the conjugation of a targeting agent, such as nonimmunogenic DNA or RNA aptamers to a nanoparticle surface, an active targeting effect could be established in order to enhance therapeutic efficiency and further modify the pharmacokinetics of drugs.²⁴⁻²⁶

As a targeting agent, aptamer (AS1411) that binds to the external domain of the membrane protein nucleolin has been well investigated because of its therapeutic potential.²⁷ Due to the unique characteristics of the aptamer and the preferential properties of nucleolin as a receptor; AS1411 is an ideal candidate for aptamer-mediated targeting for various cancers.²⁸

Previously, it has been shown that overexpression of nucleolin occurs in many cancer cell lines, including the DU145 (prostate cancer cell line), MDA-MB-231, MCF-7 (breast cancer cell lines), A549 (lung cancer cell line), and HeLa (cervical cancer cell line).²⁹

Specially, in patients with non-small cell lung cancer, nucleolin overexpression is associated with metastatic progression, poor prognosis, and, consequently, poor overall survival.^{30,31}

Accumulating evidence from other researches suggests that the AS1411-DNA aptamer can effectively target the tumor tissue in the A549 (NSCLC) carcinoma xenograft in mice.³²

While until now, AS1411 aptamer-targeted nanocarriers for the delivery of therapeutic quantities of GEM to NSCLC have not been reported in the literature.

Hence, in the current study, we explored the feasibility of employing the AS1411 aptamer-decorated PEG-PLGA nanopolymersome as a targeted delivery system for GEM in the A549

cell line. This polymeric drug delivery system was characterized for cellular uptake, internalization, and cytotoxicity *in vitro*.

2. Materials and methods

2.1. Materials

Poly(d,l-lactic-co-glycolic acid) (PLGA) (Average M_w : 10000; lactic acid : glycolic acid = 75:25), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-carboxyfluorescein (CF), N-hydroxysulfosuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N,N-diisopropylethylamine were obtained from Sigma Aldrich (Schnelldorf, Germany). A hetero-functional PEG polymer with terminal amine and carboxylic-acid functional groups (HCl, NH_2 -PEG-COOH, M_w : 3,500) was purchased from JenKem Technology USA Inc. (Beijing, China).

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS) and trypsin were purchased from GIBCO (Darmstadt, Germany). Other solvent and chemical reagents were procured from Merck & Co. (Darmstadt, Germany) without further purification.

Finally, the AS1411 DNA aptamer was employed as the targeting ligand. The 26-mer AS1411 DNA aptamer (sequence: 5'-amino-C6 linker—GGT GGT GGT GGT TGT GGT GGT GGT GG, molecular weight: 8272.3 g/mole) was custom-synthesized by Microsynth AG (Balgach, Switzerland).

2.2. Synthesis and characterization of PEG-PLGA

The conversion of PLGA-COOH to PLGA-NHS was carried out as follows:³³ One gram of PLGA-COOH (~10 kDa M_w) was dissolved in 4 mL dichloromethane and gently stirred at room temperature in the presence of N-hydroxysuccinimide (1:8 PLGA:NHS molar ratio) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (1:8 PLGA:EDC molar ratio) for 24 h.

To remove residual NHS and EDC, PLGA-NHS was precipitated with cold diethyl ether and washed three times with a cold freezing solution containing 80% diethyl ether and 20% methanol.

After drying under a vacuum, PLGA-NHS was redissolved in chloroform (5 mL) followed by the addition of HCl.NH₂-PEG-COOH (1:1.2 PLGA:PEG molar ratio) and N,N-diisopropylethylamine (0.2 mmol). The copolymer was precipitated with cold diethyl ether after 24 h and washed with methanol: diethyl ether solution (30%:70%) to take out unreacted PEG and other impurities. The final PLGA-PEG block copolymer was freeze-dried for 48 h and stored at -20°C until use.

For ¹H-NMR analysis, PEG-PLGA (5-10 mg) was dissolved in deuterated chloroform and placed in a glass NMR tube.

The PEG-PLGA copolymer was analyzed at room temperature using a Bruker Avance 400 MHz NMR spectrometer (Rheinstetten, Germany) to verify the purity and to quantify the extent of PEG conjugation to PLGA.

2.3. Preparation of blank or carboxy fluorescein-loaded nanopolymersomes

Blank or CF-loaded self-assembled structures were prepared by the film rehydration method. Briefly, 2 mL of copolymer solution (10 mg/mL) in dichloromethane was transferred into a round bottom flask. The solvent was evaporated under vacuum using a flask rotary evaporator at room temperature for 6 h.

The thin dried polymer film was hydrated by the addition of 2 mL 250 mM ammonium sulfate or a 100 μM solution of CF at 55°C and stirred overnight under 1250 rpm continuous stirring.

The polymersome dispersion was extruded 15 times at 55 ± 2°C through polycarbonate membranes of 400 nm and 100 nm sequentially using a mini-extruder device (Avantipolar

lipids Inc. USA). At the final stage, to remove free CF, the prepared nanoparticle batch was dialyzed (cut off 3.5 kD) against 3 L deionized water for 18 h.

2.4. Preparation of GEM loaded nanopolymersomes

GEM-loaded polymersomes were prepared using the pH gradient method.^{33, 34} For this purpose, the thin dried polymer film of copolymer (20 mg) was hydrated using 2 mL aqueous ammonium sulfate 250 mM at 55°C (above T_g of the copolymer) under 1250 rpm continuous stirring overnight. The polymersome dispersion was extruded 15 times at $55 \pm 2^\circ\text{C}$ through polycarbonate membranes of 400 nm and 100 nm, sequentially, using a mini-extruder device (Avantipolar lipids Inc. USA). The extruded polymersomes were kept undisturbed for 4 h in a refrigerator at 4°C.

To establish a transmembrane pH gradient, dialysis against NaCl solution 150 mM was carried out. Briefly, the dialysis tubing (cut off 3.5 kD) was filled with 2 mL ammonium sulfate polymersome suspension. The sac was then immersed in a flask containing 200 mL of NaCl solution 150 mM.

The contents of the flask were stirred at 400 rpm, and the flask was closed with Parafilm. The dialysis was carried out for 48 h to develop a transmembrane pH gradient. After the establishment of the pH gradient, volumes equivalent to 20 mg of polymersomes (10 mg/mL) were incubated with the 200 μL of GEM solution (10 mg/mL) for 18 h at $55 \pm 2^\circ\text{C}$ in bath oil. At the final stage, ultrafiltration (15 min, 1500g, MWCO: 10 kDa) (Millipore, Hampshire, UK) was used to remove free GEM.

To determine the encapsulation efficiency of GEM in polymersomes, 50 μL of GEM-loaded polymersomes were mixed with 950 μL of dimethyl sulfoxide (DMSO) to break the structure of the nanovesicles.

The solution was then analyzed by spectrophotometry measurements at $A = 270\text{ nm}$ using UV-Vis spectrophotometer (UV-160A Shimadzu, Japan).

The drug-loading content (LC%) and the encapsulation efficiency (EE%) of PEG-PLGA NPs loaded with GEM were calculated using the following equations:

$$\text{LC (\%)} = \frac{\text{mass of drug in NPs}}{\text{mass of drug-loaded NPs}} \times 100\% \quad (1)$$

$$\text{EE (\%)} = \frac{\text{amount of drug in NPs}}{\text{amount of drug used for encapsulation}} \times 100\% \quad (2)$$

Each experiment was conducted in triplicate.

2.5. Characterization of polymersomes

Suspensions of blank and GEM-loaded NPs were prepared in deionized water at a concentration of approximately 1 mg/mL.

A dynamic light-scattering detector (Zetasizer Nano ZS 3000HS, Malvern, UK) was used to analyze the average size and zeta potential of nanoparticles at 25 °C and at a scattering angle of 90° equipped with a 4-mW He-Ne laser operated at 633 nm through back-scattering detection.

All the measurements were performed in triplicate, and the average values were calculated.

Transmission electron microscopy (TEM) was performed to observe the size, morphology, and homogeneity of the produced GEM-loaded nanopolymersomes and Apt-GEM-loaded nanopolymersomes using a CM10 transmission electron microscope (Philips, Holland), operated at 80 kV. For this purpose, the suspension of GEM-NPs (0.5 mg/mL) was dropped onto copper grids coated with an amorphous carbon film and dried thoroughly at an ambient temperature.

2.6. NPs-Apt Conjugation

The 1 mL of blank, CF or GEM-loaded PLGA-PEG-COOH NPs suspensions (10 mg/mL) in DNase/RNase-free water were incubated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (400 mM, 62 mg) and N-hydroxysuccinimide (100 mM, 11 mg) for 1 h at room temperature with gentle stirring. The resulting N-hydroxysuccinimide-activated particles were washed three times with 2 mL DNase/RNase-free water to remove any remaining NHS

or EDC using an ultrafiltration device (15 min, 1500g, MWCO: 30 kDa) (Millipore, Hampshire, UK).

Then, 1 mL of the activated nanoparticles (10 mg/mL) were incubated with 100 μ L of 50 μ M stock solution of 5'-NH₂-modified AS1411 Aptamer (final aptamer concentration~ 5 μ M) at room temperature for 18 h under gentle stirring. The resulting NP-Apt were washed three times with 2 mL DNase/RNase-free water to remove any free aptamers using ultrafiltration (15 min, 1500g, MWCO: 10 kDa) (Millipore, Hampshire, UK).

2.7. Investigation of Aptamer conjugation to NPs

Agarose (2.5%) gel electrophoresis was performed to verify the amide-coupling chemistry of the DNA aptamer to the surface of the GEM-NPs. The DNA aptamer and Apt-GEM-NPs were loaded on the gel (containing 0.2 μ g/mL ethidium bromide), and the gel was run in a tris-borate-EDTA (TBE) buffer at 80 V for 40 min. Then, the DNA fragments were visualized using a standard transilluminator.

Another experiment was conducted to verify the presence of the DNA aptamer on blank nanoparticles surface by UV spectroscopy (absorbance at 260 nm) and to evaluate the conjugating efficacy.

2.8. *In vitro* drug release from GEM-loaded nanopolymersomes and Apt-GEM-loaded nanopolymersomes

The *in vitro* release of GEM from the targeted and untargeted formulations were assessed using the dialysis method.³⁵ The GEM-loaded formulation (1 mL) was introduced into a dialysis bag (MWCO 3.5 kDa) and then immersed into 50 mL of phosphate-buffered saline (PBS) (0.1 M, pH 7.4) and citrate buffer (0.1 M, pH 5.5) in a shaker incubator set at 90 rpm and 37°C.

Samples (1 mL) were withdrawn at definite times and replaced by the addition of 1 mL of fresh release medium. The samples were analyzed in a 96-well plate at an absorbance

wavelength of 270 nm using an Infinite® 200 PRO multimode microplate reader (Tecan Group Ltd. Männedorf, Switzerland).

2.9. Cellular uptake using flow cytometry

A549 cells were seeded in six-well plates at 1×10^5 cells per well and cultured for 24 h. The following day, the cells were incubated for two hours with either AS1411-aptamer-targeted CF-loaded nanopolymersome non-targeted CF-loaded nanopolymersomes, or with free CF (CF concentration: 0.1 μ M). Then, the culture medium was removed, and the cells were trypsinized and centrifuged at 200g for 10 min.

Supernatants were removed, and the resultant pellet was washed three times with 2 mL cold PBS. The cells were then suspended in an FCM buffer (0.1% sodium azide and 2% FBS in PBS), and the intensity of the cells' CF fluorescence was determined on a BD FACSCalibur equipped with a laser in the FL1 channel. The data were analyzed using WinDMI 2.9 analysis software.

Additionally, to confirm the findings that the nucleolin receptors were selectively targeting the SK-A549 cells for AS1411-targeted, CF-loaded nanopolymersomes, a competitive experiment was carried out. In it, excessive amounts (2 μ g/well) of free AS1411 aptamer were added to each well for 30 min before introducing the targeted formulation.

2.10. Cellular uptake by fluorescence microscopy

The cellular uptake of CF-loaded NPs (CF-NPs) or AS1411-aptamer conjugated CF-loaded NPs (Apt-CF-NPs) by A549 was further studied.

The collagen-treated cover slips (0.1% collagen in acetic acid) were deposited in the wells of a six-well plate. The cells at a density of 1×10^5 per well were seeded and allowed to adhere overnight. Next, the cells were incubated for 2 h with either non-targeted or targeted polymersome-encapsulated CF or free CF (concentration: 0.1 μ M) at 37°C or 4°C.

After a 2-h incubation period, the cells on glass cover slips were washed five times with cold PBS and fixed with 4% formaldehyde for 15 min. The cover slips were then placed on a slide to analyze under a Juli Smart Cell Analyzer (Baker and Baker Ruskin, USA).

2.11. Cytotoxicity studies of NPs

A549 (nucleolin over-expressed positive) and CHO (nucleolin negative) cells were cultured in the RPMI medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin at 37 °C, and 5% CO₂. A549 and CHO cells were seeded into 96-well plates at 5×10^3 and incubated for 24 h. GEM samples with different concentrations were prepared in RPMI containing 10% FBS. Targeted and non-targeted formulations containing equivalent amounts of GEM (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.25, 0.5, 1, 2, 5, 10, 20, 30, 40, 60, 80, 100 and 120 µg/mL) were also dispersed in RPMI containing 10% FBS.

The prepared drug concentration was added to each well, followed by incubation of the cells for 5 h. The medium was then removed, washed with PBS, replaced with fresh complete medium, and further incubated for 48 h at 37°C in a humidified incubator.

After incubation for 48 h, 20 µL of MTT (5 mg/mL in PBS) solution was added to each well and incubated for a further 4 h. The MTT solution was then aspirated from the wells using a vacuum, and 100 µL of DMSO was added to each well. The absorbance at 570 nm with a reference wavelength at 630 nm was measured using an Infinite® 200 PRO multimode microplate reader (Tecan Group Ltd. Männedorf, Switzerland).

In addition, to confirm the findings of the selective targeting of nucleolin receptors on the A549 cells by AS1411 aptamer-conjugated GEM-loaded NPs, a competitive experiment was carried out. In this experiment, excessive amounts (0.2 µg/well) of free AS1411 aptamer were added to each well 30 min before the addition of the targeted formulation.

2.12. Statistics

One-way analysis of variance (ANOVA) was used for data analysis. A probability value of less than 0.05 was considered significant. Results are expressed as mean $\pm$ SEM unless otherwise indicated.

3. Results and discussion

3.1. Synthesis and characterization of the PEG-PLGA copolymer

The PLGA-PEG-COOH copolymer was synthesized by direct conjugation of PLGA-COOH and NH₂-PEG-COOH. The ¹HNMR spectrum has been used previously for structure analysis of the prepared copolymer (Fig. 1).

The ¹HNMR spectrum of the PLGA-PEG-COOH copolymer demonstrated resonance peaks of protons at 1.5 ppm, 4.8 ppm, and 5.2 ppm for the PLGA block.

The resonance peak of protons at 3.6 ppm also corresponded to methylene groups of the PEG chain.

Using the integration of the relative molecular weights and peaks, the conjugation efficiency of NH₂-PEG-COOH to PLGA-COOH was estimated to be approximately 86%.

3.2. Characterization of GEM-loaded PEG-PLGA nanopolymersomes

Previously, we demonstrated that the polyethylene glycol–polylactide-co-glycolide (PEG–PLGA) block copolymer with the molecular weight of PEG₃₅₀₀-PLGA₁₀₀₀₀ formed polymersomes in aqueous solutions.³³

In this regard, there have been many reports that have demonstrated that in linear amphiphilic copolymers, the hydrophilic volume fraction (f_{EO}) of 25–40% results in self-assembled polymersomes.³⁶

Polymersomes share many similarities with liposomes; however, polymersomes have several advantages over liposomes, such as high stability and low permeability.^{37, 38}

It is also feasible that they can be conjugated with different ligands for targeting delivery and that they can provide sustained and controlled release of encapsulated drugs *in vivo*.³⁹⁻⁴¹

GEM-loaded PEG-PLGA nanopolymersomes with high drug loading (8.61 ± 0.27) and high encapsulation efficiency (95.32 ± 2.37) were fabricated by an ammonium sulfate gradient method.

In contrast to other methods, the ammonium sulfate gradient method exhibits high-yield capacity and scale-up feasibility for amphiphatic drug loading in lipid or polymeric vesicles.

The appearance of a GEM-loaded nanopolymersome solution was translucent with visible white opalescence, while the free GEM solution at the same concentration was highly transparent.

TEM images showed that the particles were dispersed individually with a well-defined spherical shape and size 120 to 130 nm in diameter (Fig. 2B).

Dynamic light scattering (DLS) measurements of the polymersomes revealed that NPs had an average size of 128 ± 5.23 and a narrow polydispersity index (PDI) of 0.12 (Fig. 2D).

The zeta potential measures of GEM-NPs and blank-NPs were found to be -24.6 and -26.7 mV, respectively, indicating that the GEM loading did not affect the surface charge of the PEG-PLGA nanopolymersomes.

The nanoparticle size is a crucial characteristic that greatly influences the circulation time and organ distribution.⁴²

The existence of a highly fenestrated blood vasculature in the tumor tissues promotes the EPR effect and enhances the uptake of nanoscale vehicles (less than 200 nm in size) into the tumor.⁴³ Moreover, the poor lymphatic flow in the tumor tissue increases the EPR effect and enhances the retention of nanoparticles within the tumor tissue.

On the other hand, vehicles less than 50 nm in size extravasate from the tumor tissue through the fenestrated vessels of the tumor blood vascular system, and consequently, their retention time in the tumor cite decreases.⁴⁴

The prepared formulation demonstrates ideal characteristics at ~130 nm in diameter with a narrow size distribution, which improves the therapeutic index of the encapsulated drug.

3.3.Characterization of aptamer conjugation on the surface of nanopolymersomes

The AS1411 aptamer was conjugated to blank, CF- and GEM-loaded nanopolymersome by the addition of EDC/NHS.

In the aforementioned reaction, EDC and NHS were added to catalyze the formation of a covalent bond between the carboxyl group of PLGA-PEG-COOH on the nanoparticle surface and the 5'-NH₂ AS1411 aptamer.

The nanoparticle size was 128 ± 5.23 nm with PDI of 0.12 before coupling to aptamers, which increased the size to 137 ± 0.21 nm with a PDI of 0.14 after the conjugation, presumably because of the added size of the AS1411 aptamer (Fig. 2A, C).

Due to the presence of the free carboxylic acid groups of PEG on the nanopolymersomes surfaces, the resultant NPs would have a negative zeta potential. This has strongly restricted non-covalent interactions between the negatively charged aptamers and the NPs' surfaces.

On the other hand, the NPs covalently conjugated with aptamer were washed three times with DNase RNase-free water in order to remove free non-targeting aptamer.

To verify that the AS1411 aptamer was covalently conjugated to the surface of the nanopolymersomes, two experiments were conducted.

To confirm the conjugation of the AS1411 aptamers to the GEM-NPs, agarose (2.5%) gel electrophoresis was used to separate the components in the coupling reaction. The gel electrophoresis data in Figure 3 demonstrated that Lane 2 (Apt-GEM-NPs) was unable to run on the gel, indicating the formation of an Apt-NPs complex. Moreover, other bands

corresponding to free aptamers were not observed in the lane of Apt-NPs, suggesting that free aptamers had been removed after the washing step.

The second experiment was conducted to examine the presence of the AS1411 aptamer on the surface of nanopolymersomes by UV spectroscopy and to calculate the conjugating efficacy. It was obviously demonstrated that more aptamers were conjugated to nanoparticles in the presence of EDC and NHS (Fig. 4). The number of conjugated aptamers on NPs was also calculated. In this reaction system, approximately 0.17 nmole aptamers were covalently coupled to 1 mg PEG-PLGA NPs.

3.4. *In vitro* release profile of GEM from NPs

The *in vitro* release profiles of the GEM from the GEM-NPs and Apt-GEM-NPs were evaluated in PBS pH 7.4 and citrate buffer pH 5.5 at 37°C up to 120 h (Fig. 5 A, B).

The release profiles of GEM-NPs and Apt-GEM-NPs were found to be virtually similar, indicating that the aptamer functionalization on the NP surface does not affect the drug release rate but facilitates cellular uptake, leading to superior therapeutic efficiency.

The release patterns of GEM from GEM-loaded nanopolymersomes (both targeted and non-targeted) demonstrated a higher release rate at a pH of 5.5 (as opposed to pH 7.4) due to the improved solubility of protonated GEM in water.

Furthermore, hydrolysis of polyesters, such as PLGA, has been shown to be accelerated by acidic pH.⁴⁵

Then, it is anticipated that at 37°C and a pH of 5.5, similar to the endolysosomal environment, the PLGA block of copolymers in nanopolymersomes structures may be hydrolysed, and this could result in a faster rate of GEM release.

An initial burst drug release was observed within 24 h in both formulations (GEM-NPs: 24% and Apt-GEM-NPs: 28%), a routine phenomenon among polymer-based matrixes.⁴⁶

The burst release is related to the diffusion of the GEM near the NP surface and/or to the release of the encapsulated drug residing on the NP surface.

Following the initial burst release, a sustained release of the drug from the polymersome through diffusion and erosion of the NPs was observed.⁴⁷

After 120 h, the amount of drug released reached 41% for GEM-NPs and 44% for Apt-GEM-NPs. In this case, we can conclude that the stability of the NP bilayer, low molecular weight of PEG, and also hydrophobicity of the PLGA (LA:GA 75:25) help in the controlled drug release by decreasing the amount of water that penetrates into the NPs, leading to a slower polymer degradation rate and consequently a sustained drug release.

In this study, GEM loading with an ammonium-sulfate gradient was driven by GEM protonation and charging within the polymersomes and by GEM precipitation in the polymersomes hydrophilic interior due to the protonated GEM interaction with SO_4^{2-} anions.⁴⁸

Then, the presence of suspended (undissolved) GEM in the form of a gel-like precipitate $[(\text{GEM-NH}_3)_2\text{SO}_4]$ in the interior compartment of the polymersome also contributes to the sustained drug release, as reported previously.⁴⁹ It would appear that the suspended drug provides a reservoir to maintain a sustained, prolonged driving force for the diffusion of cargos (GEM) out of the nanopolymersomes.

3.5. Cellular uptake experiment by flow cytometry

The most important property of a targeted drug delivery system is its specificity toward target cells. To explore the *in vitro* cancer targeting of Apt-NPs against nucleolin-overexpressing cells (A549), this study compared the cellular uptake of A549 using a flow-cytometry analysis (Fig. 6). CF-loaded NPs, aptamer-conjugated CF-loaded NPs, and free CFs were co-cultured with A549.

As a negative control, excess quantities (2 $\mu\text{g}/\text{well}$) of free AS1411 aptamers were added to the wells 30 min before the addition of Apt-NPs. The fluorescent intensity of A549 cells incubated with NPs was similar in amplitude to that of cells treated with free aptamer prior to the addition of Apt-NPs. The addition of excess aptamer as a free, competing ligand for targeted NPs prior to the cellular-uptake experiment reduced the aptamer-targeted NPs' internalization efficiency. However, for Apt-NP-treated A549 cells, the fluorescent intensity increased by 81%, while that of NP-treated cells increased by 45%. This distinction was presumably caused by the conjugated aptamers affinity to nucleolin proteins on the A549 cell surfaces, resulting in more nanoparticles binding to the cell surface and penetrating the cells. The fluorescence intensity of A549 cells exposed to free CF increased by 89%, demonstrating that cells can take up abundant free CFs. The lower cellular uptake of CF-loaded polymersomes in comparison with that of free CFs is largely due to the stability of the nanopolymersomes bilayer and this system's controlled-release characteristics.

3.6. Cellular uptake by fluorescent microscopy

After having confirmed the capability of Apt-NPs bioconjugates to selectively target nucleolin-overexpressing target cells (A549) via flow cytometry, we further examined the cellular internalization of cargos (CF) by targeted and non-targeted formulation at 37°C or 4°C because it was not entirely clear whether the Apt-NPs attached to the cells surfaces or were internalized into the cells.

To further evaluate the interaction between Apt-CF-NPs and A549 cells, fluorescent microscopy was used to illustrate the location of Apt-CF-NPs inside the cells (Fig. 7).

It was clearly demonstrated that the Apt-CF-NPs mainly accumulated within the cells at 37°C. Therefore, Apt-CF-NPs could be internalized into the target cells and could transmit the cargos into the cells. Compared to Apt-CF-NPs and free CF, far fewer CF-NPs entered

the A549 cells at 37°C, confirming that AS1411 aptamers enhance the cellular uptake of nanoparticles.

Conversely, free CF and CF-NPs cannot bind with A549 cells at 4°C but A549 cells incubated with Apt-CF-NPs exhibited a strong green fluorescence that could not be washed away. Obtained results indicated that the cell internalization of Apt-CF-NPs is not energy dependent and AS1411 aptamer interacts with nucleolin on the surface of A549 at 4°C. The obtained results confirmed that the Apt-CF-NPs can target A549 cells with high affinity and specificity.

3.7. *In vitro* cytotoxicity

To investigate the enhanced GEM anti-proliferative effect brought about by the increased cellular uptake of particles due to AS1411 aptamer conjugation, an *in vitro* cytotoxicity assay was conducted.

The A549 and CHO cells were treated with free GEM, blank NPs or Apt-NPs, GEM-loaded NPs (GEM-NPs), and GEM-loaded NPs conjugated to the AS1411 aptamer (Apt-GEM-NPs). Blank NPs or Apt-NPs did not show cytotoxicity, signifying that the carriers are relatively nontoxic to the cells and that the observed cytotoxicity certainly originated from the encapsulated GEM.

Compared to GEM-NPs, Apt-GEM-NPs exhibited enhanced cytotoxicity in A549 cells ($P<0.05$) while the toxicities of GEM-NPs and Apt-GEM-NPs were similar in CHO cell line as negative control ($P>0.05$).

The cytotoxicity results are illustrated in Figure 8. The cytotoxicity of Apt-GEM-NPs was also examined after the addition of the free AS1411 aptamer as a competing ligand to block the A549 cell receptors, and the obtained results confirmed that the cytotoxicity efficiency is similar to non-targeted GEM-NPs ($P>0.05$).

The AS1411 DNA aptamer conjugation intensified the cytotoxicity of GEM-NPs into nucleolin-overexpressing A549 tumor cells. Furthermore, the Apt-NPs increased the GEM cytotoxicity in the A549 tumor cells, while indicating no efficacy against the free aptamer-treated A549 cells and nucleoline-negative CHO cells (Fig. 8).

IC₅₀ values for free GEM, GEM-loaded polymersome (GEM-NPs) and AS1411 aptamer conjugated GEM-loaded polymersome (Apt-GEM-NPs) against A549 and CHO cell lines are represented in Table 1.

Obtained results demonstrated that the IC₅₀ value for Apt-GEM-NPs against A549 was much lower than GEM-NPs ($P < 0.05$) while the IC₅₀ values for Apt-GEM-NPs and GEM-NPs in free aptamer-treated A549 and CHO was similar ($P > 0.05$).

This is consistent with the results shown in Figure 6, in which the AS1411 aptamer increased the uptake of Apt-NPs into A549 cells, but not in A549 cells treated with the free aptamer.

Altogether, the results imply that the AS1411 DNA aptamer may selectively increase the delivery of cargos to A549 non-small cell lung cancer. The AS1411 aptamers exhibited high affinity and selectivity against the A549 cell line.

Previous studies have demonstrated that the use of AS1411 aptamer-coupled polymeric nanoparticles have improved the efficacy of encapsulated drug for the treatment of gliomas.

The conjugation of polymeric nanoparticles with aptamers as targeting ligands enables these vehicles to deliver the encapsulated drugs in a sustained and spatially intelligent pattern, which may increase the therapeutic window of drugs and reduce their systematic toxicity.

Other studies have pioneered the development of MUC-1, AS1411, EpCAM, and A10 aptamer-targeted polymeric nanoparticles for cancer therapy.^{33, 50-52}

Cellular uptake of nanoscale vehicles is managed by the internalization process, specifically via endocytosis.⁵³

Receptor-mediated cell internalization is a particular endocytosis-mediated transport involved in receptor binding and internalization, which is the basis of novel approaches for targeting drug delivery to tumor cells.

The development of next-generation targeted vehicles for drug delivery is expected to solve the systematic toxicity problem and enable researchers to develop safer and more effective therapeutic nanoparticles.

In this study, we designed a 26-mer DNA AS1411 aptamer-based targeted drug delivery system to increase the delivery of GEM to nucleolin-overexpressing non-small cell lung cancer.

The high-level expression of nucleolin is correlated with the proliferative status of cells, and nucleolin levels are higher in tumors and actively dividing cells.⁵⁴ On the other hand, it was proved that the increasing expression of nucleolin associated with aggressive characteristics of cancer tissues, and implied that nucleolin expression may serve as a promising biochemical marker for predicting the clinical outcome of patients with malignancy.⁵⁵

AS1411 is the first ssDNA aptamer to reach phase I and II clinical trials.⁵⁶ AS1411 can bind to nucleolin specifically and is subsequently internalized into the tumor cells, including in colorectal, breast, prostate, and non-small cell lung cancers.⁵⁷⁻⁵⁹

Moreover, AS1411 Apt is resistant to nuclease degradation and remains stable in plasma and serum-containing medium.⁶⁰ It has been successfully implemented as a targeting ligand in various drug delivery systems.^{61, 62} For this reason, nucleolin is ideal as a target for aptamer-based guided drug delivery to treat a various adenocarcinomas.

In this study, the AS1411 aptamer was utilized for the first time as a targeting ligand in a nanopolymersome-based GEM delivery system. The AS1411 aptamer exhibited high binding affinity and selectivity toward non-small cell lung cancer.

The prepared AS1411 aptamer-targeted nanopolymersome delivery system was assessed for its targeting capability against A549 cells *in vitro*.

Pretreatment of A549 cells with free AS1411 aptamer significantly neutralized the increased uptake of Apt-NPs. When A549 cells were pretreated with free AS1411, cellular uptakes were the same for NPs treated cells without Apt modification (Figure 6), indicating that blockade of nucleolin by free aptamer hinders the cancer cell targeting ability in Apt-NPs. Thus, the nucleolin-mediated transmembrane pathway is an important mechanism for cellular uptake of Apt-NPs. Comparable to the previously prepared targeted drug delivery system based on A10, MUC-1, and EpCAM aptamers, we observed enhanced uptake and cytotoxicity of Apt-NPs by A549 cells.^{33, 50-52}

While the nucleolin protein as a biomarker is overexpressed on the surface of numerous types of cancer cells, the prepared targeted drug delivery system may potentially improve the delivery of GEM to nucleolin-overexpressing cancer cells. Our results suggest that AS1411-targeted GEM-loaded nanopolymersomes are promising candidates for clinical translation as a first-line drug in NSCLC chemotherapy.

4. Conclusion

We have demonstrated that AS1411 aptamer-targeted GEM-loaded PEG-PLGA nanopolymersomes are able to achieve preferential receptor-mediated targeting of A549 cells *in vitro*. Furthermore, the GEM-loaded nanopolymersomes were capable of controlled and sustained release of GEM.

The unique characteristics of biodegradable PEG-PLGA nanopolymersomes, the suitability of polymeric vesicles as carriers for amphipatic drugs, the overexpression of nucleolin on non-small cell lung cancer, and the potency of nucleolin-mediated transport make this system attractive to complement and improve existing treatment methods for non-small cell lung cancer.

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Figure legends

Figure 1. ^1H NMR spectrum of the PEG-PLGA copolymer in CDCl_3 .

Figure 2. TEM image of AS1411 aptamer conjugated GEM-loaded nanopolymersomes (A); GEM-loaded nanopolymersomes (B); Size distribution of aptamer conjugated GEM-loaded nanopolymersomes (C); Size distribution of GEM-loaded nanopolymersomes (D). Scale bar in both A and B insets is 200 nm.

Figure 3. Agarose gel electrophoresis (2.5%). Lane 1: Apt-GEM-NP before washing, lane 2: Apt-GEM-NP after washing, lane 3: GEM-NPs, lane 4: Apt-GEM-NP after washing and lane 5: free AS1411 aptamer.

Figure 4. UV absorption of AS1411 aptamer at 260 nm ($n=3$) on the surface of nanopolymersomes that reacted with aptamers. $p>0.5$ consider as non-significant (ns), *** $p<0.001$.

Figure 5. Release profile of GEM-NPs (A) and Apt-GEM-NPs (B) formulations in PBS pH 7.4 and citrate buffer pH 5.5 ($n=3$, error bars represent standard deviation).

Figure 6. Flow cytometry analysis of A549 cells incubated with targeted and non-targeted carboxy fluorescein loaded nanopolymersome formulations.

Figure 7. Fluorescent microscopy images detecting cellular uptake of free carboxy fluorescein (A); carboxy fluorescein loaded nanopolymersomes (B); AS1411 targeted carboxy fluorescein -loaded nanopolymersomes (C) at 37°C and fluorescent microscopy images detecting cellular uptake of free carboxy fluorescein (D); carboxy fluorescein loaded nanopolymersomes (E); AS1411 targeted carboxy fluorescein -loaded nanopolymersomes (F) at 4°C .

Figure 8. Cytotoxicity of free GEM, GEM-loaded nanopolymersomes; AS1411 targeted GEM-loaded nanopolymersomes on the A549 and CHO cell lines after 48 h. ($n=4$, error bars represent standard deviation).

Table

Table 1. IC₅₀ values (μg/ml) for free GEM, GEM-loaded polymersome (GEM-NPs) and AS1411 aptamer conjugated GEM-loaded polymersome (Apt-GEM-NPs) on A549 and CHO cell lines after 48 h.

Cell lines	IC ₅₀ GEM	IC ₅₀ GEM-NPs	IC ₅₀ Apt-GEM-NPs	IC ₅₀ free Apt+ Apt-GEM-NPs
A549	1.75	28.9	4.9	28.3
CHO	8.05	35.7	33.2	-----

Figures

Figure 1

¹H NMR spectrum of the PEG-PLGA copolymer in CDCl₃.
117x122mm (300 x 300 DPI)

Figure 2

TEM image of AS1411 aptamer conjugated GEM-loaded nanoparticles (A); GEM-loaded nanoparticles (B); Size distribution of aptamer conjugated GEM-loaded nanoparticles (C); Size distribution of GEM-loaded nanoparticles (D). Scale bar in both A and B insets is 200 nm. 245x169mm (300 x 300 DPI)

Figure 3

Agarose gel electrophoresis (2.5%). Lane 1: Apt-GEM-NP before washing, lane 2: Apt-GEM-NP after washing, lane 3: GEM-NPs, lane 4: Apt-GEM-NP after washing and lane 5: free AS1411 aptamer.
253x129mm (300 x 300 DPI)

Figure 4

UV absorption of AS1411 aptamer at 260 nm (n=3) on the surface of nanopolymersomes that reacted with aptamers. $p > 0.5$ consider as non-significant (ns), *** $p < 0.001$.
296x314mm (300 x 300 DPI)

Figure 5

Release profile of GEM-NPs (A) and Apt-GEM-NPs (B) formulations in PBS pH 7.4 and citrate buffer pH 5.5 (n=3, error bars represent standard deviation).
827x304mm (300 x 300 DPI)

Figure 6

Flow cytometry analysis of A549 cells incubated with targeted and non-targeted carboxy fluorescein loaded nanopolymersome formulations.
332x247mm (300 x 300 DPI)

Figure 7

Fluorescent microscopy images detecting cellular uptake of free carboxy fluorescein (A); carboxy fluorescein loaded nanopolymersomes (B); AS1411 targeted carboxy fluorescein -loaded nanopolymersomes (C) at 37°C and fluorescent microscopy images detecting cellular uptake of free carboxy fluorescein (D); carboxy fluorescein loaded nanopolymersomes (E); AS1411 targeted carboxy fluorescein -loaded nanopolymersomes (F) at 4°C.

250x149mm (300 x 300 DPI)

Figure 8

Cytotoxicity of free GEM, GEM-loaded nanopolymersomes; AS1411 targeted GEM-loaded nanopolymersomes on the A549 and CHO cell lines after 48 h. (n=4, error bars represent standard deviation).
611x428mm (300 x 300 DPI)