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Shell cross-linked polyethylenimine-modified micelles for temperature-triggered drug release and gene delivery

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Abstract

Stimuli-responsiveness is an important characteristic of nanocarriers that can deliver therapeutic molecules in a controlled and on-demand manner. In particular, temperature-responsive micelles have recently gained increasing attention for use in diverse therapeutic areas, including hyperthermia therapy. Herein, we developed novel shell cross-linked Pluronic F127 (PF127) polymers with branched polyethylenimine (PEI), called FPEI, for temperature-triggered drug delivery and gene transfection. The successful synthesis of FPEI copolymers was confirmed by Fourier-Transform Infrared spectroscopy, with the weight percent of PEI in the FPEI copolymers being 7.5%. The critical micelle concentration of FPEI and PF127 copolymers was shown to be 0.013 and 0.03 mg/ml at room temperature, respectively. When Nile Red (NR), used as a hydrophobic model drug, was loaded into FPEI copolymers, the appropriate concentration of NR was 3 and 5 μg/ml in 5 and 10 mg/ml of FPEI, respectively. Interestingly, the loading capacity of FPEI increased with an increase in the micelle concentration. As temperature increased, the NR release from the micelle increased, while the micelle radii of FPEI, NR-loaded FPEI, and NR-loaded FPEI/DNA decreased. The DNA binding ability of FPEI copolymers increased with an increase in the N/P ratio. FPEI cytotoxicity in HeLa cells showed a similar tendency, when equivalent PEI amount in FPEI was treated. While further in-depth studies remain as to the in vitro and in vivo biological functions, the FPEI system demonstrated well temperature-triggered NR release into HeLa cells, along with sufficient transfection of plasmid green fluorescence protein, which suggested potential usefulness in temperature-responsive therapy.

Keywords: Temperature-responsive, Micelle, Multifunctional delivery, Transfection, Polyethylenimine, Pluronic F127, Gene delivery, Hyperthermia, Critical micelle concentration, Nile red

1. Introduction

The use of stimuli-triggered micelle delivery has received great attention in active targeting therapeutics, due to the unique properties of responding to specific stimuli and the protection of normal tissues or cells from the damaging effects of loaded biomolecules. The stimuli-triggered delivery system is designed to act by specified stimuli, such as temperature,[1] pH,[2] ionic strength,[3] magnet,[4] glutathione,[5] and light.[6] Among these stimuli, temperature has long been used as one of the most-effective signals due to the ease of manipulation and safety in medical applications, such as the hyperthermia treatment of solid tumors. For example, poly(N-isopropylacrylamide) (PNIPAAm) and Pluronics have been widely used for targeting therapeutics with local hyperthermia,[7, 8] owing to the self-assembled micelle forming property in aqueous solutions.[9, 10] Self-assembled micelles with nanoreservoir structure are able to encapsulate poorly water-soluble drugs in the hydrophobic core above the critical micelle concentration (CMC), leading to improved water solubility and efficiency of the release of therapeutic drugs. However, micelles composed of PNIPAAm or Pluronics can dissociate into polymeric units at low concentrations, below the CMC, upon dilution by body fluids during blood circulation, consequently losing their drug-loading capacity.[11] In addition, the nonionic nature of these polymers results in hardly forming gene complexes or facilitating cellular uptake.[12] Recently, one challenging approach to crosslinking the micellar shell to cationic polymers has been suggested not only to avoid micellar dissociation in the *in vivo* condition but also to facilitate gene complex formation,[13-17] which include chitosan,[18] poly-L-lysine[19] or polyethylenimine (PEI).[20] Of these, branched PEI containing primary, secondary, and tertiary amines is well-accepted as a non-viral gene carrier with high buffering capacity over a wide pH range. The unique structure and properties of PEI made possible to facilitate immediate formation of gene complexes, cellular uptake, and further endosomal escape.

In this work, Pluronic F127 (PF127)-crosslinked PEI (FPEI) nanoparticles were produced for temperature-triggered micellar release of drugs and gene transfection. The resultant FPEI nanoparticles were characterized with a focus on co-delivery of drugs and genes by thermal transition reaction.

2. Materials and methods

2.1. Activation of PF127 with 4-nitrophenyl chloroformate (4-NPC)

PF127 (MW 12500 Da, Sigma-Aldrich, USA) was activated with 4-NPC (Sigma-Aldrich, USA) as described by a previous report.[21] Briefly, a solution of PF127 (1 g) dissolved in 3 ml benzene was slowly added to a stirred solution of 4-NPC (0.05 g) in 3 ml benzene. After stirring for 24 h, the resultant was precipitated at least twice using an eight-fold excess of petroleum ether, and recovered by filtration by removal of the remaining solvent by evaporation under vacuum overnight. The substitution degree of *para*-nitrophenolate ions was determined in an alkaline solution (0.1 N NaOH) by spectrophotometry at 402 nm.

2.2. Synthesis and characterization of FPEI

FPEI was synthesized by a modified method as previously reported.[21] Briefly, activated PF127 (600 mg) was added to 1.2 g of branched PEI (MW: 1,800 Da, Polysciences, Inc. USA) in 15 ml of 0.1 N NaOH. The reaction mixture was stirred for six hours at room temperature, and purified by a dialysis membrane with a molecular weight cut-off (MWCO) of 3,500 against deionized water for 3 days for freeze-drying. The synthesis of FPEI copolymers was confirmed by Fourier-Transform Infrared Spectrometry (FT-IR, Perkin Elmer, Spectrum BXII spectrometer, USA). The conjugated PEI content was calculated by a TNBS (2,4,6-trinitrobenzene sulfonic acid) assay.[22]

2.3. Determination of critical micelle concentration (CMC)

CMC was measured by a modified method as described by previous reports.[23-25] Briefly, a 0.5 mg/ml stock solution of Nile Red (NR) fluorophore (Sigma-Aldrich, USA) in acetone was prepared and stored at 4^{o}C until use. NR is hardly water soluble, but exhibits strong fluorescence intensity even in tiny amounts. For the CMC measurement, FPEI in the aqueous solution was prepared in its

concentrations ranging from 0.0001 mg/ml to 1 mg/ml. To these polymer solutions, NR dissolved in acetone was added to become a final concentration of 5 μg/ml. The resulting solutions were placed into a 96-well plate for a fluorescence measurement on a fluorescence plate reader (Spectramax M2, Molecular Devices, USA) with λ_{ex}=544 nm and λ_{em}= 612 nm at room temperature. The CMC was determined as the intersection point on the two linear lines fitting the plots of the concentration-dependent section and the concentration–independent section. The hydrodynamic radii of FPEI, NR-loaded FPEI, and NR-loaded FPEI complexes with DNA at the concentrations exhibiting their morphological changes were measured on the basis of the principle of dynamic light scattering (DLS) using a Zetasizer (Malvern, Worcestershire, UK).[26]

2.4. NR loading efficiency and release

FPEI solutions (1, 5, and 10 mg/ml) containing NR over the concentration range of 0.1-10 μg/ml were prepared to measure the NR loading efficiency. The mixtures were incubated at 60°C for 1 h, cooled down to room temperature for 1 h to reach micellar equilibrium, and placed into a 96-well plate for measurement of fluorescence after filtration with a 0.45 μm syringe filter. Loading efficiency was analyzed by measuring the fluorescence intensity of NR in the micelle solutions.

In order to assess temperature-dependent micelle release behaviors, the NR release study was performed using a dialysis method. In brief, 0.2 ml of 10 mg/ml of FPEI solutions loading 3 μg/ml of NR fluorophore was placed into D-tube™ Dialyzer mini tubes with an MWCO of 6-8 kDa (EMD Millipore, Damstadt, Germany). Then, each group of samples was incubated at 25, 37, and 40°C. The released NR extract from the tube was then collected and replaced with fresh water (0.5 ml) after given periods of incubation time. The NR concentration in the released extract was determined by correspondence to standard curves of fluorescence for the molecules dissolved in 1 % acetone. Unreleased NR content in the dialysis tubes was measured to further analyze total NR amounts more precisely. The cumulative NR release (%) was calculated as the weight of the released NR to the weight of the total NR amounts fed.

2.5. Gene complexes with FPEI copolymers

FPEI complexes with plasmid DNA were confirmed by agarose gel retardation. FPEI/DNA complex over an N/P ratio range up to 7.5 was loaded on a 1.5 % agarose gel containing 0.1 μg/ml of Red Safe (Intron, South Korea). The resulting DNA migration pattern was exhibited under UV irradiation. The surface charge of the FPEI/DNA complex over an N/P ratio range up to 100 was measured by the DLS method using a Zetasizer (Malvern, Worcestershire, UK).

2.6. Cell culture for toxicity

For the *in vitro* cytotoxicity study of the FPEI copolymers, the human cervical cancer cell line (HeLa) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cells were maintained at 37 ^{o}C in an atmosphere of 5 % CO_2 in DMEM containing 10 % fetal bovine serum (FBS, Gibco, USA) and 1 % penicillin-streptomycin. The medium was regularly refreshed and the cells were passaged at confluence. The cells were plated in a 96-well plate at a density of 5 x 10^3 cells per well with 100 μl culture medium and cultured for 24 h. The sterilized PF127, FPEI, and PEI samples at various concentrations were added to each well of the plates, and incubated at 37 and 40^{o}C for 1 h. Next, the plate that was incubated at 40^{o}C was returned to an incubator at 37^{o}C. At 48 h after treatment of the samples, the culture medium was replaced with fresh medium containing 10 % cell counting kit-8 solution (CCK-8, Dojindo, Japan), and incubated for another 4 h at 37^{o}C. At the end of CCK-8 treatment, a 100 μl aliquot of each cell culture supernatant was collected and measured at 450 nm using an iMark microplate reader (BioRad, USA).

2.7. In vitro cell transfection and NR release

The cells were seeded into a 24-well plate at a density of 2.5 x 10^4 cells per well with 0.5 ml of culture

medium. Plasmid green fluorescence protein (GFP) (1 μg) was added to NR (1 ng)-loaded FPEI micelles (0.04 mg), and incubated at room temperature for 30 min. Then, the complexes were added into each well and incubated at 37 and 40°C for 1 h. Next, the plate that was incubated at 40°C was returned to an incubator at 37°C. After 36 h of treatment of the samples, the culture medium was replaced with fresh medium and the cells were observed by an inverted fluorescence microscope (IX-71, Olympus, Japan).

3. Results and discussion

3.1. Synthesis of shell cross-linked FPEI micelles

In this study, PEI was covalently conjugated with the end group of PF127 copolymers following 4-NPC activation, to produce a dual carrier that can facilitate drug loading and DNA complex formation with PEI. **Figure 1a** depicts the molecular structure of the synthesized FPEI, and a schematic diagram exhibiting the temperature-triggered drug release by the squeezing effect of the FPEI micelle complexes with the genes. The conjugation of PEI with PF127 was analyzed by FT-IR spectra over the range of 400-4000 cm^{-1} for the PF127 and FPEI copolymers, as shown in **Figure 1b.** Distinctive amine (-NH-) stretching at 3450 cm^{-1} and amine (-NH-) bending at 1630 cm^{-1} detected were assigned to the amine groups of PEI in the FPEI spectrum, indicating covalent conjugation of PEI and PF127. The weight content of the total nitrogen molecules in the FPEI copolymer calculated by a TNBS assay was 7.5 %, possibly forming gene complexes and facilitating cellular uptake.

3.2. Self-assembled micelle formation of FPEI copolymers

The CMC is a determinant parameter determining the relative thermodynamic stability of self-organized micelle aggregates in aqueous solutions. The polymeric CMC is determined by the fluorescence measurement of increasing polymer concentrations.[27, 28] Self-assembled micelles possessing the spherical core-shell structure are formed above the CMC, and fluorescence probes

can be used to determine the CMC.[29] The CMC of FPEI copolymers was found by a modified method using an NR fluorophore as a fluorescence probe.[23] **Figure 2a** shows the CMC measurement by the fluorescence intensity of the NR fluorophore, indexing arbitrary units as a function of copolymer concentrations. The micelle formation of FPEI and PF127 was achieved at concentrations above 0.013 and 0.03 mg/ml, respectively. The CMC of FPEI micelles was approximately 2.3-fold lower than that of PF127, providing direct evidence for an increase in the colloidal stability upon shell cross-linking.[30, 31]

The hydrodynamic radii of free FPEI, NR-loaded FPEI, and NR-loaded FPEI complexes with DNA were measured by a DLS at increasing temperatures to examine the temperature-triggered behavior of the FPEI micelles (**Figure 2b**). Temperature-dependent decreases in particle diameter were monitored for the different types of samples with an increase in temperature. The diameter of the FPEI micelles was reduced from hundreds of nanometers at room temperature, to a few tens of nanometers above body temperature. Conversely, the size reduction degree of the NR-loaded FPEI micelles and their complexes with DNA was less than that of free FPEI micelles, owing to the presence of NR loaded in the micelle core. The temperature-dependent size change was attributed to the hydrophobic interaction by rigid PPO chains in the micelle core,[23, 32] while the hydrophobically condensed micelles may effectively release NR molecules.

3.3. In vitro drug loading and release of thermosensitive FPEI micelles

The loading efficiency of NR into FPEI micelles at the given concentrations (1, 5, and 10 mg/ml) was investigated as a function of NR concentration, to understand the hydrophobic drug loading behavior of FPEI micelles. **Figure 3a** shows the NR loading efficiency, indicating the influence of FPEI concentration on how much payload of NR was possible. The loading efficiency was determined by fluorescence intensity quantification of the amount of NR which the FPEI micelles were capable of loading. The NR payload achievable without significant loss was shown to be appropriate at 3-5 μg/ml after the purification process, i.e., the best aqueous stability was exhibited at the highly

achievable concentration of NR payload in the FPEI system. NR loading efficiencies of the FPEI micelles were dramatically improved with an increase in NR concentration (3-5 μg/ml), but were notably reduced above that concentration. This was possibly due to the precipitation of insoluble NR aggregates in aqueous media that might not be loaded into the FPEI micelles, indicating the loading capacity limit, which would decrease the room for drug entrapment.[33-35] From these results, it is important to note that the drug-loading capacity of FPEI micelles was significantly increased up to the extent of the optimal drug concentration, upon finding the appropriate drug payload. The results implied that drug-loading capacity was largely dependent on hydrophobic interaction with PPO chains in FPEI copolymers.

For the cumulative NR release test, NR-loaded FPEI micelles were incubated at three temperature points, 25, 37, and 40^{o}C, on the basis of the results of the NR loading efficiency. **Figure 3b** shows the temperature-dependent release behaviors of NR-loaded FPEI micelles in aqueous media as a function of the release periods. With an increase in temperature, cumulative NR release from the FPEI micelles was improved. This is because the stronger hydrophobic interactions between PPO chains and NR in the core caused the micelles to squeeze together further at higher temperatures, thereby resulting in accelerated NR release.[23, 36] These results also suggested that the FPEI micelles showed a potential for use in the temperature-triggered delivery of hydrophobic drugs.

3.4. Gene complexes with FPEI copolymers

Having confirmed the effective loading and delivery of hydrophobic drugs in the developed nanocarriers, we next aim to co-deliver negatively-charged genetic molecules. The co-delivery of genes with drugs in the same pot has often been shown to synergize the therapeutic efficacy, including anticancer effects.[37] First, we sought to achieve high loading capacity of both therapeutic molecules within the nanocarrier depots, which is required for subsequent efficient release. In order to determine the DNA binding ability of FPEI, the formulation of NR-loaded FPEI complexes with pDNA was confirmed at different N/P ratios by an agarose gel retardation assay (**Figure 4a**), and their

surface charge was analyzed by zeta potential measurement (**Figure 4b**). These DNA complexes were electrophoresed down at an N/P ratio of 1.5, but not above an N/P ratio of 3.0. Likewise, the surface charge of the complexes increased with an increase in the N/P ratio. These results indicated that the DNA binding ability increased with an increase in N/P ratio, which was consistent with a number of typical analyses of PEI-DNA complexes.

3.5. Cytotoxicity of FPEI micelles

A CCK-8 assay was conducted to evaluate the *in vitro* cytotoxicity of FPEI micelles over a wide range of concentrations on HeLa cells at 37 and 40^{o}C, as shown in **Figure 5**. After 1 h, both were incubated at 37^{o}C for two days. FPEI samples below a concentration of approximately 250 μg/ml did not induce cytotoxicity, but those above 250 μg/ml began to show severe cytotoxicity, compared to the PF127 cytotoxicity results. A similar tendency was shown at both temperatures observed. We assumed that these results were caused by the amine groups of PEI conjugated with PF127, rather than the incubation temperature. Thus, the cytotoxicity test of PEI itself was assessed over the range of 0.5-50 μg/ml, concentration range of which was equivalent to that of PEI contained in the FPEI copolymers. The resulting PEI cytotoxicity showed a similar tendency to the results obtained for the FPEI samples. This suggested that a concentration range of FPEI micelles from the CMC up to approximately 250 μg/ml was a safe window for use in *in vitro* delivery.

3.6. Temperature-dependent NR release with pGFP transfection

From our preliminary experiments, the optimal N/P ratio for the best gene transfection was found at nearly 20-30 (figure not shown), and the NR concentration to make a staining difference between 37 and 40^{o}C was approximately 10 ng/ml underlying the FPEI system. Thus, pGFP (1 μg) was added into 1 ng NR-loaded FPEI micelles (80 μg/ml) and incubated for 30 min to form stable gene complexes. The complexes were added into the HeLa cell-populated plate. At 1h after delivery,

a few cells with weak red fluorescence were observed in the cultures under the condition of 40°C, but not at 37°C, despite the equal exposure time (**Figure 6**). At 36 h, however, the red and green fluorescence intensity denoting NR-staining and GFP expression, respectively, became almost similar at both incubation temperatures. In comparison to these results in the presence of FPEI carriers, almost all HeLa cells exposed to free NR showed red fluorescence independently of incubation time, implying that free NR might explosively diffuse into the cytosol through the cell membrane adsorption as soon as the treatment. These results indicated that the FPEI system demonstrated a temperature-triggered NR release into the cytosol, consistent with the cumulative NR release test, along with pGFP transfection, probably following the endocytosis by PEI in the FPEI copolymers.

4. Conclusion

Thermosensitive and shell cross-linked micelles composed of PF127 grafted to PEI were successfully prepared to facilitate hydrophobic drug encapsulation and formation of gene complexes. In this study, the FPEI system was logically characterized by performing sequential measurements, including the temperature-dependent size, drug loading and release behaviors, DNA binding ability, and surface charge. The FPEI system showed a potential for temperature-responsive drug release along with gene transfection in HeLa cells. Development of thermosensitive and shell cross-linked FPEI micelles suggests a promising possibility for temperature-responsive therapeutics, while further in-depth biological studies on practical therapeutic drugs and genes are needed.

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Figure captions

Figure 1. (a) Schematic diagram showing the temperature-triggered FPEI micelle system, including its molecular structure. (b) FT-IR analysis of F127 and FPEI copolymers.

Figure 2. (a) CMC measurement of F127 and FPEI copolymers at room temperature. Arrows indicate the respective CMC. (b) Temperature-dependent size measurement of FPEI, NR-loaded FPEI, and NR-loaded FPEI complexes with DNA (n=3).

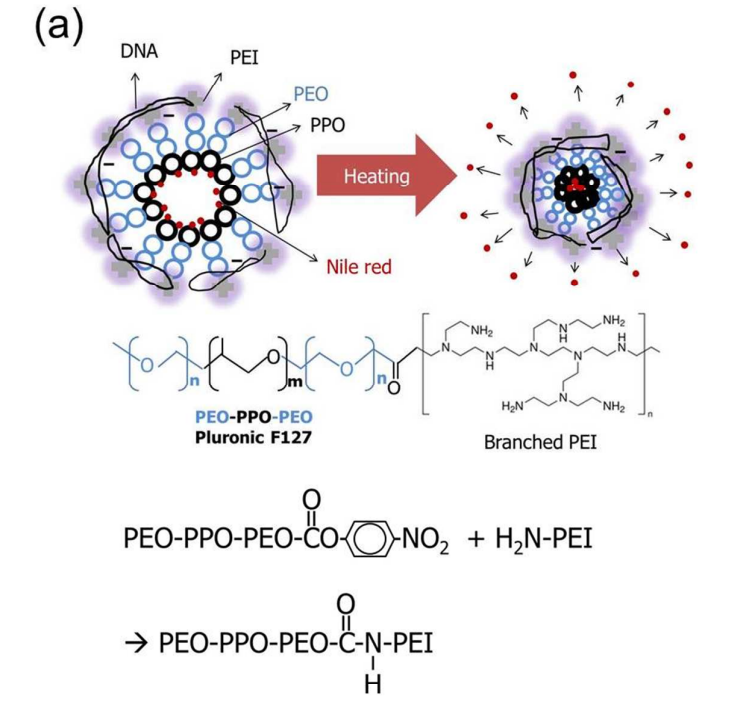
Figure 3. (a) NR loading efficiency in FPEI micelles (1, 5, and 10 mg/ml). (b) Cumulative NR release from the FPEI micelle system at 25, 37, and 40^{o}C, based on the results of NR loading efficiency (n=3).

Figure 4. (a) Gel retardation assay and (b) zeta potential measurement to understand the DNA binding behavior of FPEI copolymer with an increase in N/P ratio (n=3).

Figure 5. *In vitro* cytotoxicity of F127, FPEI, and PEI, conducted on HeLa cells at 37 and 40^{o}C. Results were presented as mean ± SD (n=5).

Figure 6. Fluorescence images of HeLa cells treated with GFP transfection and NR release underlying the optimized FPEI delivery system at 37 and 40^{o}C after 1 and 36 h incubation.

342 Fig. 1.



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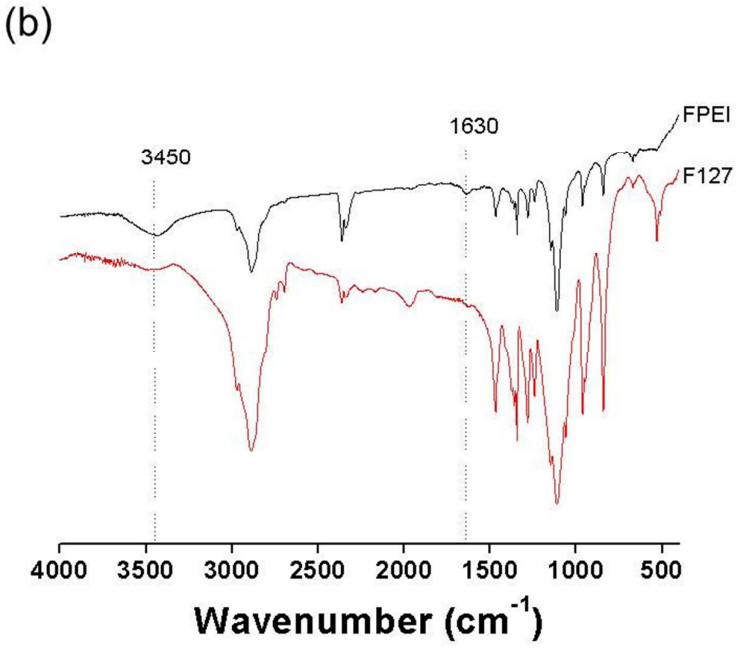

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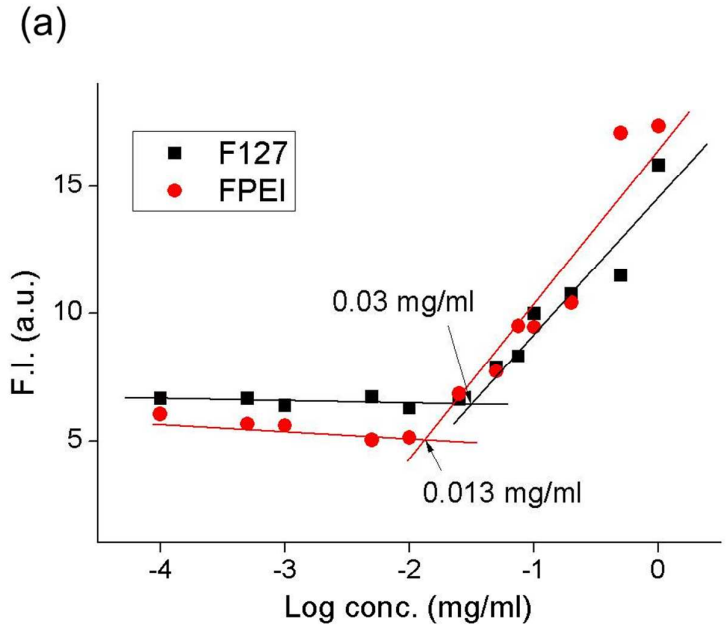

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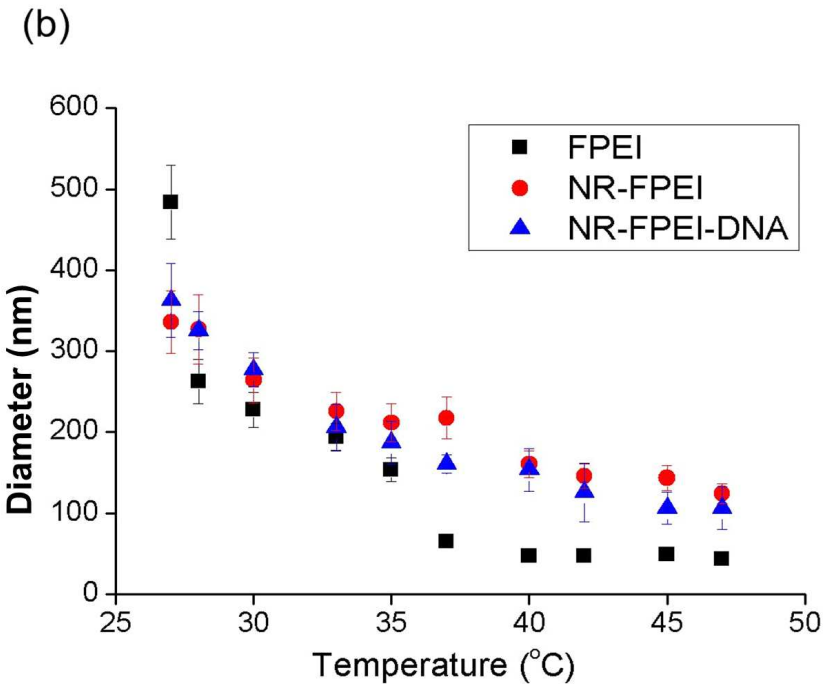

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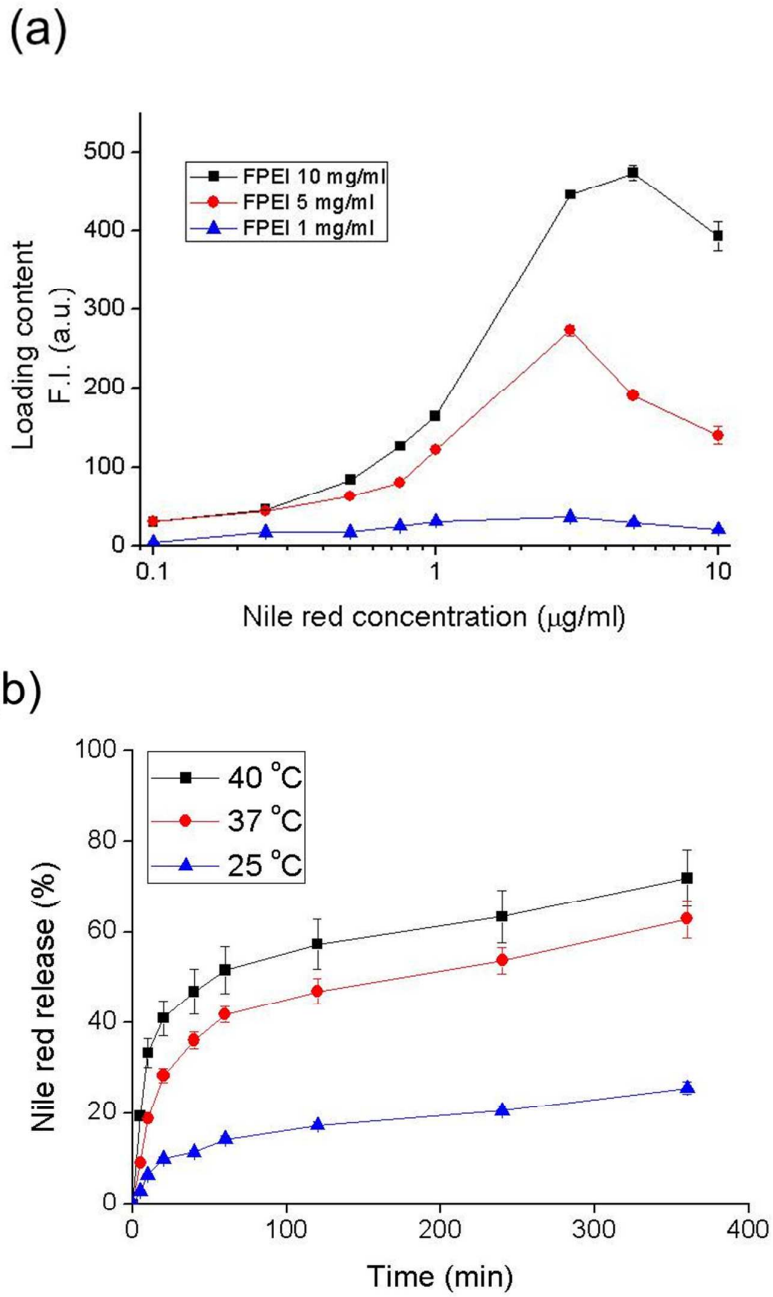
350 Fig. 3



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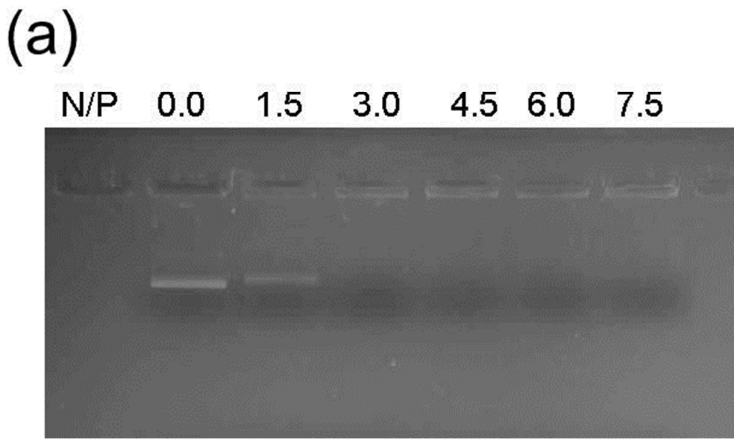
354 Fig. 4.



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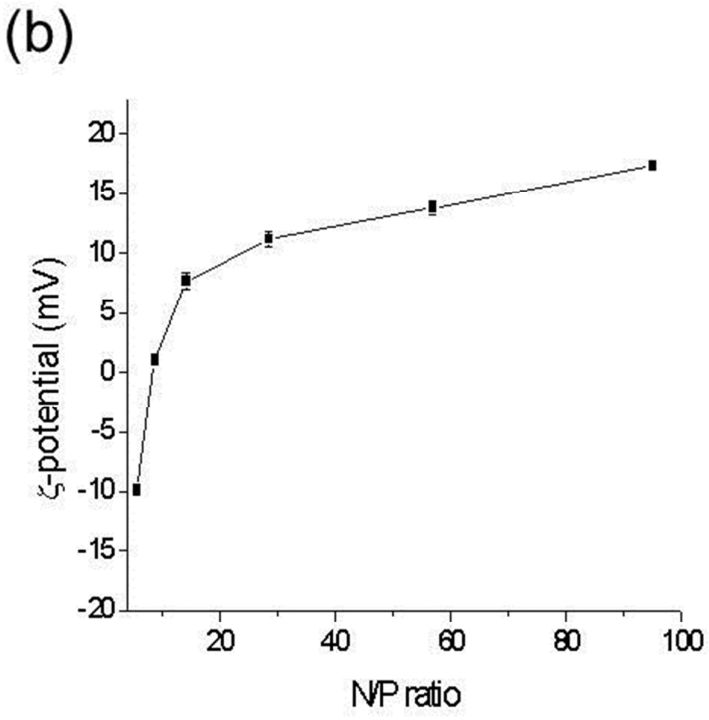

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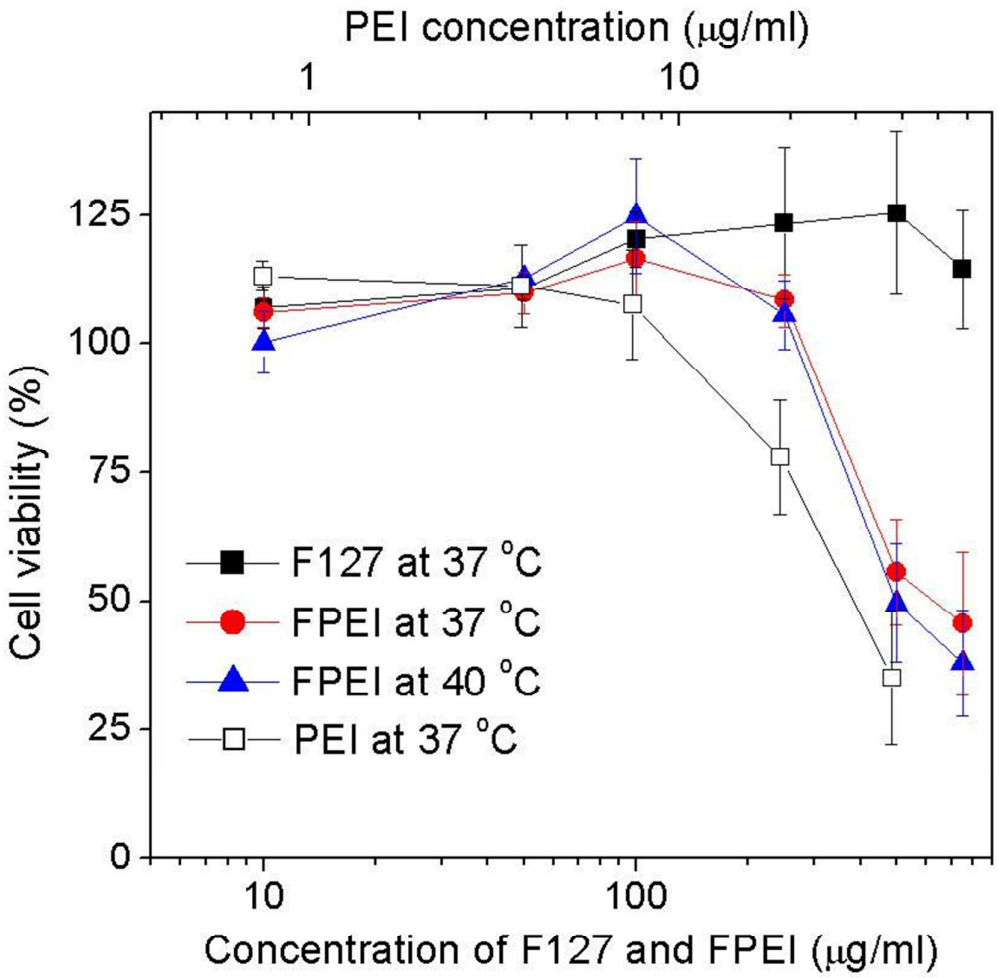
358 Fig. 5.



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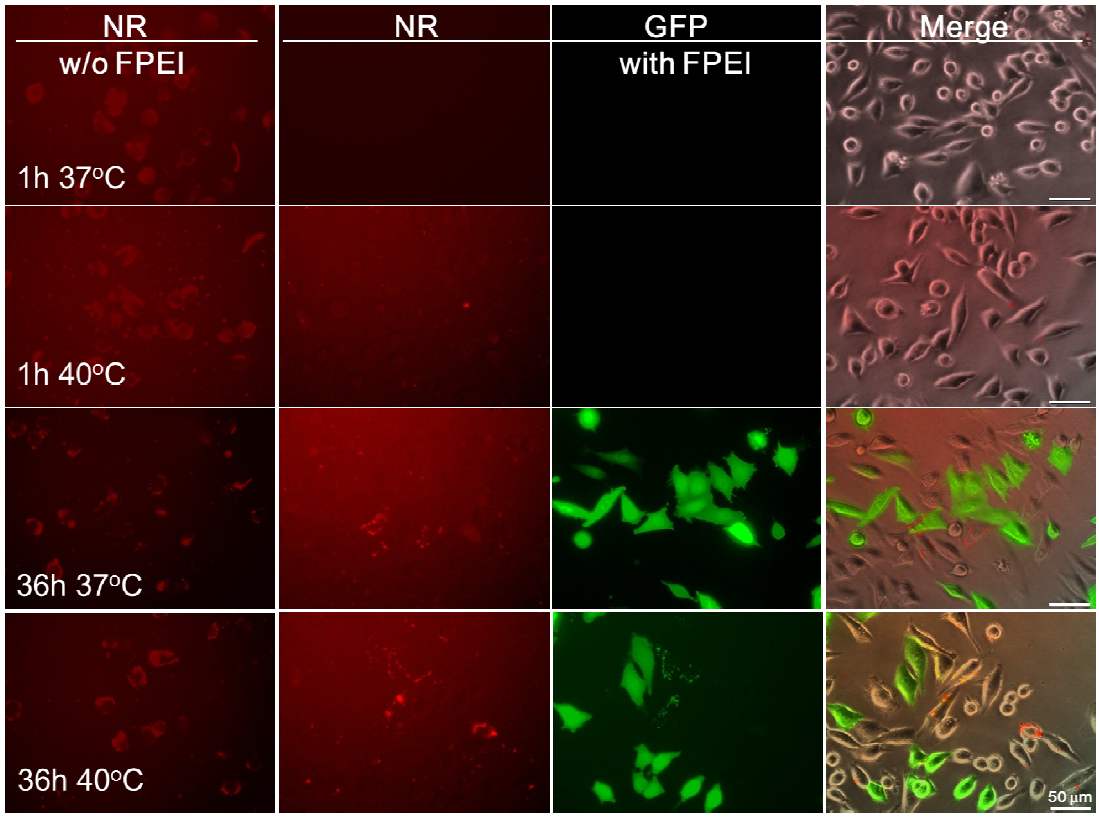
361 Fig. 6.



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