Construction of an Effective Delivery System for DNA Vaccines Using Biodegradable Polylactic Acid Based Microspheres

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Research

Keywords: PLA/PEI microspheres, delivery system, DNA vaccines, foot-and-mouth disease

Posted Date: December 4th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-118223/v1

Abstract

Background: Nanotechnology represents a new impetus for biomedical research applications, especially using nanotechnology to formulate microspheres or nanospheres based delivery system for treatment of infectious diseases in animals.

Results: Polylactic acid (PLA) microspheres with an average size of 156nm were prepared by combining emulsion polymerization coupled with emulsion–solvent evaporation. Coating with three different molecular weights of polyethylenimine (PEI) polymers increased the surface charges of the resulting PLA/PEI microspheres. Electrostatic interactions enabled plasmid DNA to adsorb tightly to the microspheres. After pig kidney-15 cells were cultured with the PLA/PEI/DNA complexes for 48 h, all three PLA/PEI microspheres successfully transferred plasmid DNA into the cells with high transfection efficiency. The protection rate of PLA/PEI microspheres loaded with DNA vaccine against foot-and-mouth disease in guinea pigs reached 87.5%, which was significantly higher than that of the control group injected with only DNA vaccine.

Conclusions: In this work, PLA/PEI microspheres were constructed by preparing PLA microspheres and modifying with PEI polymers, and were shown a great potential in improve the efficacy, biosafety and economic effects of DNA vaccines. The results indicated PLA/PEI microspheres were expected to be an effective delivery system for DNA vaccines.

Background

A DNA vaccine is a recombinant eukaryotic expression vector that encodes a specific protein antigen and is injected directly into an animal to enable the foreign gene to be expressed *in vivo*. The antigen is produced and then activates the immune system of the animal, thus inducing specific humoral and cellular immune responses. Compared with traditional vaccines, DNA vaccines have several advantages: (1) the potential to induce a broad immune response without the risks observed using replicating microorganisms[1]; (2) the ability to simultaneously stimulate humoral and cellular immunity[2]; (3) the process of purifying plasmid DNA is simple, inexpensive, and suitable for mass production[3]; (4) DNA molecules are stable, therefore easy to transport and preserve[4]; and (5) by simply mixing multiple plasmid DNA samples, antigens with similar biochemical properties can be combined to form a multivalent vaccine[5]. Based on these advantages, DNA vaccines may be substitutes for traditional vaccines and may have a wide range of applications in the treatment of infectious diseases in humans and animals[6]. For example, Zhang et al. have developed a DNA vaccine for *Clostridium difficile*, which encodes two *C. difficile* toxin receptor binding domains and showed good results in mouse and hamster model experiments[7]. Liu et al. have constructed a DNA vaccine encoding *Toxoplasma gondii* superoxide dismutase[8], which provided immune protection against acute toxoplasmosis in mice. However, many clinical studies have shown that DNA vaccines have weak immunogenicity and low transfection efficiency[9], which are key factors preventing the further application of DNA vaccines. Therefore, it is

desirable to improve the efficacy of DNA vaccines by developing vaccine delivery systems, adding appropriate adjuvants, and altering the vaccination route[10].

In biomedicine, the small size and large surface energy of nanoparticles has enabled their wide use to improve the pharmacokinetic properties, bioavailability and targeting ability of molecules, pharmaceuticals and drugs [11, 12]. PLA is a polyester polymerized from lactic acid, which has excellent biodegradability and compatibility[13]. PLA-based nanomaterials and composite nanomaterials have been used successfully as drug delivery systems[14]. For example, Rohollah Ghasemi has synthesized three PLA-polymerized nanoparticles by controlling the ratio of PLA to polyethylenimine glycol and used these nanoparticles to encapsulate human growth hormone[15]. The nanoparticles demonstrated good sustained-release ability and high drug-loading capacity[15]. Fatemeh Zabihi has prepared water-soluble poly (lactic acid–glycerol) granules by the ring-opening polymerization of glycerol and lactide monomers, which could improve the transdermal transfer rate and treatment efficiency of tacrolimus[16]. DNA vaccines can also be delivered using PLA as a carrier, but the delivery system has some problems, including instability and facile degradation[17]. Therefore, optimization of the delivery system, the search for effective combinations of vectors and DNA vaccines, and improvement of the stability and transfection efficiency are currently the main areas of DNA vaccine research.

PEI, one of the most widely investigated gene transfer polymers[18], is a cationic polymer with a high cationic charge density that can be attached to proteins or DNA by electrostatic attraction[19], PEI can act as an oligonucleotide delivery vector by forming complexes with oligonucleotides and then entering cells through endocytosis, and this delivery ability has been widely demonstrated *in vivo* and *in vitro*[20, 21, 22]. In the present study, PLA microspheres were prepared by controlling the water-to-oil ratio and shear rate, and the surfaces were coated with PEI to form a carrier system that could deliver DNA. Using this system, we transfected plasmids encoding genes for green fluorescent protein, and the gene was well expressed in mammalian somatic cells. The PLA/PEI microspheres with the highest transfection efficiency were used to load a DNA vaccine against foot-and-mouth disease (FMD) (Fig. 1).

Materials And Methods

2.1 Materials

Polyvinyl alcohol (PVA) was purchased from J&K Science Co., Ltd. (Beijing, China) and dichloromethane (DCM) was purchased from Sinopretics Co., Ltd. (Shanghai, China). The pEGFP-n1 plasmid expressing enhanced green fluorescent protein was purchased from BD Biosciences Clontech (Palo Alto, CA, USA). Porcine kidney-15 (PK15) cells were purchased from the National Science and Technology Laboratory Cell Resource Platform (Beijing, China). 24-well plates, 96-well plates, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco (Darmstadt, Germany).

2.2 Methods

2.2.1 Preparation of PLA microspheres

PLA microspheres were prepared by a combination of emulsion polymerization technology and an emulsion-solvent evaporation method, and the optimum shear rate and oil ratio of the PLA microspheres were investigated. To form the aqueous phase, 1 g PVA was added to 100 ml distilled water and stirred with a magnetic agitator at 500 rpm and 80 °C until completely dissolved. To prepare the oil phase, PLA (15 mg/ml) was completely dissolved in DCM using ultrasonication. Using a shear rate of 8000 or 10000 rpm, the oil phase was added into the water phase drop by drop at a ratio of oil phase: water phase = 1:3 or 1:5. After the addition of all the oil, the oil phase was continuously cut for 3 min. Then, the rotation speed of the shear machine was adjusted to 3000 rpm, and the oil was cut and mixed evenly to obtain a water-in-oil emulsion. The emulsion was ultrasonicated for 5 min and then the methylene chloride was volatilized overnight with a magnetic agitator at 500 rpm. The obtained PLA microspheres were dissolved in water and centrifuged at 10000 rpm for 10 min. The supernatant was discarded, and the PLA microspheres were obtained after freeze drying.

2.2.2 Synthesis of PLA/PEI microspheres

An appropriate amount of PEI polymers with molecular weights of 800, 10000, and 25000 were added into distilled water (1% solutions). The PEI polymers were added to the PLA microspheres at a PLA:PEI mass ratio = 1:3 and the mixture was stirred at 500 rpm for 4 h at room temperature, then, the solution was centrifuged at 8000 rpm for 10 min and the supernatant was discarded. Distilled water was added and the PLA/PEI microspheres were dissolved by ultrasonication, the solution was centrifuged at 8000 rpm for 10 min and after three or four repetitions of this washing process, the deposits were collected.

2.2.3 PLA/PEI microspheres loading DNA

Plasmid DNA containing green fluorescent protein (GFP) gene was dissolved in in DMEM solution and stirred evenly. Then, PLA/PEI microspheres were added at a mass ratio of PLA/PEI microspheres: plasmid DNA = 2:1 and the mixture was mixed well by oscillation. The mixtures were incubated at 37 °C for later use.

2.2.4 Particle size and Zeta potential measurements

The particle size and zeta potential were measured by the laser particle size analyzer (Malvern ZetasizerNano ZS, UK). At room temperature, the PLA microspheres and the PLA/PEI microspheres were diluted to 1 μg/mL in an aqueous solution. The average value of three measurements was used for the end result.

2.2.5 Morphology characterizations

The prepared PLA and PLA/PEI microspheres were dissolved with an appropriate amount of water, a drop of the solution was placed on a silicon wafer with a pipette. After completely dried at room temperature, they were observed under a scanning electron microscope (SEM, Hitachi SU8010 Japan). The PLA/PEI

microspheres loaded with DNA were dissolved in water. Drop an appropriate amount of solution on the mica sheet. After air drying for 10 minutes, gently rinse the mica sheet with ultra-pure water, and then put it into the mica sheet until it is fully dried. Record the results at the scanning speed of 0.99 Hz under an atomic force microscope (AFM, Bruker Multimode Ⅷ, USA).

2.2.6 Agarose gel electrophoresis

Agarose was placed in a conical flask, then electrophoresis buffer was added to prepare an agarose gel with a mass fraction of 1%. The conical flask was rotated, and the solution was mixed well. After heating and dissolving, ethidium bromide, a nucleic acid stain, was added. When the temperature had dropped slightly, the gel was poured into the groove and allowed to set.

A series of PLA/PEI microspheres and plasmid DNA composite solutions were prepared with mass ratios of PLA/PEI microspheres: plasmid DNA = 2:1, 1:1, 1:2, 1:4, 1:6, and 1:8. 30 μl solution was taken into the sample tank. Electrophoresis was performed for 40 min at 100 V.

2.2.7 Cell transfection experiments

PK15 cells were cultured at 37 °C, 5% CO_2, and a particular humidity in DMEM medium supplemented with 10% FBS and 1% antibiotics. The medium was changed after culturing for 48 h. An appropriate amount of DEME solution containing 10% FBS was added to a 24-well cell culture plate. Approximately 10^5 porcine kidney cells were inoculated into each well and cultured until the fusion rate reached 70%. The old culture medium was discarded, and the cells were rinsed once with PBS buffer. The medium was replaced with a medium that did not contain fetal bovine serum. Then, 200 μl of PLA/PEI/DNA complexes solution were added to each well. After a period of incubation at 37 ℃ and 5% CO_2, the expression of GFP was observed under a fluorescence microscope (Olympus IX70, Japan).

2.2.8 Cytotoxicity test

The cytotoxicity of the microspheres was evaluated by the 3-(4, 5- dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. PK15 cells were seeded into a 96-well plate at a concentration of 10^4 cells/well and cultured at 37 °C and 5% CO_2 for 24 h. Then, the PK15 cells were treated with PLA/PEI/DNA complexes and PLA/PEI microspheres. Untreated PK15 cells were used as a control. After incubation for 6 h, the medium was removed. The PK15 cells were cultured at 37 °C and 5% CO_2 for 24 h, and were cultured for another 4 h in DMEM containing MTT (0.5%), then the medium was carefully removed. Dimethyl sulfoxide (150 μl/well) was added and the solution was oscillated gently to dissolve the crystals. The absorbance at 490 nm was measured using a microplate reader. The cell viability was expressed as [OD490(sample)/OD490(control)] × 100.

2.2.9 Animal experiments

The DNA vaccine against foot-and-mouth disease (FMD), experimental animals (guinea pigs) and test sites were provided by the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The guinea pigs were monitored to be pathogen-free and all animals were treated according to

the regulations of Chinese law and the local ethical committee. Guinea pigs were given regular food and water to drink.

The study consisted of three groups with eight animals in each group: guinea pigs treated with PBS; guinea pigs treated with foot-and-mouth disease viral DNA; and guinea pigs treated with the PLA /PEI/DNA complexes. The weight of the guinea pigs was controlled to be approximately 300 g. Each guinea pig was given 200 μl of the vaccine, which contained 100 μg of DNA. Twenty-eight days after immunization, each guinea pig was injected with 200 μl of foot-and-mouth disease virus and the protection rate for each group was observed.

Results

3.1 Preparation of PLA microspheres

Nanoparticles with a size of less than 200 nm have been reported to be appropriate for cell adsorption and DNA transfection [23]. Therefore, the preparation of PLA microspheres with an appropriate particle size and good uniformity is required for use as vaccine carriers. However, there are many factors that can affect the preparation of PLA microspheres. Here, we explored the optimal shear rate and the optimal water-to-oil ratio for preparing PLA microspheres. The structure and morphology of single PLA nanometer-sized microspheres were observed directly by a scanning electron microscope (Fig. 2). The PLA microspheres prepared using four different conditions had good homogeneity, smooth surfaces, and no depressions. The shear rate and water-to-oil ratio mainly affected the particle size of the PLA microspheres. Therefore, the particle size of the PLA microspheres was measured by a Malvern particle size analyzer (Table 1 and Fig. 3). The average particle size of the PLA microspheres was the smallest (156 nm) with a shear rate of 10000 rpm and an oil-to-water ratio of 1:5, and the largest (449 nm) with a shear rate of 8000 rpm and an oil-to-water ratio of 1:3. These results indicated that the ratio of water to oil had a greater influence on the particle size of the PLA microspheres than the shear rate, and the subsequent ultrasonication was a critical factor in controlling the particle size.

Table.1. Average particle sizes of PLA microspheres under different preparation conditions.

Conditions	Average size(nm)
oil phase: water phase = 1:5, 8000 rpm	276
oil phase: water phase = 1:3, 8000 rpm	449
oil phase: water phase = 1:5, 10000 rpm	156
oil phase: water phase = 1:3, 10000 rpm	394

3.2 Preparation of PLA/PEI microspheres

The amount of cationic charge on the surface of nanoparticles affects the efficiency of their condensation with DNA[24, 25]. The purpose of modifying PLA nanometer-sized microspheres with PEI was to distribute more positive charge on the surface of the PLA microspheres to enable better loading of DNA by electrostatic attraction. Here, we selected three types of PEI with molecular weights of 800, 10000, and 25000. According to the results of zeta potential measurements, the average zeta potential of PLA microspheres increased, depending on the PEI molecular weights (Fig. 4); this was due to the electric negative PLA microspheres complexing with electric positive PEI. Especially, PLA/PEI microspheres modified with molecular weight of 10000 have the highest positive charge on the surface, with the average potential of 44.6 mV. So we predicted that it would have the strongest DNA adsorption capacity. Observation of the PLA/PEI microspheres indicated that there was no appreciable difference in morphology or particle size between the PEI-linked microspheres with different molecular weights (Fig. 5).

3.3 PLA/PEI microspheres loaded with DNA

PEI modification covers the surface of the PLA/PEI microspheres with a large number of positively charged groups, which provides a theoretical basis for the loading of negatively charged plasmid DNA. To verify the DNA loading capacity of PLA/PEI microspheres and investigate the optimal mass ratio, we prepared PLA/PEI/DNA composite solutions with mass ratios of microsphere/DNA = 2:1, 1:1, 1:2, 1:4, 1:6, and 1:8. In the electrophoresis of these solutions in agarose gel there were no bands from DNA at the mass ratios of 2:1, 1:1, 1:2, 1:4, and 1:6 which indicated that the DNA bound well to the PLA/PEI microspheres (Fig. 6). The PLA/PEI microspheres bind nucleic acid effectively, protecting the loaded nucleic acid from degradation by nucleases, thus ensuring the stability of the nucleic acid in transfection[26].

In order to observe the self-assembly of PLA/PEI microspheres and DNA, AFM was used to observe DNA-loaded PLA/PEI microspheres. As shown in Fig. 7, PLA/PEI microspheres present as individual spherical nanoparticles, with several DNA strands attached to each spherical particle. An area of the dotted box in Fig. 7(a) was selected to measure the height of DNA strands in PLA/PEI/DNA complexes. The line traces between two blue plus signs drawn in Fig. 7 (b) is about 4 nm in height, indicating that the selected sample was two strands of DNA. The red curve in Fig. 7 (b) showed that the height of one peak was maintained at 4 nm, indicating DNA strands, and the other peak is about 74 nm in height, which implied this red plus point is located in the surface of PLA/PEI microsphere. The results of AFM correspond to those of agarose gel electrophoresis that PEI modification can enhance the ability of PAL/PEI microspheres to adsorb DNA and DNA arranged radially with nanoparticles as the center, which laid a good foundation for plasmid DNA transfection.

3.4 Cytotoxicity test

The cytotoxicity of the PLA/PEI microspheres was evaluated by the MTT assay. After the cells were incubated with a series of PLA/PEI/DNA complexes with different mass ratios, the absorbance at 490 nm was measured using a microplate reader. The absorbance of five wells was randomly selected and one-

way analysis of variance was performed. As shown in Fig. 8, the survival rate of cells in each group was over 80% and there were no significant differences between the groups ($p > 0.05$), which indicated that the PLA/PEI microspheres had good biocompatibility.

3.5 Efficiency of cell transfection

Studies have shown that the GFP gene has the highest transfection efficiency after incubation for 24 to 48 h[27]. Therefore, we observed the expression of GFP in PK15 cells 48 h after transfection under a fluorescence microscope (Fig. 9). The expression rates of GFP in PK15 cells incubated with DNA and PLA–DNA complexes were 3.01% and 10.3%, respectively, while the expression rates of that incubated with the PLA/ PEI/DNA complexes all exceeded 70%. In particular, the PLA/PEI microspheres modified with PEI with a molecular weight of 10000 had the strongest DNA transfection ability with an expression rate of green fluorescent protein 80.6%, which was consistent with the amount of positive charge on the surface (Fig. 10). This may be due to the differences in the PEI content and molecular weight in the prepared PLA/PEI polymers.

3.6 Animal immunity experiment

Animal testing is an important means to evaluate the effectiveness of vaccines. We used the PLA/PEI microspheres with the highest transfection efficiency for green fluorescent protein (the microspheres modified with PEI with a molecular weight of 10000), to load the DNA vaccine against foot-and-mouth disease (FMD) for the animal experiments. To reduce experimental error, we selected eight male guinea pigs of similar quality for each group. The protective rates of the vaccine for each group of guinea pigs were shown in Table 2. All the guinea pigs injected with PBS died. Five of the guinea pigs injected with the pure DNA vaccine died, the protection rate of the pure DNA vaccine was 37.5%. The effect of the DNA vaccine loaded by PLA/PEI microspheres was the greatest, only one guinea pig died and the vaccine protection rate was 87.5%. These results indicated that PLA/PEI microspheres enhanced the effectiveness of DNA vaccines and showed the potential of an ideal delivery system.

Table 2
The protection rates against guinea pig foot-and-mouth disease virus.

Groups	The total number	Number of healthy guinea pigs	Vaccine protection rate
PBS	8	0	0%
pure DNA	8	3	37.5%
PLA/PEI/DNA	8	7	87.5%

Discussion

The clinical application of DNA vaccines cannot be achieved without the use of a safe and efficient gene delivery system. Non-viral vector systems have attracted extensive attention in recent years because of

their advantages over traditional systems, including high targeting, low cost, and good reproducibility[28, 29]. PEI, one of the most studied non-viral vectors, has high gene transfection efficiency, but high toxicity has restricted its further application[30]. Although PLA and PLGA have good biocompatibility, the DNA encapsulation rate and the gene transfection efficiency need to be improved [31, 32]. Therefore, research on polymers, such as PEI and PLA, has mainly focused on modification with other chemical groups to improve the DNA transfection ability and reduce the toxicity. For example, Terry et al. have modified DNA with PEI, prepared a PEI-DNA polymer, and used PLA modified with polyethylene glycol as the carrier to achieve a high encapsulation rate of DNA[33]. Gwak has used cholesterol-modified PLGA nanospheres as vectors to transfect pDNA *in vivo* and *in vitro* with high efficiency and high safety[34]. However, there are few reports regarding the integration of PLA and PEI[35].

In the present study, PLA microspheres with uniform sizes were prepared and modified using three types of PEI with different molecular weights. PLA/PEI/DNA complexes were prepared by loading plasmid DNA to these three PLA/PEI microspheres. Agarose gel electrophoresis and AFM morphology characterization verified the self-assembly of PLA/PEI microspheres and DNA, confirming that PEI modification enhanced the ability of PAL/PEI microspheres to adsorb DNA. In addition, the potential of PLA/PEI microspheres as DNA carriers was evaluated by observing the transfection in PK15 cells and animal experiments. After culturing PK15 cells for 48 h in medium mixed with the PLA/PEI/DNA complexes, the transfection efficiency of the three PLA/PEI microspheres exceeded 70%. Furthermore, the PLA/PEI microspheres with the highest transfection efficiency were used to load a DNA vaccine against FMD, and the protection rate of guinea pigs reached 87.5%, which was much higher than that of the DNA vaccine group without the vector. Moreover, an MTT assay indicated that the PLA/PEI microspheres were biocompatible with low cytotoxicity. The results indicated that PLA/PEI microspheres were promising delivery system and potential adjuvant for DNA vaccines.

Conclusions

We construct an effective delivery system for DNA vaccines, PLA/PEI microspheres, by preparing PLA microspheres with uniform sizes of 156 nm and modifying with PEI polymers. As expected, PLA/PEI microspheres adsorbed plasmid DNA effectively, improved the plasmid transfection rate in PK15 cells and the DNA vaccine protection rate in of guinea pigs, and showed good biocompatibility. In conclusion, our study gives an insight on the performance of PLA/PEI microspheres delivery system, and represents a step forward for the application of using this delivery system as DNA vaccine vehicles.

Declarations

ACKNOWLEDGMENTS

This paper was supported by the National Key Research and Development Program (2017YFD0500900), the Agricultural Science and Technology Innovation Program (CAAS-ZDRW202008), the Basic Scientific

Research Foundation of National non-Profit Scientific Institute of China (BSRF201907, BSRF202006), and the National Key Research and Development Program (2017YFD0501100, 2016YFE0204100).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

Authors' contributions

RS and GL contributed equally to this work. RS and GL performed the experiments and drafted the manuscript. WC assisted in synthesized particles and performed biophysical characterization. WY, SC and CH analyzed the data and gave direction for experiments. RJ, YY and GH provided the DNA vaccine, animals and test sites for animal immunity experiments. ZX conceived, supervised and designed the experiments. All authors read and approved the final manuscript.

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Figures

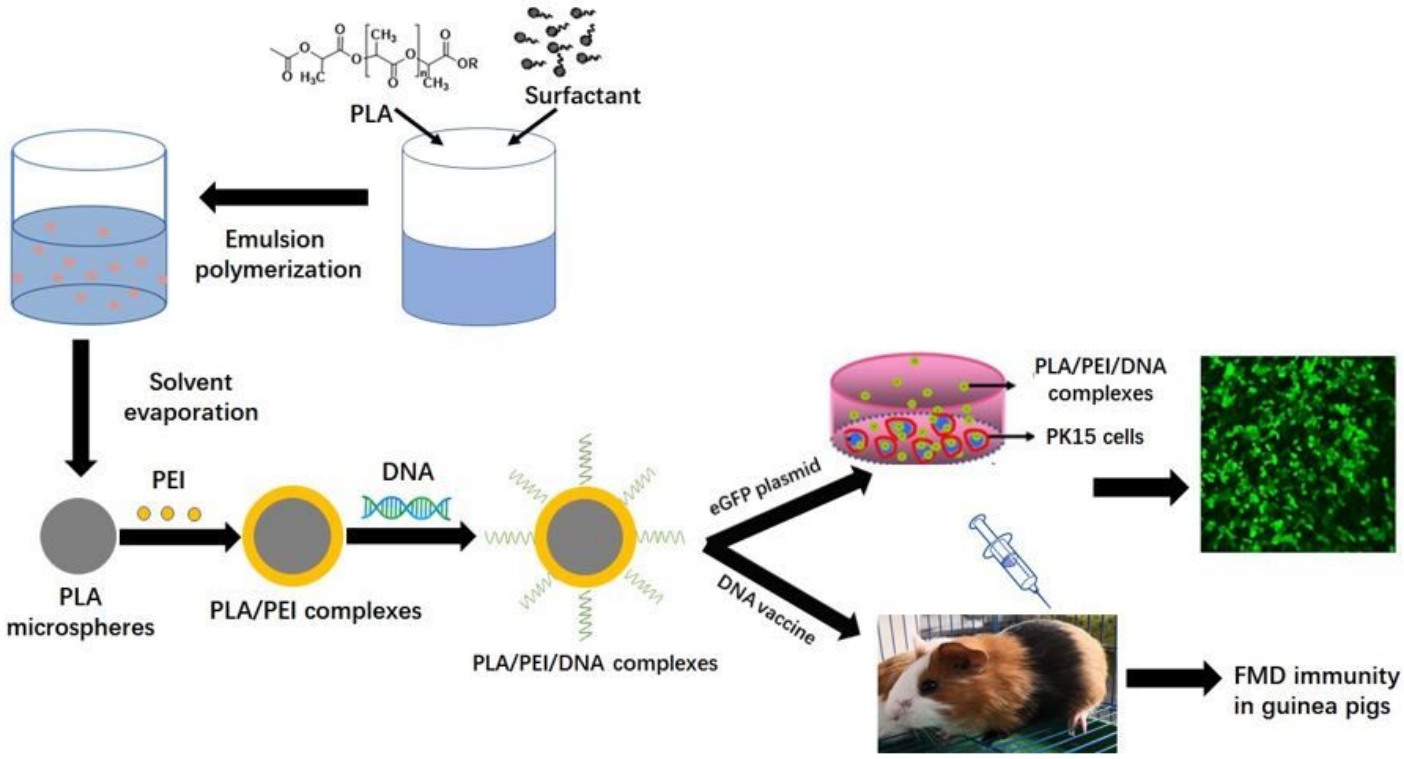
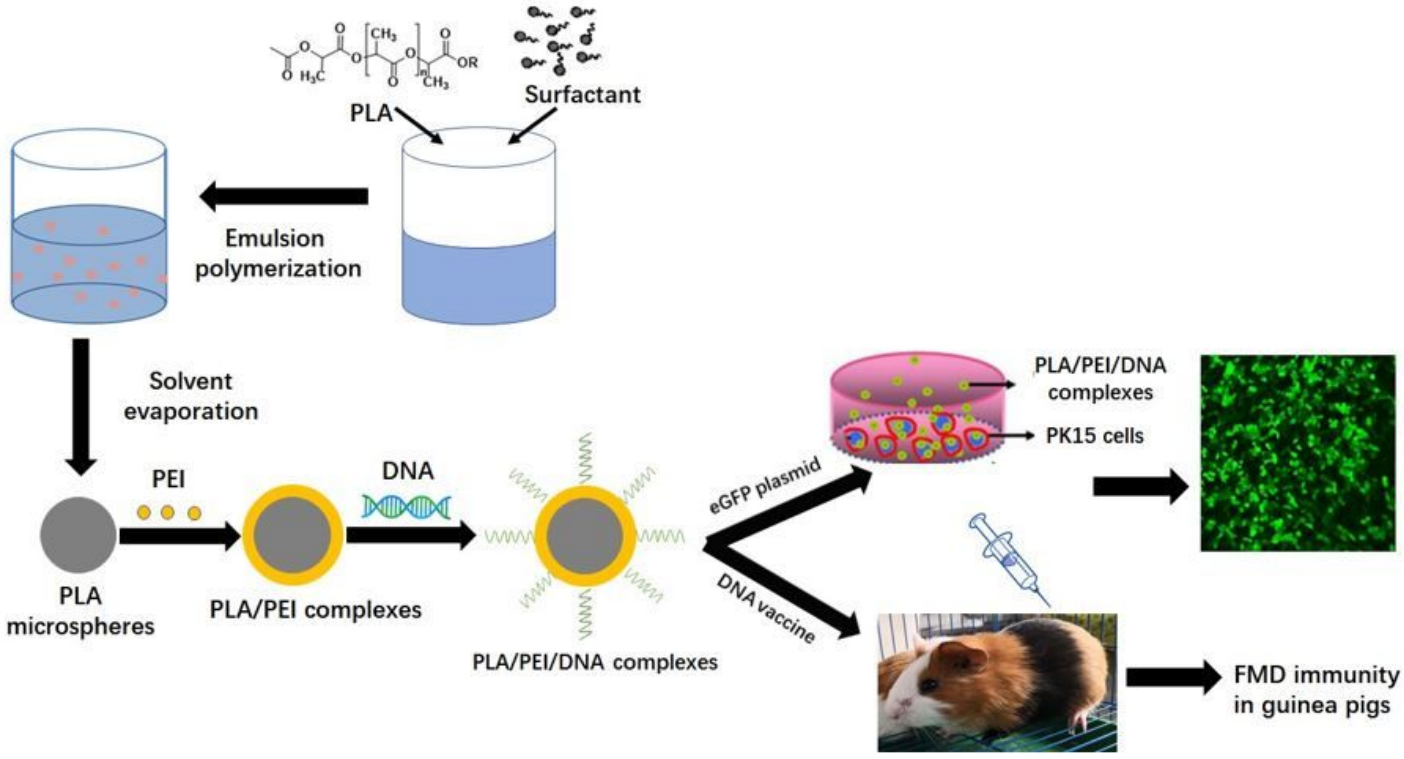


Figure 1

Schematic representations of construction of PLA/PEI microspheres for DNA vaccines



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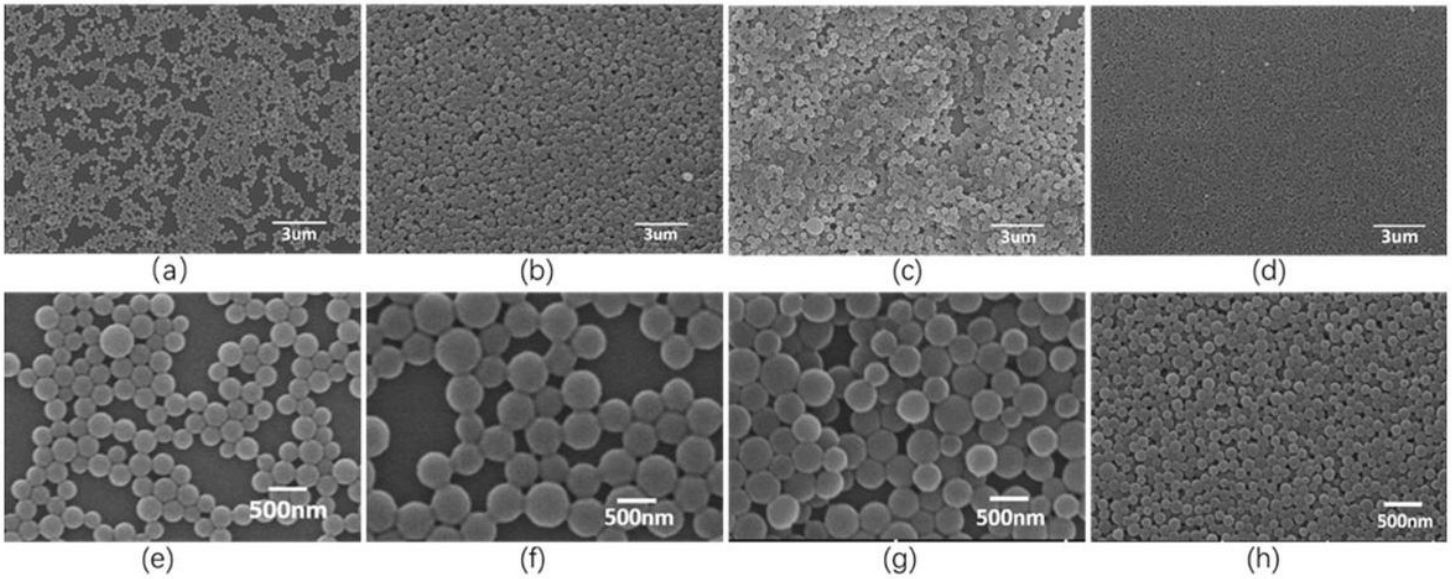
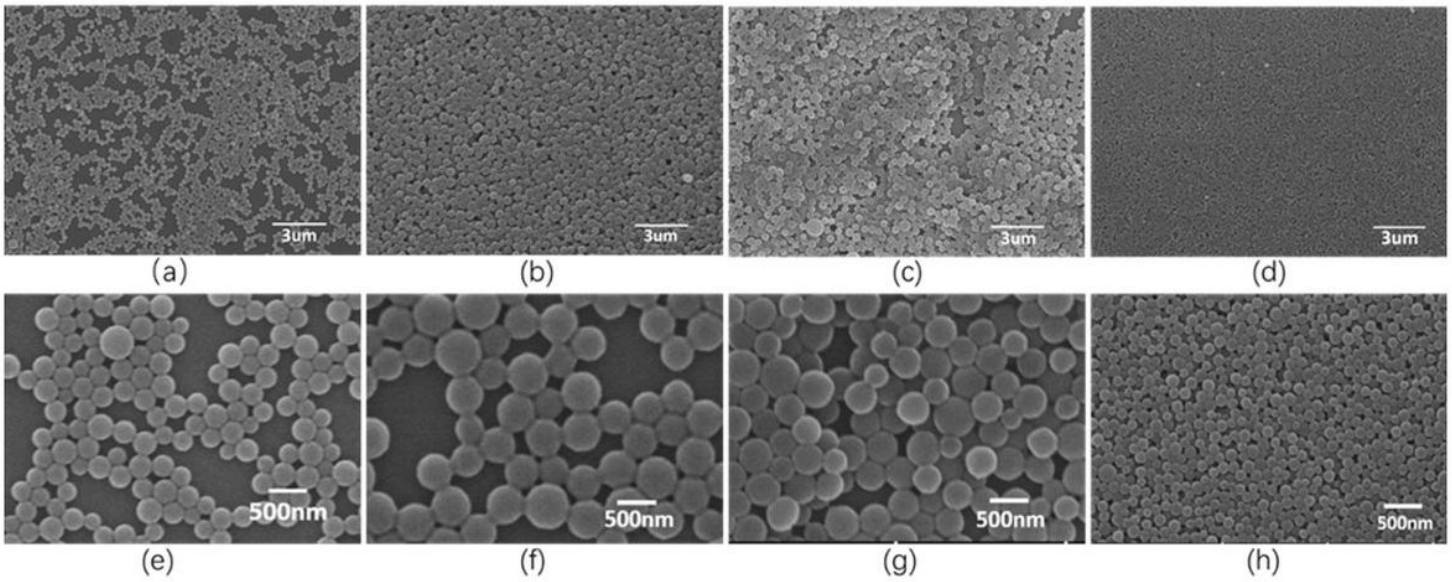


Figure 2

SEM images of PLA microspheres. (a): Shear rate: 8000 rpm; oil phase: water phase = 1:5; (b): Shear rate: 8000 rpm; oil phase: water phase = 1:3; (c): Shear rate: 10000 rpm; oil phase: water phase = 1:3; (d): Shear rate: 10000 rpm; oil phase: water phase = 1:5; (e)~ (h): a higher magnification under the condition of (a)~(d), respectively.



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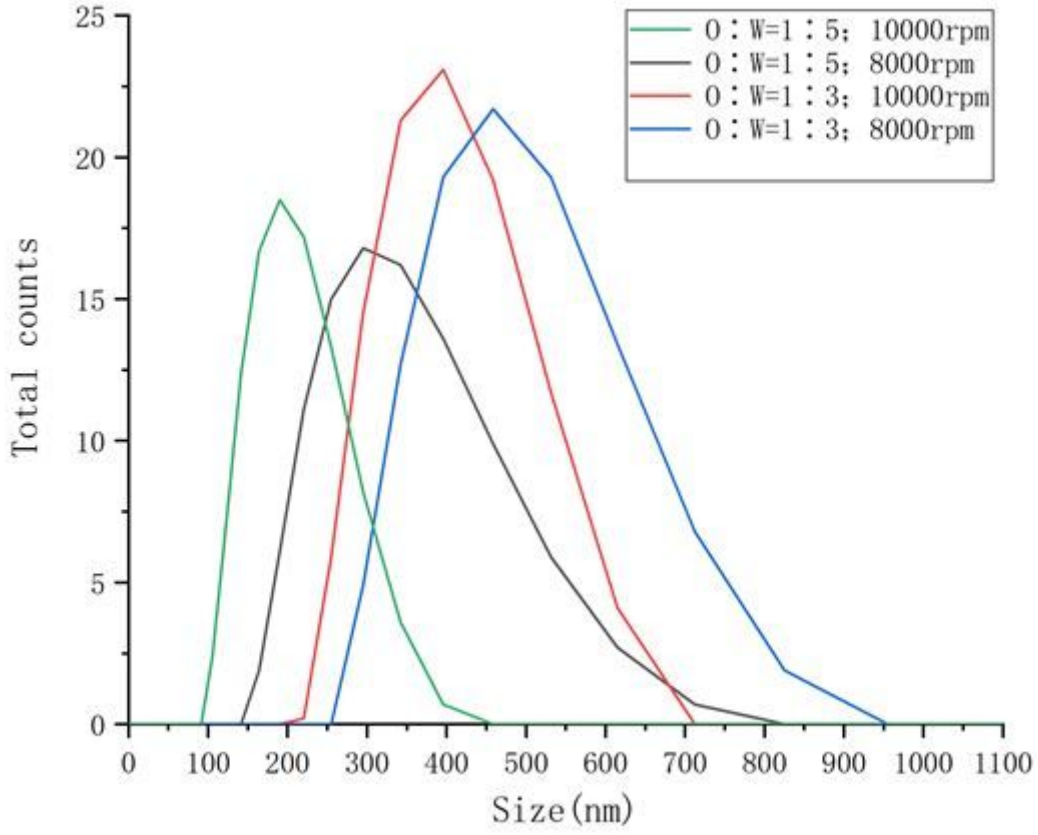
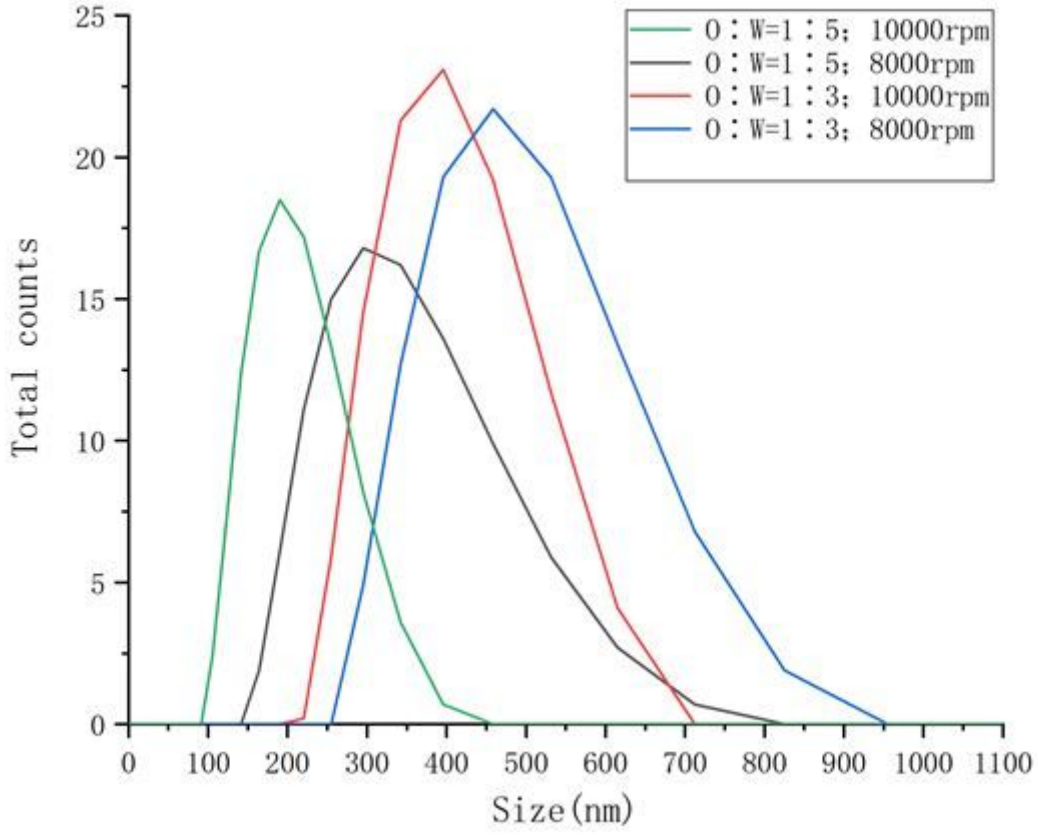


Figure 3

Size distributions of PLA microspheres under different preparation conditions.



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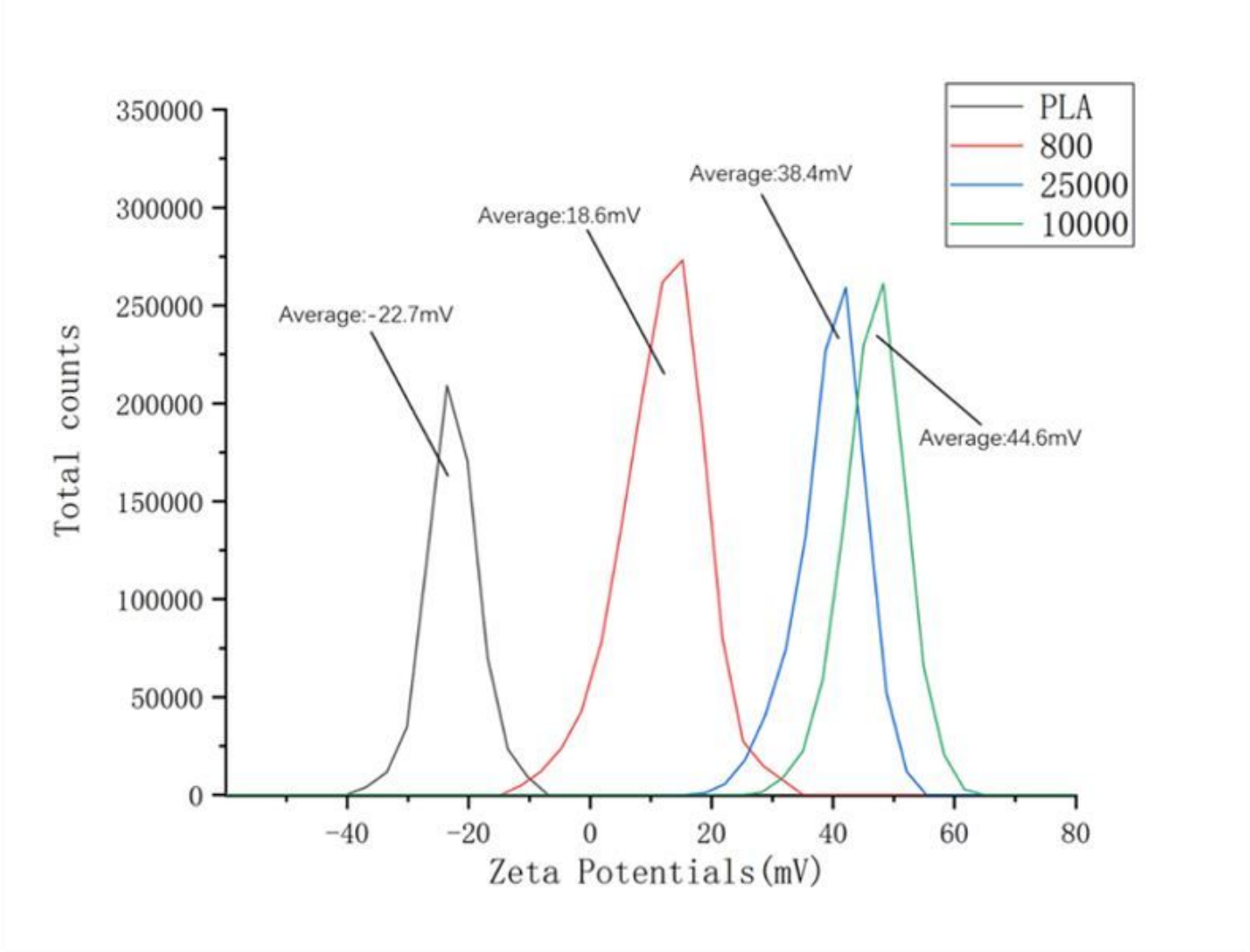
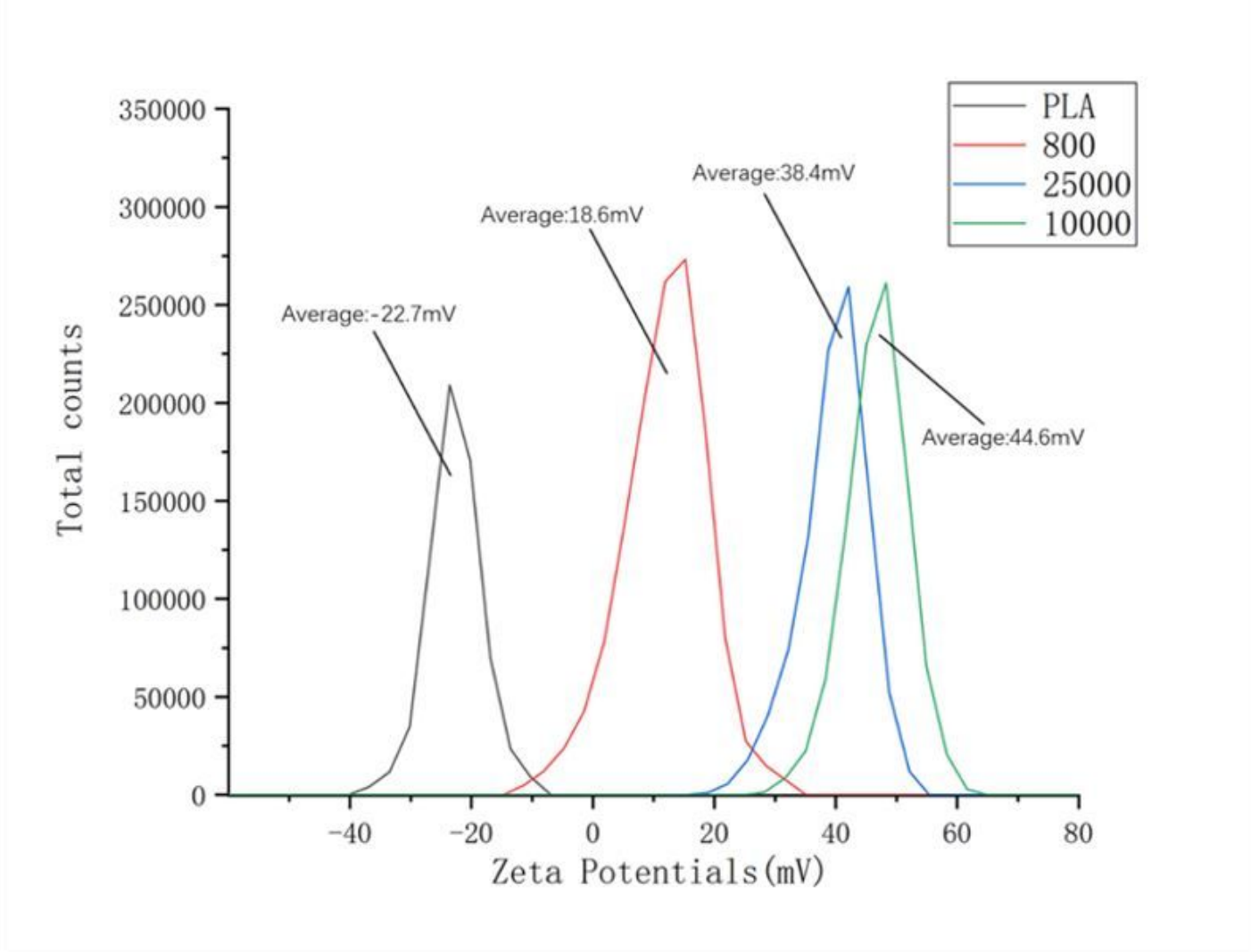


Figure 4

Zeta potentials of PLA microspheres and PLA/PEI microspheres.



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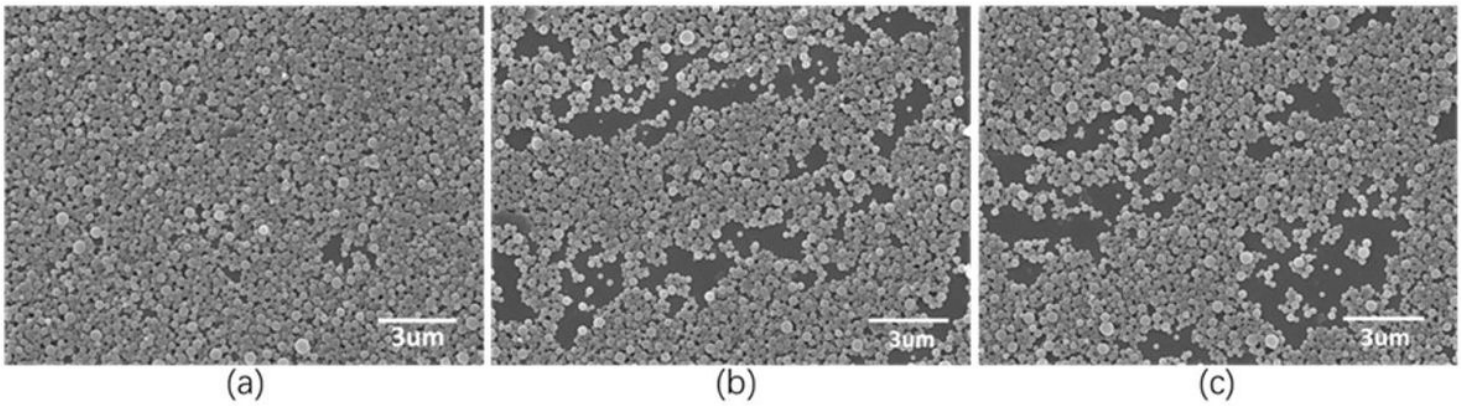
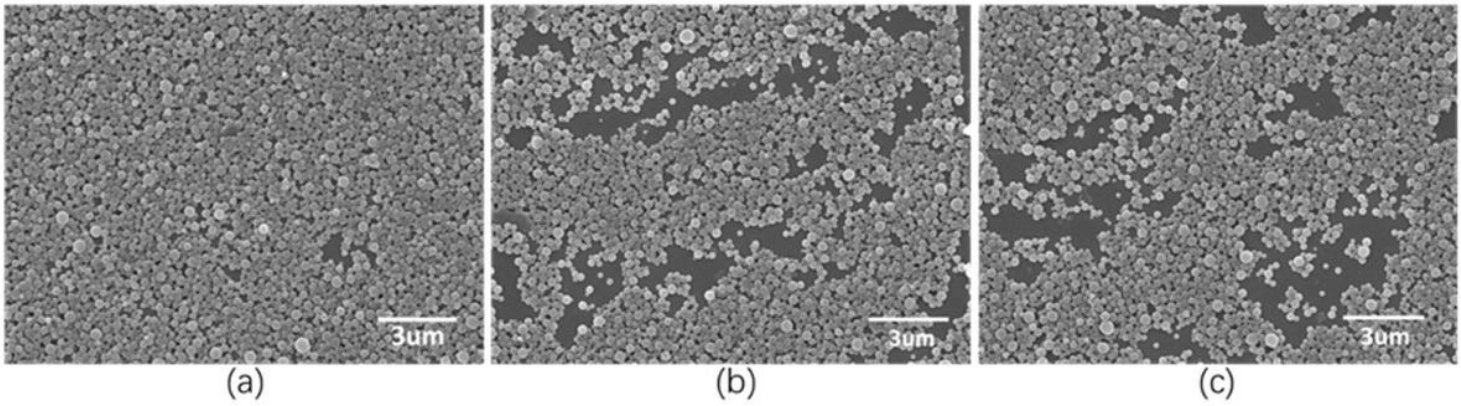


Figure 5

SEM images of PLA/PEI microspheres. (a): PLA/PEI microspheres modified with PEI with a molecular weight of 25000; (b): PLA/PEI microspheres modified with PEI with a molecular weight of 10000; (c):

PLA/PEI microspheres modified with PEI with a molecular weight of 800.



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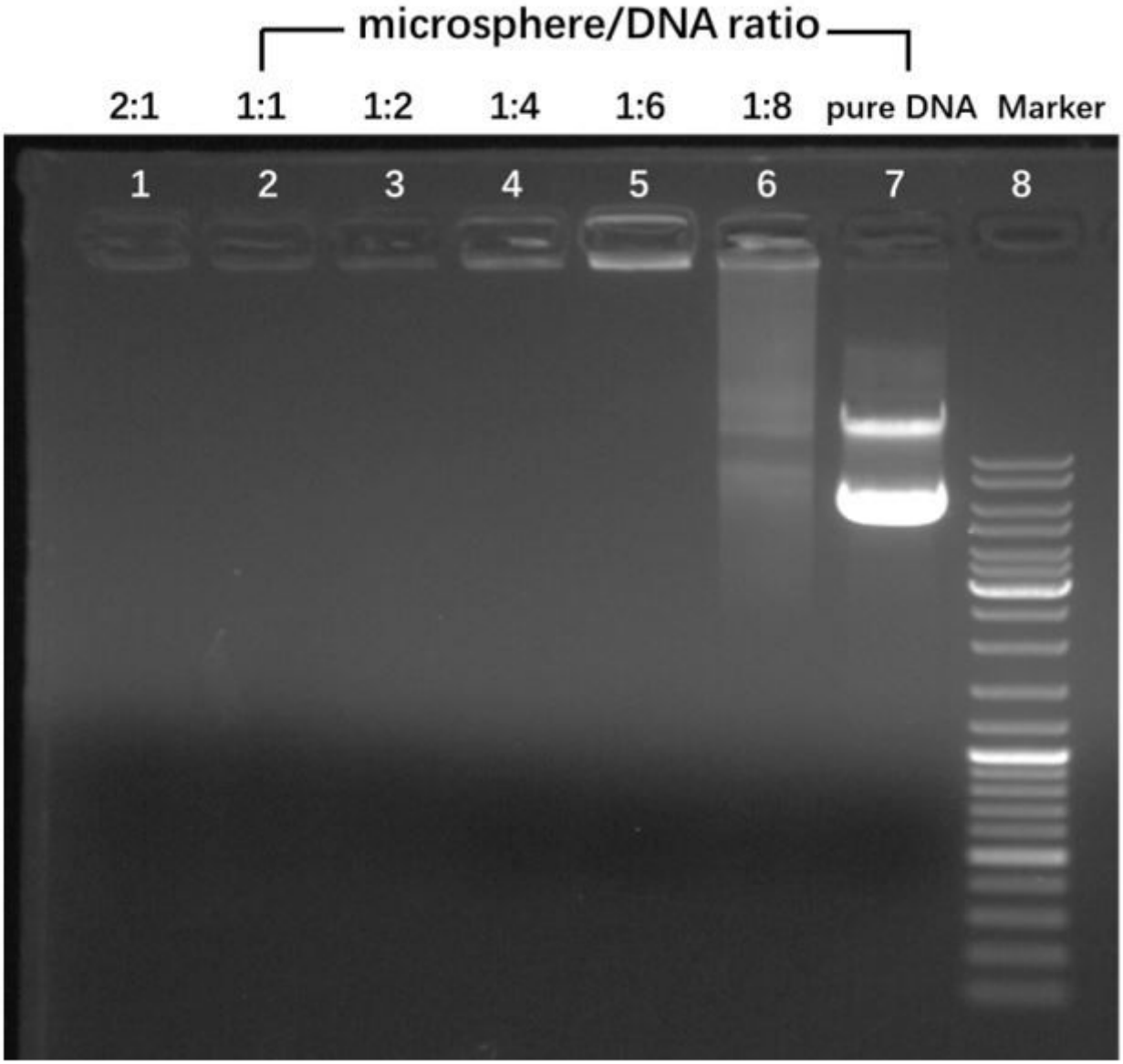
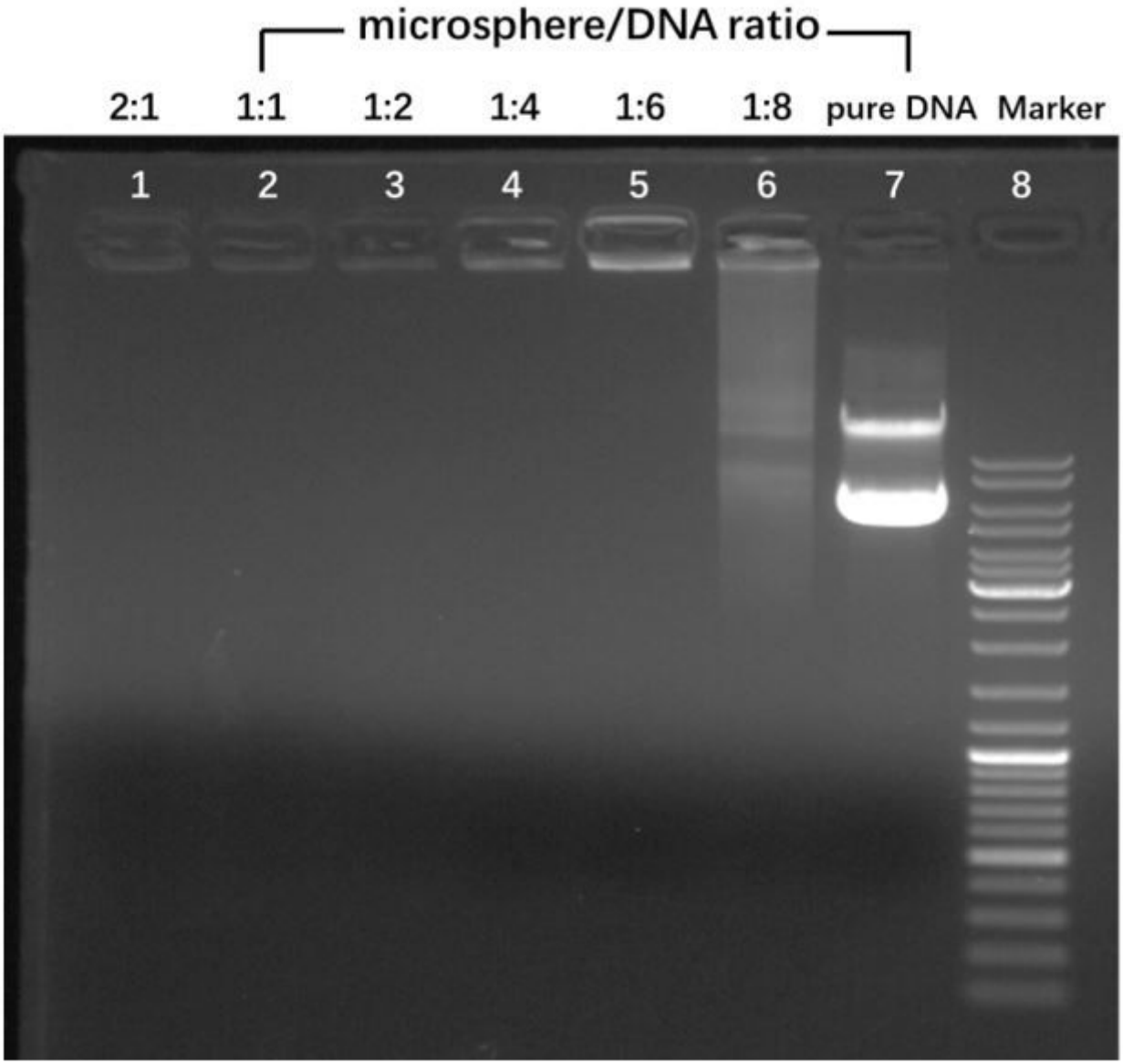


Figure 6

Agarose gel electrophoresis of DNA and PLA /PEI/DNA complexes.



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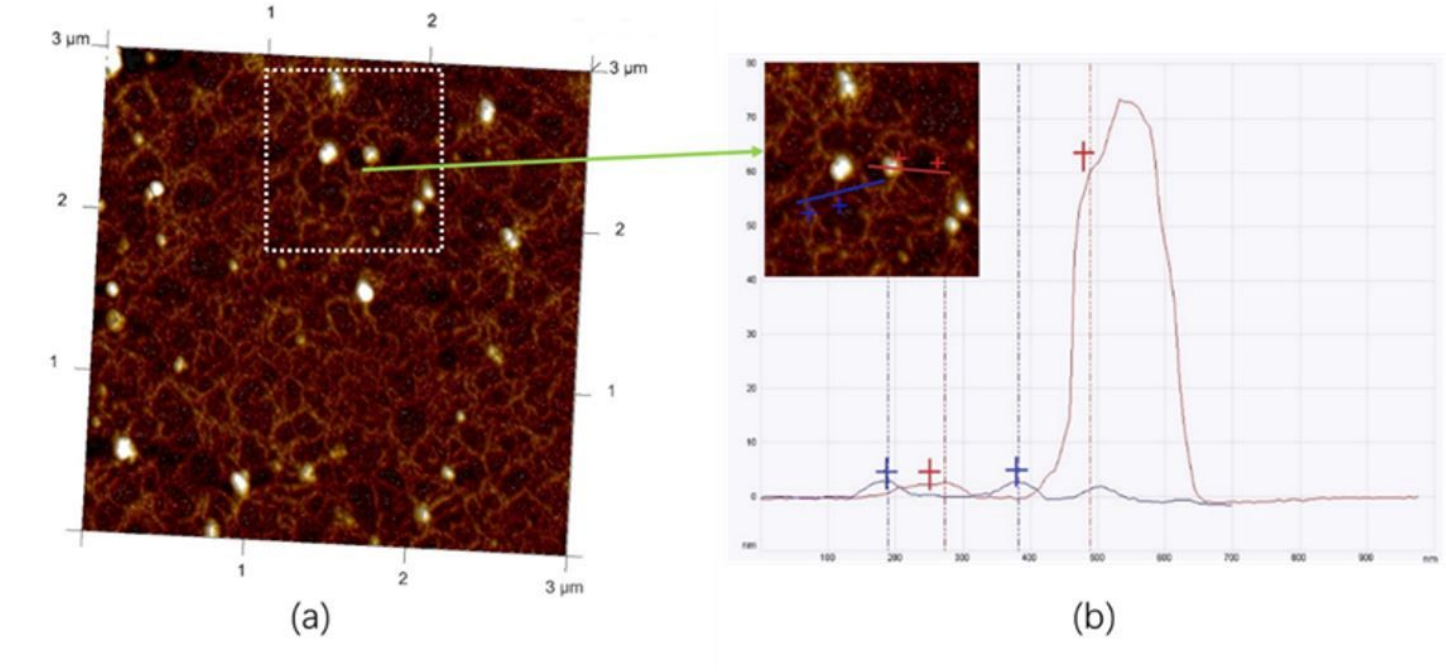
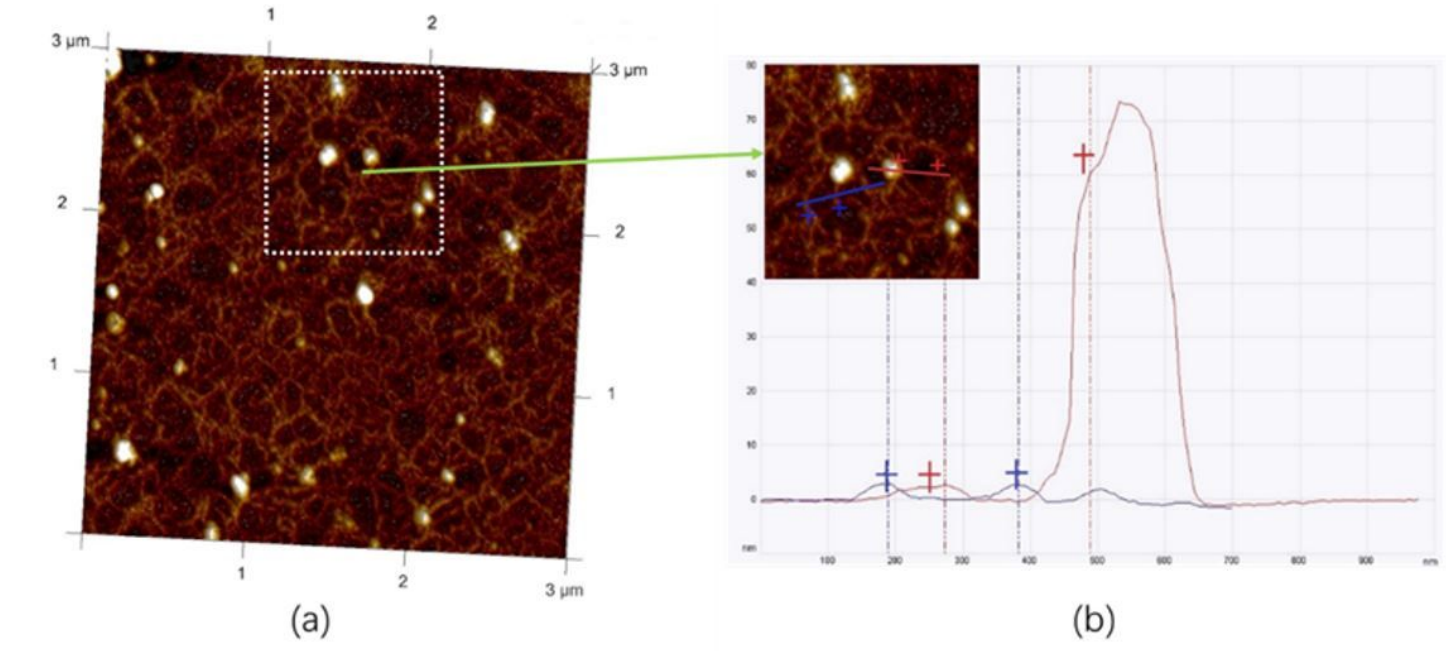


Figure 7

Shape and surface topography of the PLA/PEI/DNA complexes. (a): morphology of PLA/PEI microspheres under AFM; (b): the topographic distance profile corresponding to the region between the arrowheads in the area of dotted line in (a).



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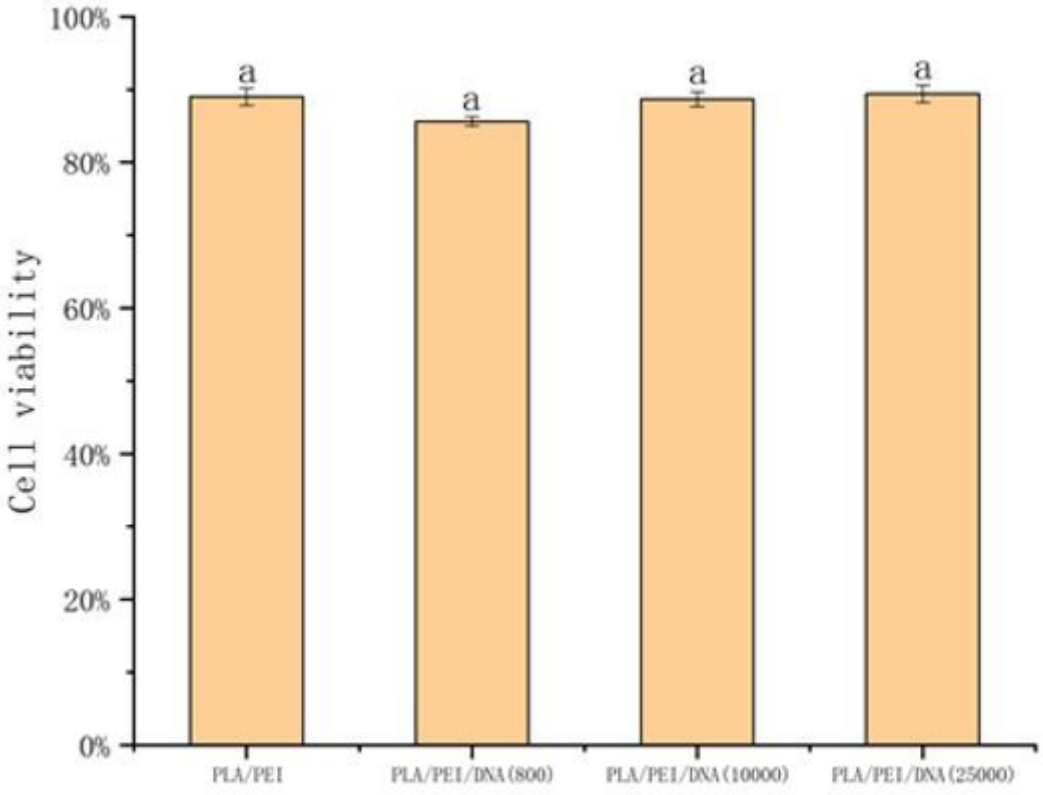
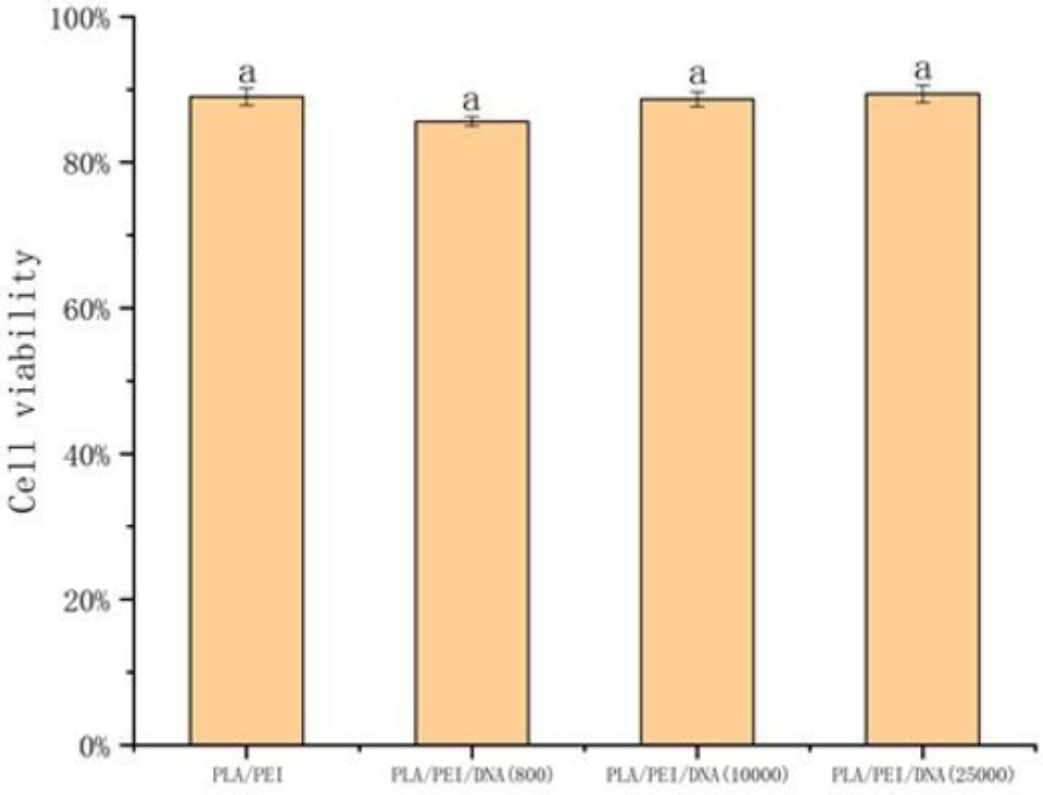


Figure 8

Cell viability after treatment with PLA/PEI and PLA/PEI/DNA complexes. Error bars represent standard deviations from three replicates ($p>0.05$).



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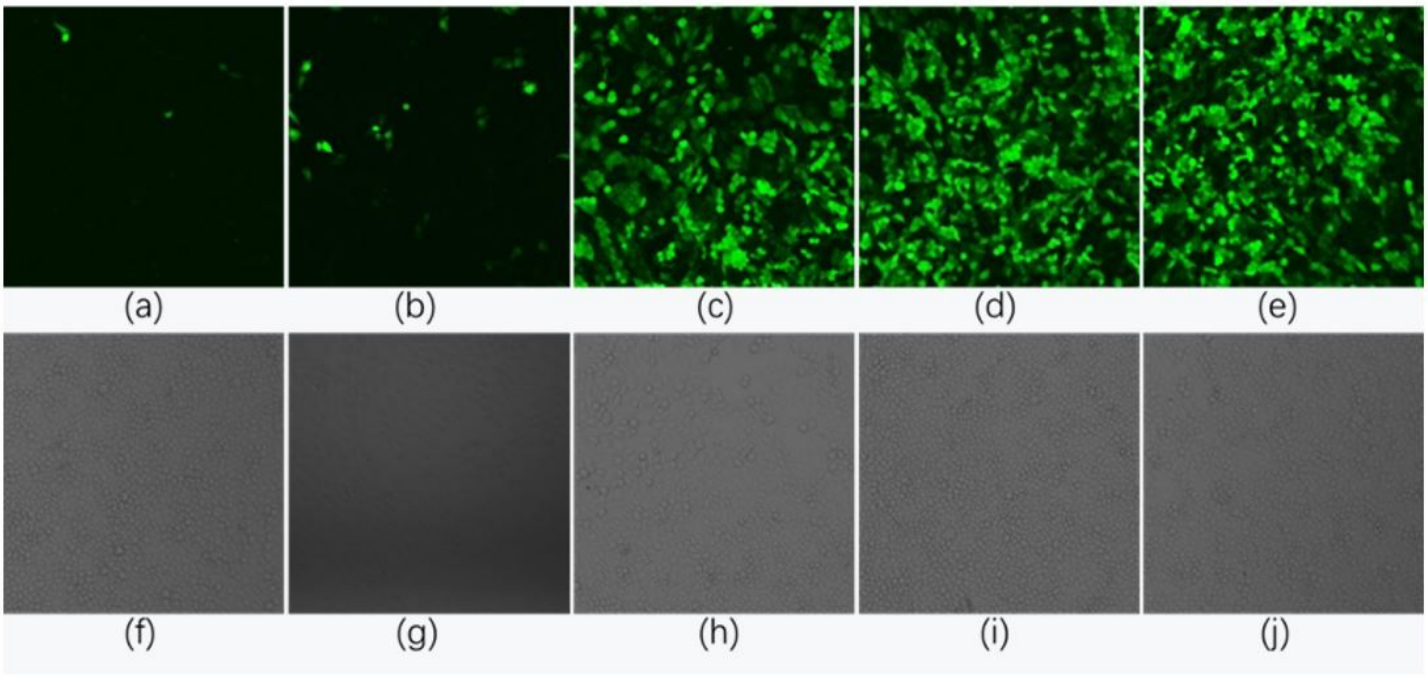
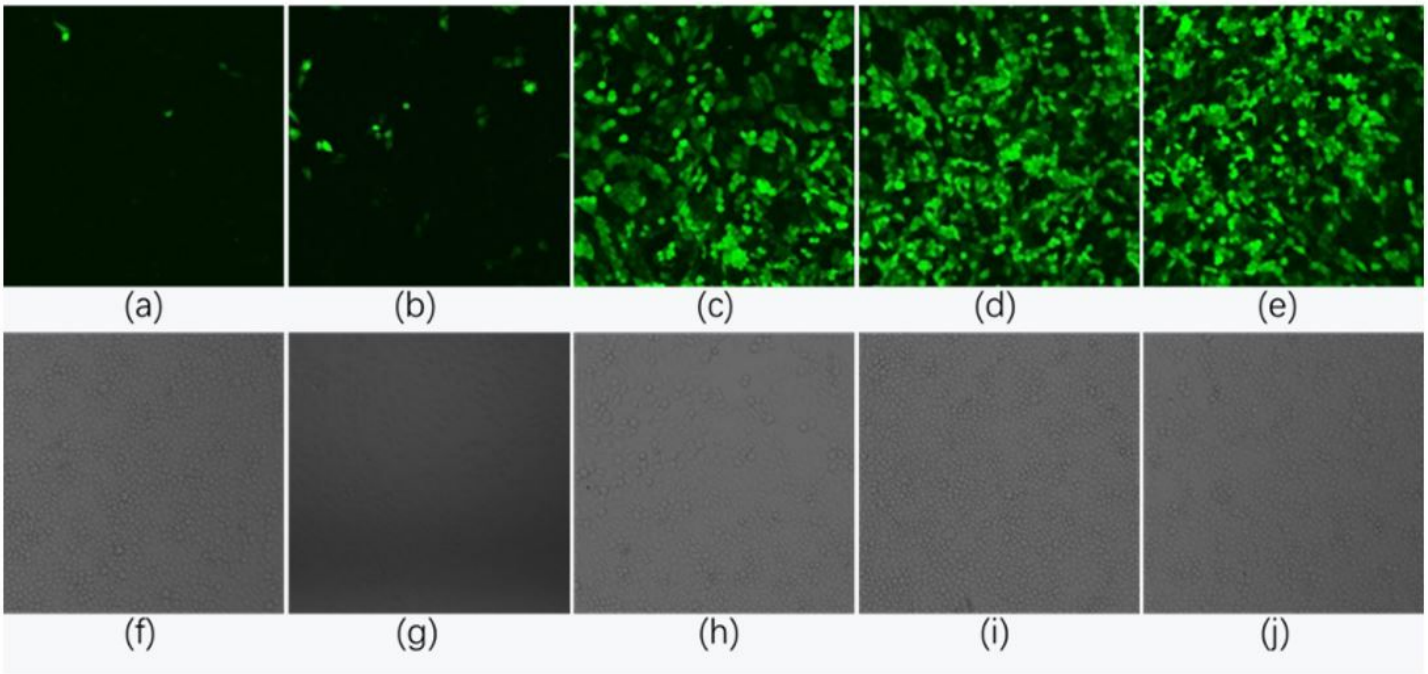


Figure 9

Green fluorescent protein luminescence images in PK15 cells. (a):fluorescent after transfected by plasmid DNA;(b):fluorescent after transfected by plasmid DNA+PLA; (c):fluorescent after transfected by plasmid DNA+PLA/PEI(800); (d):fluorescent after transfected by plasmid DNA+PLA/PEI(10000); (e):fluorescent after transfected by plasmid DNA+PLA/ PEI(25000);(f)~(j): bright field images of (a)~(d), respectively.



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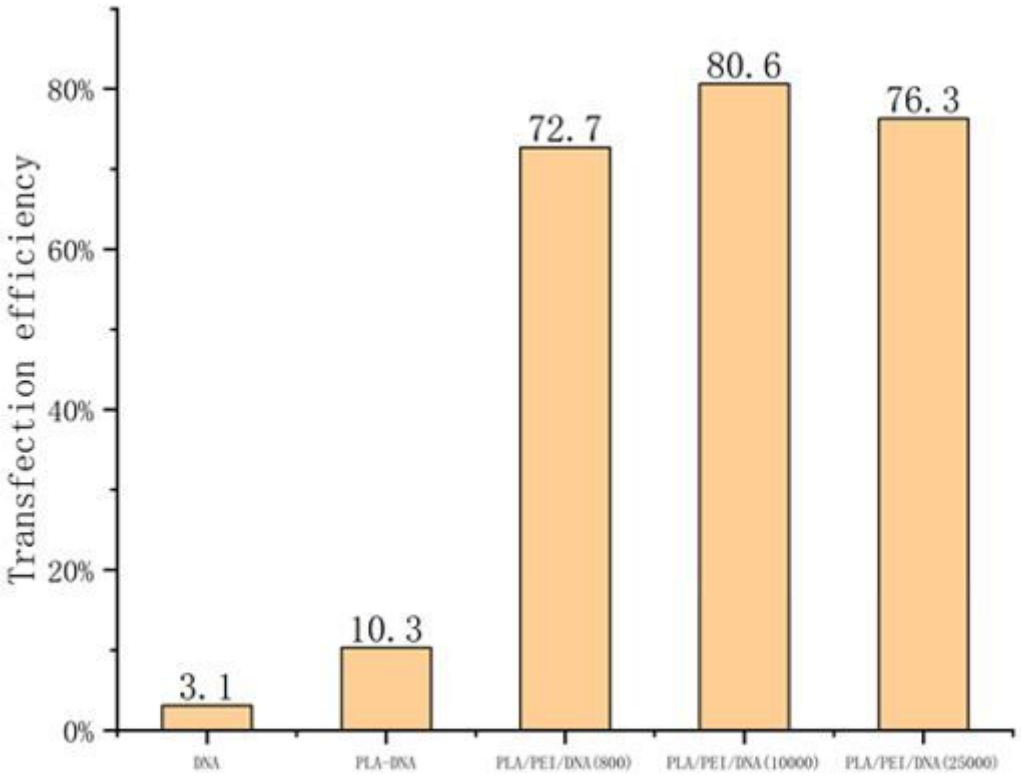
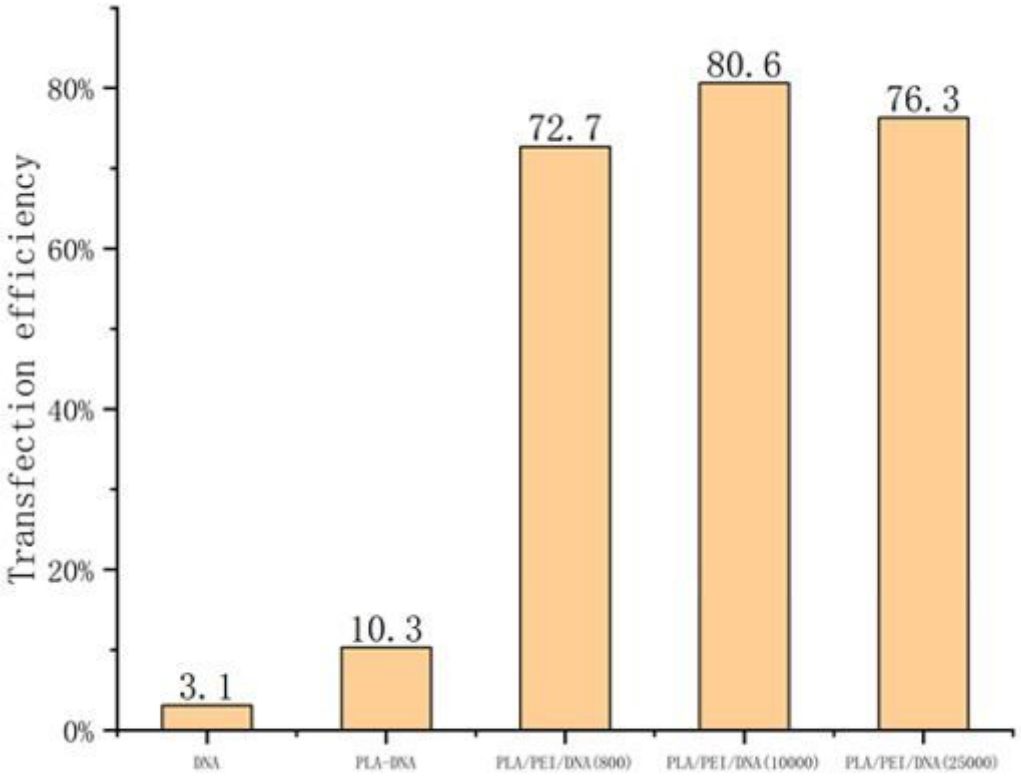


Figure 10

Expression efficiency of green fluorescent protein in PK15 cells.



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