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# Synthesis of Polyethyleneimine Functionalized Metal Organic Framework MIL-100(Fe) for Gene Delivery Application

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# Abstract

Metal–organic frameworks (MOFs) are porous polymers composed of metal centers and organic linkers that offer high surface area, tunable porosity, and excellent drug-loading capacity, making them attractive for biomedical applications. Among them, MIL-100(Fe) is particularly suitable for in vivo use because of its low toxicity, water stability, biocompatibility, and environmentally friendly characteristics. In this study, a surface-modified MIL-100(Fe) nanocarrier was developed by conjugating 10 kDa polyethyleneimine (PEI) with 10kDa MW through 6-bromohexanoic acid (Hex) as a linker. The resulting PEI10-Hex-MIL100 nanoparticles measured approximately 138 nm in diameter with a positive surface charge of + 17.23 mV and effectively condensed plasmid DNA at low concentrations. Characterization demonstrated improved buffering capacity, reduced cytotoxicity, and enhanced cell viability in CT26 mouse colon cancer cells. Notably, transfection efficiency increased at a C/P ratio of 2 compared with unmodified PEI10kDa, indicating that chemical modification of MIL-100(Fe) with PEI creates a promising and more effective nonviral gene delivery system.


# Introduction

Gene therapy is a fascinating method that involves transferring foreign nucleic acids into cells to stimulate the expression of therapeutic proteins in pathological conditions. It has been introduced as an attractive approach for treating various diseases (1). However, the development of efficient and safe delivery vehicles for gene-based therapies is a significant challenge which needs to be dissolved.

Although viral vectors have made remarkable progress in the field of gene therapy, limitations of this method, including carcinogenicity, immunogenicity, limited DNA loading capacity, and difficulty in producing the proper carrier, have hampered their use(2). On the other hand, nonviral gene therapy has the potential to overcome many of these limitations, especially concerning immune system stimulation (immunogenicity). These systems are highly regarded due to their potential ability to deliver large genetic particles and their ease of synthesis compared to viral vectors. So far, a diverse range of synthetic carriers have been developed to deliver therapeutic nucleic acids to their targeted site (3, 4).

Among synthetic carriers, cationic polymers have shown impressive success in delivering nucleic acid. These systems can facilitate the uptake of nucleic acid across cellular and intracellular membranes (such as endosome membranes) through their positive surface charge, compacting nucleic acid, and creating nanoscale polyionic complexes. This mechanism enhances electrostatic interactions with the plasma membrane and leads to more effective delivery (5). A recently emerging and developing class of hybrid materials consisting of organic and inorganic components is that of metal-organic frameworks (MOFs), also known as porous coordination polymers (PCPs)(6). Generally, these structures are constructed from metal ions/clusters and organic linkers. Due to their intrinsic properties, including uniform porous networks, adjustable pore sizes, tunable chemical environments, and large surface areas, these systems have attracted much attention in medical research. One interesting application of

MOFs is their capacity to encapsulate huge biomolecules, such as proteins, vitamins, lipids, nucleic acids and oligopeptides.

Additionally, innovative MOF-biomolecule hybrid structures, have shown promising prospects as antitumor therapies, gene delivery systems and biomolecule detection (7). Moreover, particle morphology, size distribution, and chemical properties also play an important role in MOF applications (8). So far, various forms of MOF have been designed and evaluated in several studies. MIL-100 is one of the different forms of MOFs with a wide range of applications which synthesized by Fer´ey and his colleague for the first time (9, 10). The presence of organic linkers enables MIL to undergo reactions with several trivalent entities, for example, Fe, Sc, and Al. Especially, the MIL-100(Fe) with the formulation [Fe3O(NO3){C6H3(CO2)3}2] has received much attention. MIL-100(Fe) possesses a number of notable characteristics, including a high surface area, favorable colloidal stability, lack of toxicity, and biodegradability (11). In the present work, polyethylenimine-functionalized MIL-100(Fe) was synthesized and subsequently employed for gene delivery purposes.

## Results

# Morphological analysis of MOF by FE-SEM

The morphology and structures of PEI10kDa-Hex-MIL100 were examined and depicted in Fig. 1. As shown, the synthesized nano-carrier formed three-dimensional (3D) nano clusters with spherical shapes. Also, according to the FE-SEM images, the average diameter of the nanoparticles was calculated to be about 120.57 ± 7.44 using the ImageJ analysis software.

FTIR analysis determined the chemical composition of the MOFs, and Fig. 2, presented the corresponding results. Consistent with previous studies, the obtained data showed that MIL-100(Fe) exhibited absorption peaks for N–H stretching (near 3400 $cm^{-1}$), C = O stretching (around 1640 $cm^{-1}$), and C–O stretching (approximately 1400 $cm^{-1}$) (12). The PEI 10kDa has absorption peaks about the N-H Stretching (around 3300–3400 $cm^{-1}$), C-N Stretching (around 1200–1300 $cm^{-1}$), C-H Bending (around 1450 $cm^{-1}$)(13).

After the post-synthetic modification of MIL-100(Fe) particles with polyethyleneimine (14), distinct peaks at 3427/3279, 2940/2824, 1575/1364, 1467, and 1088/1015 $cm^{-1}$ confirmed the presence of PEI on MIL-100(Fe). In PEI-Hex-MIL-100, the presence of peaks related to MIL-100 indicated that PEI-Hex attached to the MIL-100.

## Size and zeta potential measurement

The polyplexes' size and their surface charge (zeta potential) significantly affect cellular uptake and also enhance transfection efficiency within the host cells. Table 1 summarized the measurements from the particle size and zeta potential experiments. According to the data in the table, the incorporation of PEI

onto the MOF surface led to a significant reduction in size compared to the MOF alone, while showing an increase relative to the base PEI. This modification also resulted in an increased surface charge of the MOF after functionalization, attributed to the presence of the cationic polymer PEI. After the polyplexes formed, an electrostatic attraction between the carrier's surface charge and the plasmid's negative charge triggered a compaction event, which consequently reduced the modified polyplex's size.

Table 1
Size and surface charge of PEI10 kDa,MOF and modified MOF/pDNA complexes at C/P ratio of 2 (w/w) (mean ± standard deviation, n = 3)

| Type | size | Polydispersity index (PDI) | zeta potential |
|---|---|---|---|
| **PEI 10** | 87.5 ± 2.4 | 0.29 ± 0.02 | + 24.2 ± 1.07 |
| **MIL100** | 347.7 ± 137.7 | 0.19 ± 0.03 | -30.8 ± 0.07 |
| **PEI10-Hex-MIL 100** | 138.2 ± 31.8 | 0.25 ± 0.01 | + 17.23 ± 1.25 |

## Gel mobility experiment

Gel mobility experiment
This test was conducted to investigate the ability of nanocarriers to condense plasmid DNA. Functionalized MOFs, due to their positive charge, can electrostatically interact with negatively charged DNA, forming a nano-sized structure known as a polyplex. Consequently, the absence of plasmid migration in electrophoresis indicates the successful formation of a stable nanocarrier. If the plasmid is condensed by the nanocarrier, no band will be observed on the gel. As illustrated in Fig. 3, almost no bands appear on the gel for most of the C/Ps 2, 4, and 6, indicating that the PEI10000-Hex-MIL 100 nanocarriers have effectively condensed the plasmid in these specific C/Ps.

## Transfection efficiency assay

To investigate the transfection ability of the modified metal-organic framework (PEI 10kDa-Hex-MIL100), we examined the GFP expression levels in the mouse colon cancer cell line (CT26) at different carrier-to-plasmid (C/P) ratios of 2, 4, and 6. We observed that the PEI10000-Hex-MIL-100 carrier improved the transfection efficiency about 1.5-fold ($P < 0.01$) relative to unmodified PEI 10 kDa at a C/P ratio of 2. Nevertheless, this improvement did not reach statistical significance when we compared it with the standard PEI 25 kDa under the same C/P conditions

Figure 4 Comparison of transfection efficiency in CT26 cells for PEI-Hex-MIL-100 and PEI 10 kDa polyplexes at C/P ratios of 2, 4, and 6, along with PEI 25 kDa. Data are shown as mean ± SD (n = 3)

## Cell viability assay

This experiment aimed to evaluate the percentage of cell survival following exposure to polyplexes. We examined the cytotoxic effects of the modified carrier and the PEI polymers on the mouse colon cancer cell line by performing the MTT assay at three different C/P ratios (2, 4, and 6). Our results indicated that as the C/P ratio increased, cell viability decreased significantly ($P < 0.01$). Compared to standard PEI 25 kDa, the PEI10000-Hex-MIL-100 nanocarrier showed enhanced cell viability at a C/P ratio of 2 and also at all other C/P ratios examined. In other words, the toxicity of the PEI10000-Hex-MIL-100 nanocarrier was significantly reduced, with the percentage of metabolic activity and overall cell survival exceeding approximately 70% (Fig. 5).

# Discussion

Gene therapy has shown tremendous progress in recent years and has become an innovative approach for treating a variety of diseases and genetic problems. In order to replace or repair damaged genes, this novel method entails adding, deleting, or changing genetic material inside a patient's cells(15). However, the field has a lot of obstacles to overcome, especially when it involves developing safe and effective ways to deliver therapeutic genes. Although viral vectors are frequently used to transfer genes, their efficacy may be hampered by problems like immunogenicity and poor packaging capacity (16).

Metal-organic frameworks (MOFs) have absorbed attentions as promising gene/drug carriers because of their special structural properties including exhibit high surface areas and porous structures(17). The MOFs composed of metal ions linked to organic ligands, MOFs such as zinc-based (e.g., ZIF-8)(18), iron-based (e.g., MIL-100(Fe)), copper-based (e.g., HKUST-1)(19), cobalt-based (e.g., Co-MOF-74)(20), and aluminum-based (e.g., MIL-53(Al))(21). Although many studies have investigated the delivery of low molecular oligonucleotides using complex surface binding to MOFs, the safe and effective delivery of high molecular weight nucleic acids, such as plasmids, remains a challenge (22). The MOFs considered as excellent candidates for gene and drug delivery applications because they revealed high loading capacities for pharmaceuticals and can protect biological molecules from external conditions.

Polyethyleneimines (PEIs) are widely applied as gene delivery vectors because of their unique properties, which depends significantly on their molecular weight (MW). Due to their proton sponge ability, the PEIs facilitate cellular uptake and endosomal escape efficiently, so enhance efficiency of nucleic acid transfection. PEIs with a low molecular weight such as 600 OR 1800Da are frequently less cytotoxic, which makes them suitable for use in vivo applications. High molecular weight PEIs, on the other hand, revealed more cytotoxicity but also have ability to form more stable complexes with nucleic acids and improve gene expression (23). The branching structure of PEIs has a tendency toward functionalization, which can enhance their biocompatibility and targeting capabilities. However, with considering variation in molecular weight (MW) that introduce challenges, develop a carrier which can optimize the balance between transfection efficiency and cytotoxicity is crucial for successful gene delivery (24).

Recent investigations on MOFs functionalized or modified with polyethyleneimines have shown their potential and flexibility as efficient gene delivery carriers. For example, piperine, a chemotherapeutic

substance, has been administered to breast cancer cell lines using chitosan-coated MIL-100(Fe) nanoparticles, demonstrating less cytotoxicity than free piperine (25). Furthermore, when plasmids were introduced into different types of cancer cells, PEI (1.8 kDa) infused with fluorine and saponin and chemically linked to zirconium-fumarate MOF (MOF-801) exhibited minimal toxicity (26). The metal-organic framework functionalized by gold nanoflower/polyethyleneimine (AuNF-PEI-MIL-101(Cr)) nanoparticle was designed and also thiolated STR aptamers immobilized on it, this nanocomposite served as an aptasensor for the sensitive detection of STR(27). Furthermore, Fe3O4@MIL-100(Fe) functionalized with PEI has been applied for the extraction of pesticide residues from food matrices, showing the ability to quantify four triazole fungicides using HPLC-UV for separation and detection(28).

So, there are ongoing researches aim to optimize the structures and functionalization strategies of these carriers to enhance transfection efficiency, reduce toxicity, and improve targeting capabilities, thereby addressing the need for safe and effective clinical applications.

In our study, we developed a novel iron-based MOF designed to achieve higher transportability and lower toxicity. We modified the MOF's surface by incorporating PEI via a 6-bromohexanoic acid linker to enhance plasmid transportability. DNA condensation by nanocarriers is critical for enhancing the stability and delivery efficiency of genetic material, as it protects DNA from degradation and facilitates cellular uptake, thereby improving the efficacy of gene therapy applications(29). Our modified carrier efficiently condensed plasmid DNA at a carrier-to-plasmid (C/P) ratio of 2; however, at a C/P ratio of 1, it failed, likely due to insufficient surface charge.

The geometric properties of nanoparticles, including size, shape, and surface morphology, significantly influence their cellular uptake and internalization, as these characteristics determine the interactions between nanoparticles and cell membranes(30). Field emission scanning electron microscopy (Fe-SEM) imaging revealed that the nanoparticles were predominantly spherical and quasi-spherical, further emphasizing the importance of geometric properties in cellular uptake. We performed FTIR analysis on the synthesized MOFs. The resulting spectra showed the characteristic functional groups associated with both the wet impregnation and the covalent linking strategies. These observations confirmed the successful conjugation of PEI to the MOF structure.It has been demonstrated that larger particles are meant to enter by the micropinocytosis pathway, whereas nanoparticles smaller than 200 nm are mainly absorbed by endocytosis (31) .the size of obtained nanoparticle in our study was below 200nm about 138.2 ± 31.8 nm and expected enter the cell through the endocytosis pathway.

As the C/P ratio increased, the toxicity of the PEI-based nanocarriers also rose, attributed to the increase in positively charged molecules while maintaining a constant amount of DNA. The positive charge on the surface of nanoparticles creates nonspecific electrostatic interactions with the cell membrane, contributing to cytotoxicity(32). Additionally, one proposed mechanism for these cytotoxic effects involves PEI particles entering the cell and interfering with host gene expression within the nucleus(33).

Notably, the cell viability of the PEI10000-Hex-MIL-100 nanocarrier at a C/P ratio of 2 was enhanced compared to standard PEI (25 kDa) across all C/P ratios. The PEI10000-Hex-MIL-100 demonstrated

superior cell transfection compared to the PEI 10000 Da polymer, particularly at a C/P ratio of 2, while its cytotoxicity was lower than that of the PEI 10000 Da polymer at a C/P ratio of 6, and even lower than that of PEI 25000 Da at a C/P ratio of 2. Therefore, the post-synthesis modification approach and the selected MOF for fabricating PEI10000-Hex-MIL-100 nanocarriers resulted in enhanced efficiency. Consequently, PEI10000-Hex-MIL-100 can be introduced as a promising candidate for further studies in gene therapy. Additionally, due to its well-structured internal pores, it may serve as a suitable carrier for studies in drug delivery alongside nucleic acids.

## Conclusion

The investigation of metal-organic frameworks (MOFs) in conjunction with polyethyleneimines (PEIs) has created new opportunities for improving gene delivery systems. This research aimed to utilize the advantages of PEI and MOF-MIL100 for gene delivery while addressing their limitations. Therefore, the modified iron-based MOF, PEI10000-Hex-MIL-100, effectively condenses plasmid DNA and exhibits enhanced transportability and lower toxicity, making it a suitable candidate for future gene therapy applications. The main limitations of this study include evaluation of transfection and cytotoxicity in only one cell line (CT26), lacking in vivo validation and mechanistic endosomal escape data. Moreover, comprehensive characterization of MOF crystallinity (PXRD), porosity (BET), and PEI grafting quantification (TGA) was not provided. Future work should address these gaps using multiple cell lines, in vivo models, and advanced physicochemical assays Furthermore, the geometric properties of PEI10000-Hex-MIL-100 were optimized, resulting in enhanced cell viability and transfection rates compared to standard PEIs. This novel carrier not only shows great promise for gene therapy but also has the potential to serve as a versatile platform for drug delivery applications. Continued research into the in-vivo experiment optimization or targeting of MOFs and PEIs will be crucial for advancing clinical applications and achieving safe, effective therapeutic outcomes in gene therapy.

## Experimental

The CT26.WT cell line (ATCC® CRL-2638™), which originates from a murine colorectal carcinoma in a BALB/c mouse, was obtained from the Pasteur Institute of Iran. Culture media components, namely Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS), were supplied by GIBCO (Gaithersburg, USA). Several chemical reagents including dimethyl sulfoxide (DMSO), 6-bromohexanoic acid, MTT dye (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), anhydrous methanol, 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were acquired from Sigma–Aldrich (Germany). Branched polyethyleneimine (b-PEI, 10 kDa) was purchased from Polysciences Inc. (Warrington, PA, USA), while EGFP encoding plasmid DNA was obtained from Promega [12]. Terephthalic acid and 2-aminoterephthalic acid ligands were sourced from Merck (Germany). Additionally, 96% ethanol, N,N dimethylformamide (DMF), and 30% hydrochloric acid (HCl) were also ordered from Merck (Germany).

## Synthesis of MIL-100

A MOF nanocomposite was prepared using the following steps. First, an aqueous solution containing 40 mL of $Fe(NO_3)_3 \cdot 9H_2O$ (7.5 mmol) and benzene-1,3,5-tricarboxylate (BTC, 7.5 mmol) as the organic linker was prepared, giving a molar ratio of 9:10 (metal to linker). The mixture underwent stirring at 95°C for 24 h, after which the temperature was increased to 50°C and maintained for 90 min to allow further reaction. The obtained solid was washed with deionized water and ethanol at 69°C for 24 h, followed by vacuum drying at 150°C for 10 h[9]. The product was stored for later use after lyophilization.

# Synthesis of PEI-MIL-100

Initially, hydrocarbon chains of 6-bromohexanoic acid were integrated into the functional groups of the MOF. For this purpose, MOF (100mg) was dissolved in 2 ml DMF under sonication for 5min. simultaneously, 68 mg of 6-bromohexanoic acid was also dissolved in 2 ml DMF and placed in a bath sonicator. Subsequently, the solution was dripped slowly into the MOF solution, and the reaction proceeded over a period of 24 hours. In the next step, the excess 6-bromohexanoic acid were removed via dialysis, and the purified product was freeze-dried for 24h. Following this, PEI10000 was added to the ends of the hydrocarbon chains of the product obtained from the previous stage to functionalize MOF. Herein, 20 mg of the final product was dissolved in distilled water. Also, two substances EDC and NHS were employed to activate the carboxyl groups. PEI10000 was dissolved separately with EDC and NHS was added dropwise to the solution obtained from the previous stage (MOF-Hex acid) via magnetic stirring. After 24 hours, to purify the final product, the resulting solutions were transferred to a dialysis bag, distilled water was used as the washing buffer and the solvent was removed via freeze-drying. Finally, the product, which was called PEI10000-Hex-MIL 100, was stored in a -20 freezer.

# Characterization of nanoparticles

Examination of the functionalized MOF's morphology and structure was carried out using FE-SEM and FTIR (Perkin Elmer, Spectrum GX, USA). Aqueous powder samples were generated by means of an ultrasonic probe. Following this, the material was transferred onto a foil sheet and left to dry for 18 hours at room temperature before being evaluated.

# Preparation of polyplexes

Different concentrations of PEI-MOFs nanoparticles (carrier:C) were separately diluted into 50 µl of HBG buffer (HEPES-buffered glucose, 20 mM HEPES, 5% glucose, pH 7.4) and added to 50 µl solutions of the pEGFP- plasmid (P) in the same solvent (4 µg/50 µl), at different weight ratios (C/P) and mixed. Then, complex of PEI-MOFs/pDNA were incubated at room temperature for 20 min to form polyplexes. The prepared C/P ratios were at 2, 4, and 6 (weight/weight ratios).

# Plasmid DNA amplification

We used the CMV-EGFP gene, which contains a synthetic green fluorescent protein gene along with a cytomegalovirus promoter. The plasmid was amplified in DH5α E.coli strain and purified using the Qiagen plasmid extraction kit (QIAGEN, Valencia, CA) in accordance with the provided instructions.

# Size and surface charge measurement

Measurements of the zeta potential and size distribution for the complexes were carried out with a Zetasizer Nano ZS (Malvern Instruments, UK) at a charge ratio (C/P) of 2. For polyplex formation, specific quantities of PEI-functionalized MOFs were first dissolved in 125 µL of HBG buffer. This solution was then combined with an identical volume of HBG buffer containing DNA, resulting in a final DNA concentration of 5 µg/mL. The resulting mixture was allowed to stand at room temperature for 20 minutes to enable complexation. All experiments were repeated three times per sample, and the data are expressed as mean ± standard deviation (SD).

# Gel mobility experiment

The pDNA condensation efficiency of the polymers was evaluated using agarose gel electrophoresis. Complexes formed between PEI-modified MOFs or PEI (10 kDa) with pDNA at various C/P ratios were subjected to the gel retardation test. A 1% (w/v) agarose gel containing 0.1 µL/mL of Green Viewer (Biotium, Fremont, CA, USA) in 1× TBE (Tris borate EDTA) buffer was prepared. Following sample loading, electrophoresis was run at 70 V for 45 minutes. DNA bands were then visualized with a gel documentation system (Gel Doc).

# In vitro transfection assay

CT26 mouse colon cancer cells were seeded into 96 well plates at a density of 10,000 cells/well and allowed to incubate for 24 h before transfection. The culture medium was then aspirated and exchanged with 100 µL of fresh medium lacking FBS. Polyplexes were administered to the cells at three different C/P ratios (2, 4, and 6), and PEI (10 kDa) at identical ratios served as a control. Following a 4 h incubation, the medium was replaced with fresh complete growth medium supplemented with 10% FBS, and the cells were incubated for another 24 h. For the transfection assay, the medium was removed, and 50 µL of lysis buffer was introduced into each well. From each well, 40 µL of the resulting lysate was transferred to a fluorescent plate for fluorimetric analysis. The proportion of transfected cells was assessed with a fluorescent plate reader (Victor X5, PerkinElmer, USA), and GFP fluorescence was recorded using bandpass filters centered at 485 nm and 535 nm.

# Cytotoxicity assessment (MTT assay)

CT26 cells were seeded into 96 well plates at a density of 10,000 cells per well and maintained for 24 h. Following this initial incubation, the growth medium was exchanged for serum free medium. Polyplexes prepared at charge ratios (C/P) of 2, 4, and 6 were then introduced to the cells, and the plates were incubated under standard conditions for 4 h. The medium was subsequently replaced with fresh medium, and after an additional 18 h of incubation, MTT reagent (5 mg/mL) was dispensed into each well. Following a 4 h incubation at 37°C, the MTT containing medium was aspirated, and the formazan crystals generated by viable cells were solubilized in 100 µL of DMSO. Absorbance was then measured using an ELISA reader at 540 nm. Cell viability was calculated as the percentage of metabolically active cells relative to control cells that received no polyplex treatment.

# Statistical analysis

GraphPad Prism (Version 6.01) was used for statistical analysis of the data. A one way ANOVA was performed, considering P values below 0.05 as statistically significant. The findings are presented as the mean ± SD.

## Declarations

# Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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# Figures

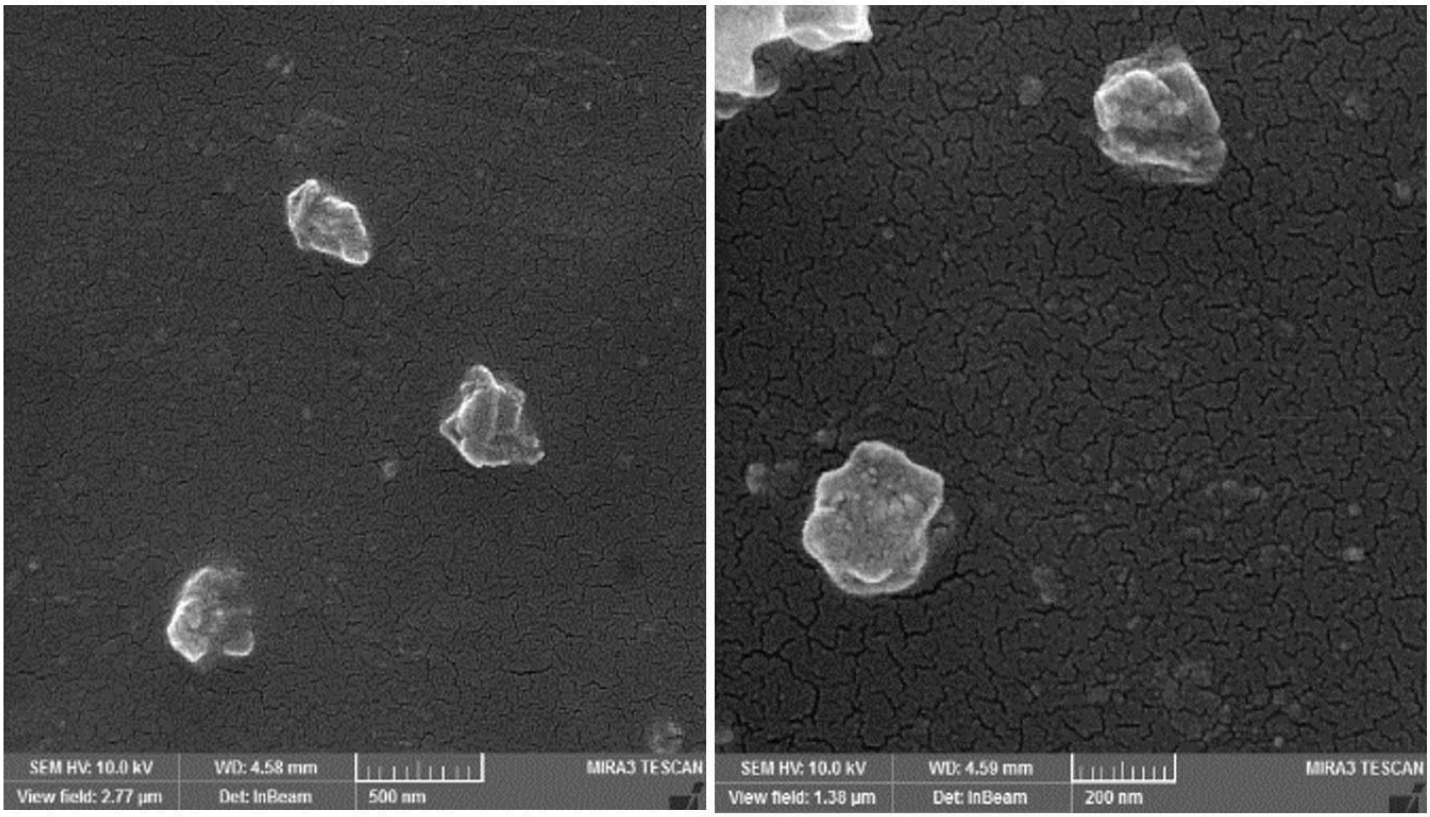



**Figure 1**

FE-SEM image of PEI10kDa-Hex-MIL100 modified with PEI10kDa and MIL-100(Fe)

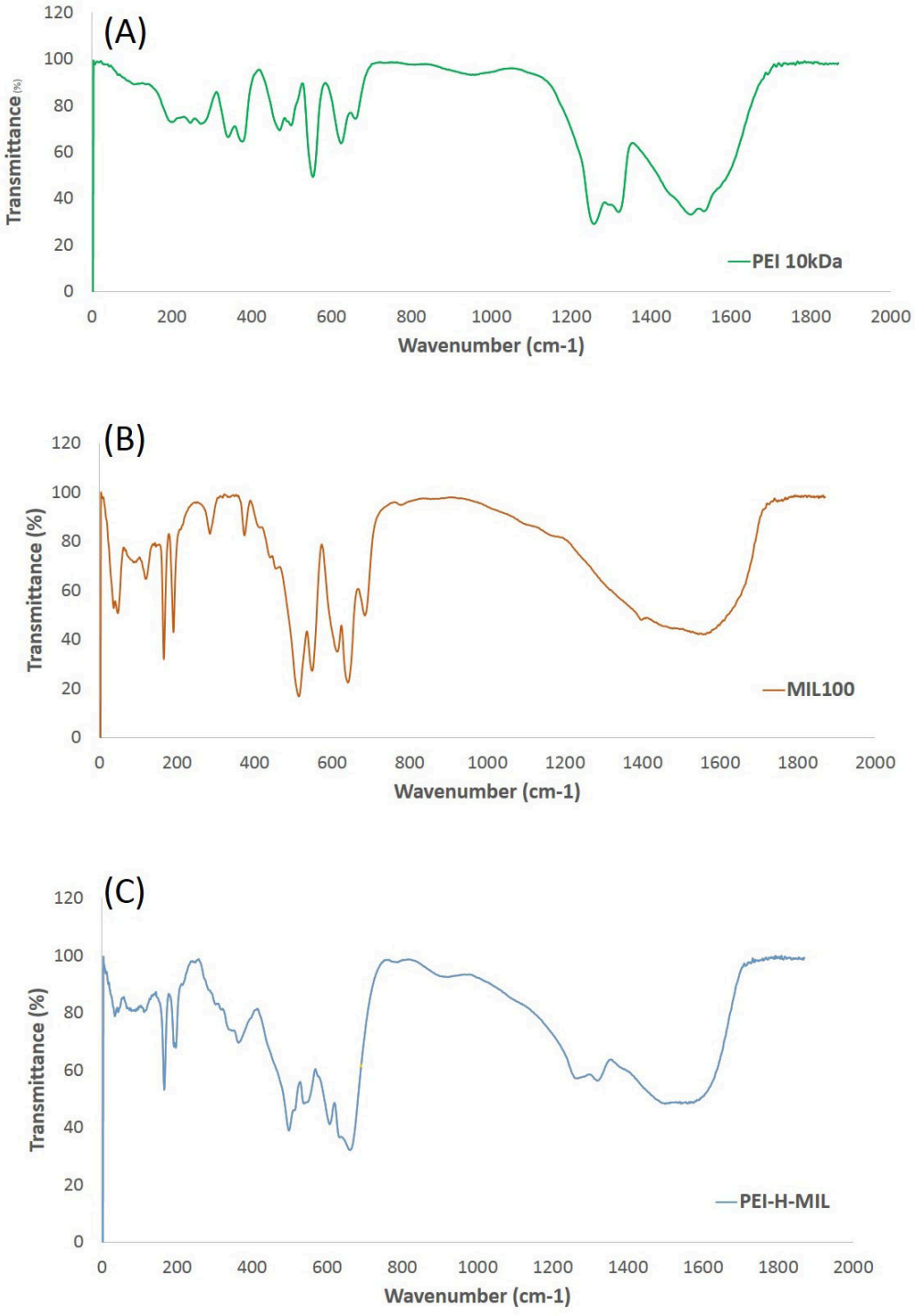



**Figure 2**

illustrated the FTIR spectra of (A) PEI 10kDa and MIL-100(Fe), and (B) PEI 10kDa-Hex-MIL-100(Fe) (PEI-H-MIL)

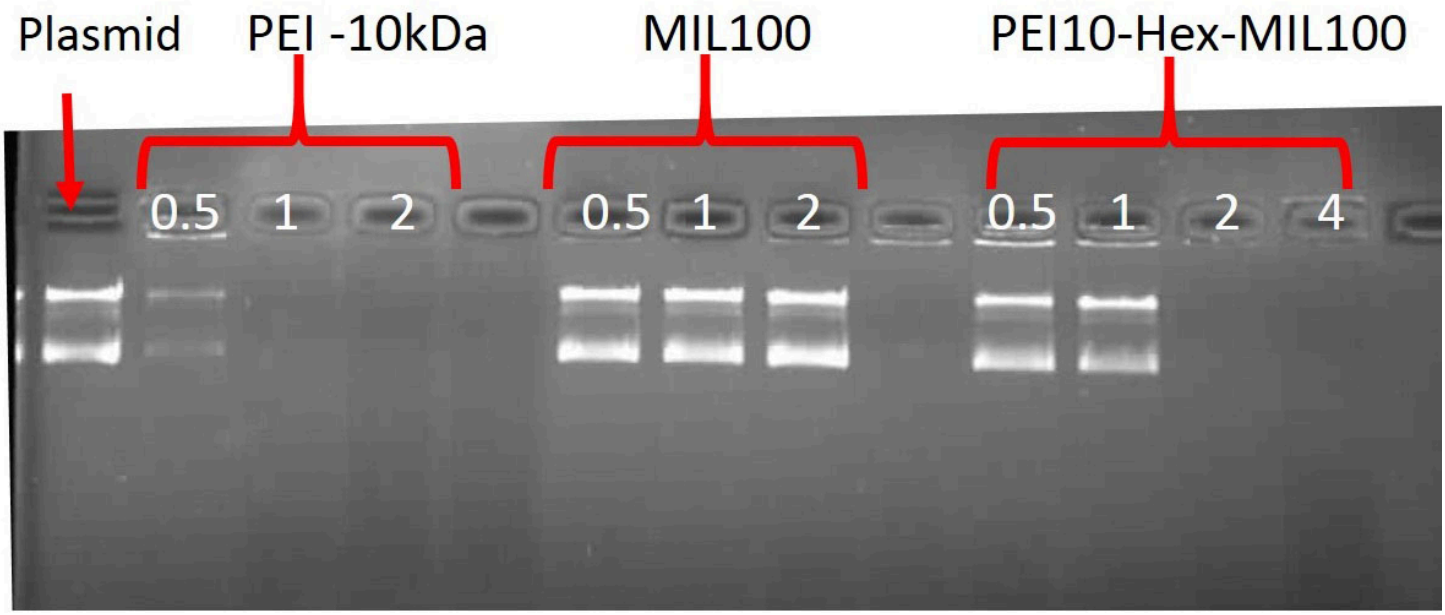



**Figure 3**

Plasmid compaction capability of PEI 10kDa,MIL100 and PEI10000-Hex-MIL-100 nanocarrier at different C/P, the numbers indicated C/P 0.5,1,2 and 4

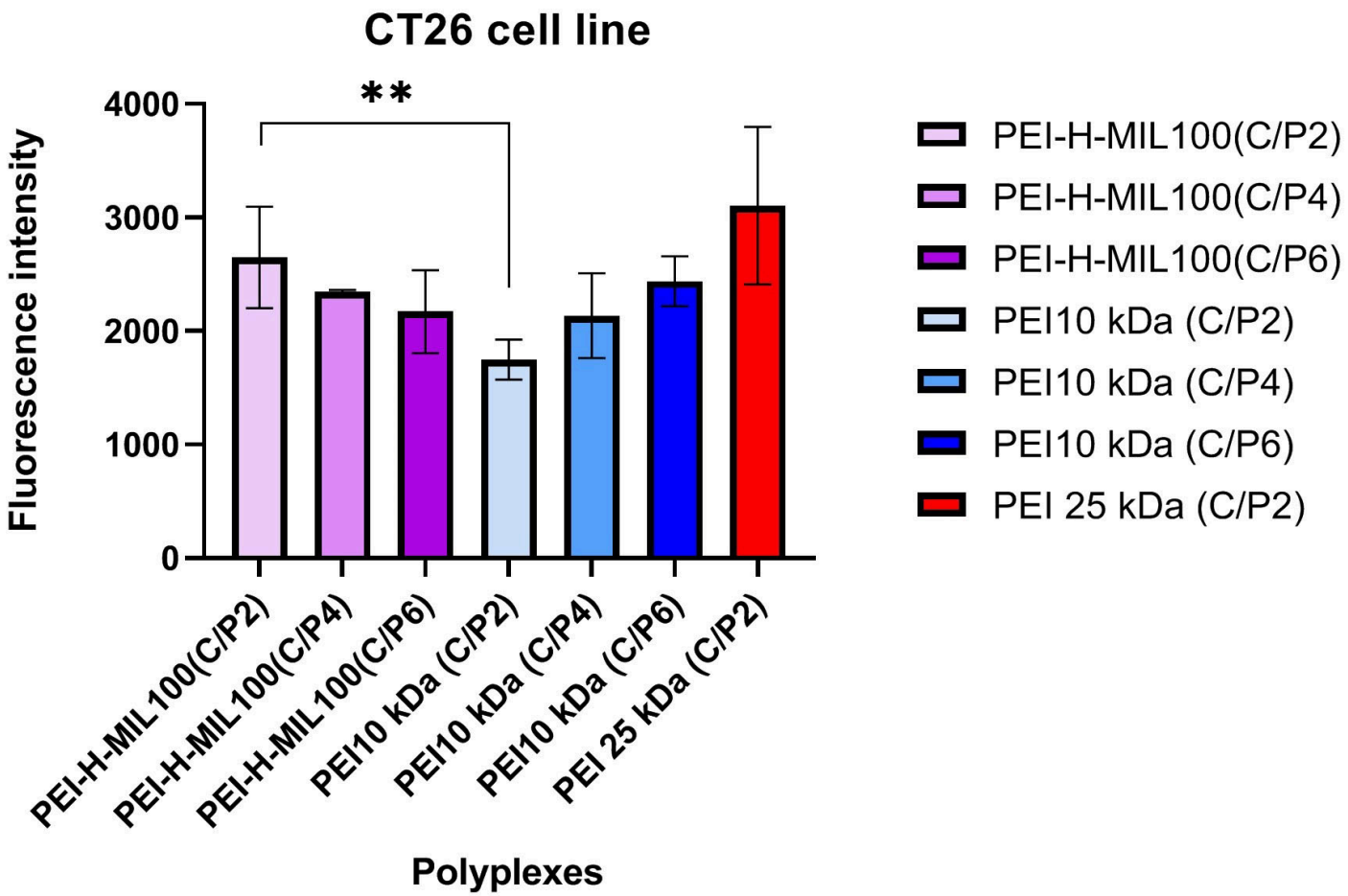



**Figure 4**

Comparison of transfection efficiency in CT26 cells for PEI-Hex-MIL-100 and PEI 10 kDa polyplexes at C/P ratios of 2, 4, and 6, along with PEI 25 kDa. Data are shown as mean ± SD (n = 3)

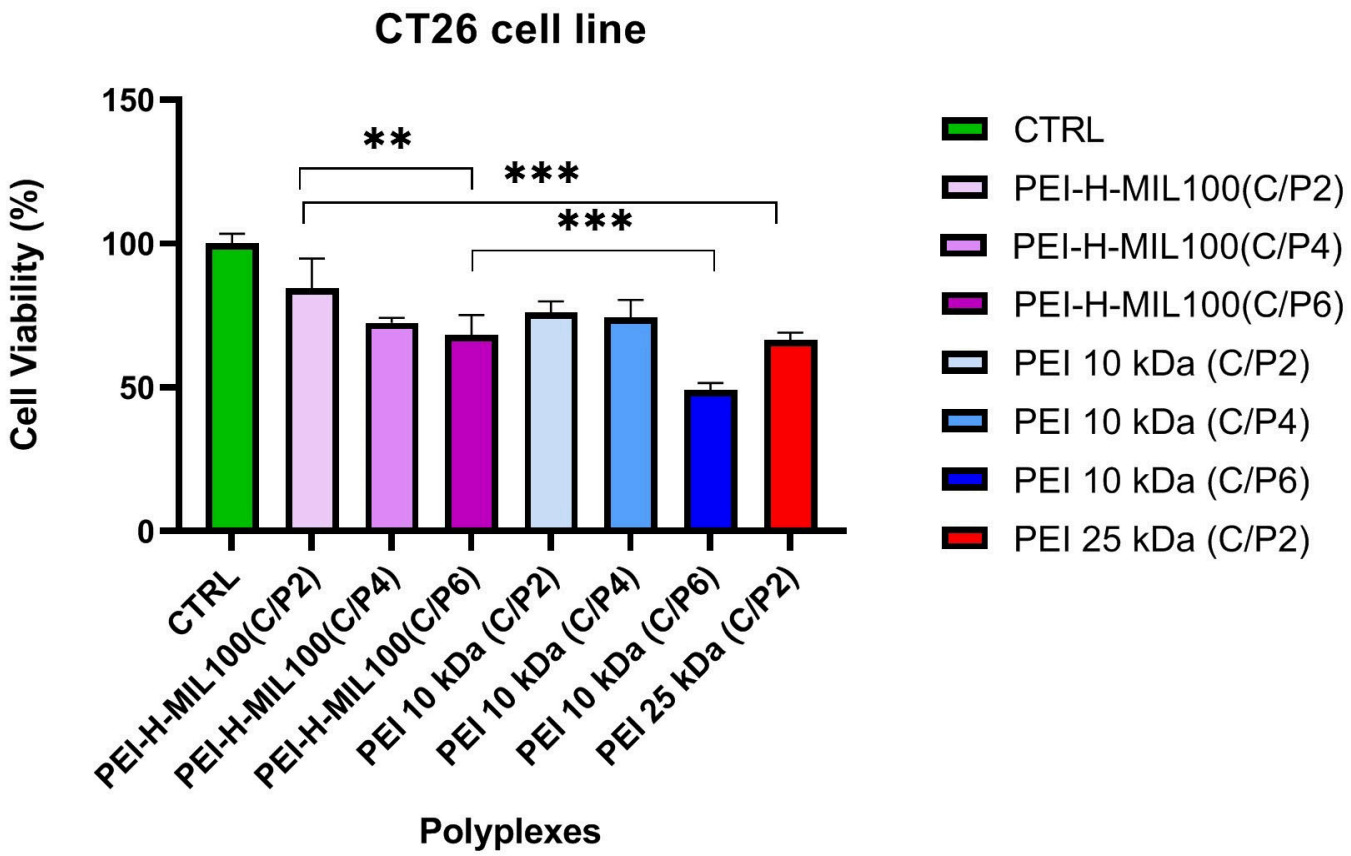



**Figure 5**

The MTT assay measured the metabolic activity of CT26 cells after exposure to PEI-Hex-MOF100 (modified MOF), PEI 10 kDa polyplexes, and PEI 25 kDa polyplexes. We tested three carrier-to-plasmid ratios (C/P = 2, 4, 6). Data represent mean ± SD (n = 3). Significance levels: **p < 0.01, ***p < 0.001

## Supplementary Files

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