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Biodegradable cyclen-based linear and cross-linked polymers as non-viral gene vectors

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ABSTRACT

Several 1,4,7,10-tetraazacyclododecane (cyclen)-based linear (**3a–c**) and cross-linked (**8a–d**) polymers containing biodegradable ester or disulfide bonds were described. These polymeric compounds were prepared by ring-opening polymerization from various diol glycidyl ethers. The molecular weights of the title polymers were measured by GPC. Agarose gel retardation assays showed that these compounds have good DNA-binding ability and can completely retard plasmid DNA (pDNA) at weight ratio of 20 for linear polymers and 1.2 for cross-linked polymers. The degradation of these polymers was confirmed by GPC. The formed polyplexes have appropriate sizes around 400 nm and zeta-potential values about 15–40 mV. The cytotoxicities of **8** assayed by MTT are much lower than that of 25 KDa PEI. In vitro transfection toward A549 and 293 cells showed that the transfection efficiency (TE) of **8c**-DNA polyplex is close to that of 25 kDa PEI at **8c**/DNA weight ratio of 4. Structure–activity relationships (SAR) of these linear and cross-linked polymers were discussed in their DNA-binding, cytotoxicity, and transfection studies. In addition, in the presence of serum, the TE of **8**/DNA polyplexes could be improved by introducing chloroquine or Ca^{2+} to pretreated cells.

1. Introduction

Gene therapy has gained significant attentions over the past two decades, and it has become a potential method for treating genetic disorders such as severe combined immunodeficiency,[1] Parkinson's disease,[2] and atherosclerosis[3] as well as an alternative method to traditional chemotherapy used in treating cancer.[4] The success of gene therapy largely depends on the availability of suitable delivery vehicles. Although viral vectors display rather good transfection properties both in vitro and in vivo, there are many problems associated with the use of these vectors.[5] To avoid these limitations, various non-viral cationic gene vectors[6] such as polyethylenimine (PEI), poly(2-dimethylaminoethyl methacrylate) (pDMAEMA) and poly-L-lysine (pLL), etc, were developed. Among these polymers, PEI is the most studied material for DNA delivery because of its strong buffering capability in the pH range of 7.4–5.1 together with high binding ability toward DNA and relatively high transfection efficiency (TE). Therefore, PEI has been considered as the gold standard of gene transfection. However, the high cytotoxicity partly caused by its high charge density seriously hampered its therapeutic use. Similar status exists in the employment of other cationic polymeric vectors, which are non-degradable and might lead to consequent risk of accumulation in the body. Therefore, the use of biodegradable polymers, which can break down into low molecular weight (LMW) segments, is a logical choice. Some typical biodegradable gene vectors including poly(β-amino ester) (PBAE),[7] poly(4-hyroxy-L-proline ester),[8] small-molecule end-groups of linear polymer determine,[9] branched poly(ethylenimine sulfide),[10] polyethylenimine-grafted polycarbonates,[11] reduction–responsive cross-linked polyethylenimine,[12] and oligoethylenimines grafted to PEGylated poly(β-amino ester)s,[13] were reported in recent years. Some of these examples gave higher TE and lower cytotoxicity comparing to 25 kDa PEI. Our group also prepared cross-linked biodegradable polymers based on LMW PEI 600 by using ester bond-containing diol glycidyl ethers, and 5 times higher TE than PEI was obtained in the in vitro transfection using these materials.[14]

Increasing knowledge about the crucial role of 1,4,7,10-tetraazacyclododecane (cyclen) in cell biology stimulated wide basic and applied research interests. Cyclen has four nitrogen atoms in one cycle. The unique macrocyclic structure endues the four N atoms with much different properties including basicity. Meanwhile, one, two or more N atoms can be easily modified with various groups. Many cyclen-based molecules have been synthesized and applied to the studies of their interaction with plasmid DNA in our lab.[15] Considering that the N atoms with different pK_a may

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have special 'proton sponge' effect that would benefit endosomal escape and lead to higher transfection, we have applied cyclen derivatives to the field of non-viral gene delivery. More recently, we testified that some cationic lipids with cyclen as hydrophilic group have better gene transfection ability than commercially available transfection agent lipofectamine 2000.[16] Moreover, our group also prepared some cyclen-based linear[17] and reticular[18] polymers which has in vitro TE close to PEI. However, these polymeric materials are not biodegradable, and we considered that better TE and lower cytotoxicity can be achieved by introducing appropriate biodegradable groups to these polymers. Qiao and co-workers also reported cyclen-grafted chitosan which could transfer DNA into HepG2 cell.[19] However, in their structure, the cyclen moiety acted only as an 'arm group' but not the component of polymer backbone, and the TE was moderate.

In this report, we would like to introduce series of novel biodegradeble linear and cross-linked cyclen-based polymeric compounds. Their interaction with plasmid DNA was investigated, and the in vitro TEs with or without serum were studied in different cell lines. Relative quantitative structure-activity relationships (QSAR) were discussed.

2. Results and discussion

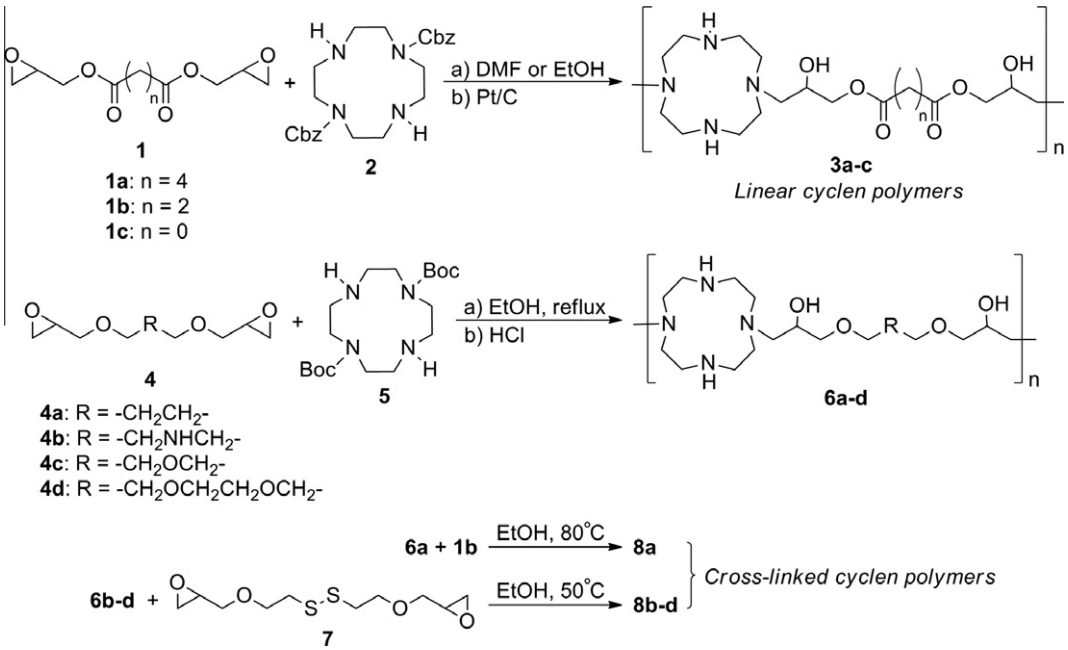
2.1. Synthesis of cyclen-based linear (3a–c) and cross-linked (8a–d) polymers

The synthetic routes for the title polymers are shown in Scheme 1. The ester bond-containing diglycidyl ester linkers **1a–c** with different chain length were prepared from adipic acid, succinic acid and oxalic acid, respectively.[14] Linear polymer **3a–c** could be simply prepared by stirring the mixture of **1a–c** and di-protected cyclen in ethanol or DMF at 80 °C. The solvent for polymerization seemed to have no obvious effect on the reaction, and little MW difference was found between the products obtained by the reactions in EtOH, $CHCl_3$ or DMF. Therefore we chose DMF as reaction solvent for its higher boiling point. Because the ester bonds might be hydrolyzed in the Boc-deprotection-alkalization process, benzyloxycarbonyl (Cbz) group was chosen to take place of Boc as protecting group. But unfortunately, the side-effect of Cbz group is the relatively low degree of polymerization. It was also reported that N-substituents with formula weight larger than approximately 100 Da might influence the polycondensation.[20] GPC gave the M_w of **3a–c** as 2450 (PDI = 1.82), 1725 (PDI = 1.72) and 819 (PDI = 1.29), respectively. For comparison, M_w of >7000 could be obtained with the use of diBoc-protected cyclen.[17]

As low M_w always leads to lower DNA condensation ability which may impair gene transfection, some previously studied non-biodegradable linear low polymers[17] were further cross-linked by biodegradable ester or disulfide bonds-containing linker to produce materials with higher M_ws. As shown in Scheme 1, **8a–d** were synthesized from different type of biodegradable linkers **1b** or **7**. We had found that disulfide compound **7** is more liable to the ring-opening polymerization than ester-containing compound **1**. On the other hand, for another starting material in the preparation of **8**, **6a** was found to have much higher reactivity than **6b–d**. As a result, **1b** could only react well with **6a** to give polymer **8a**, and no cross-linked product was found for **1b** and **6b–d**. Meanwhile, **8b–d** were obtained from the cross-linking between **6b–d** and **7**, and the reaction between **6a** and **7** led to viscous insoluble materials. Compounds **8a–d** showed good water solubility, which is required for subsequent studies. The M_ws of **8a–d** were measured by GPC as 12202, 16751, 5680 and 13978, respectively.

2.2. Interaction with DNA and the formation of polyplexes

DNA condensation capability is essential for polymeric gene vectors. Firstly, the DNA-binding ability of **3a** was investigated by gel retardation assay. As shown in Figure 1, the electrophoretic mobility of DNA was retarded by the introduction of **3a**, and total DNA retardation was detected at and above weight ratio of 20. The results suggested that **3a** can bind to DNA through electrostatic interactions between DNA backbone and the cationic nitrogen atoms in **3a**. However, the DNA-binding ability is weak. For **3b** and **3c**, poor DNA-binding abilities were also found, and nearly no DNA condensation was observed in the gel retardation assays involving **3c**. The weak DNA affinity of **3** might be attributed to their low molecular weights and the resulted weak cationic property, especially for **3b** and **3c**.



Scheme 1. Synthetic route of liner and cross-linked cyclen based polymers.

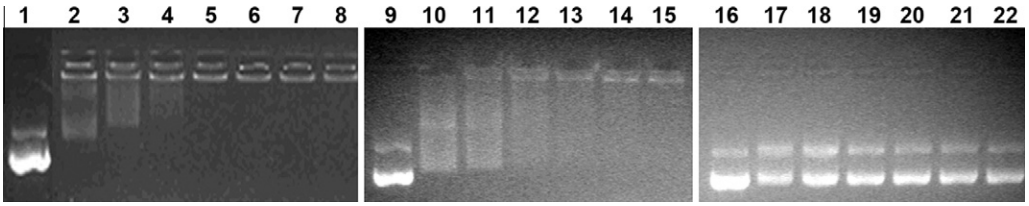


Figure 1. Electrophoretic mobility of pEGFP-N1 in the presence of **3** (ethidium bromide staining). Lane 1, 9 and 16: DNA control; Lanes 2–8: The weight ratios of **3a**/DNA were 0.5, 5, 10, 20, 40, 60, 80, respectively. Lanes 10–15(**3b**) and 17–22(**3c**): The weight ratios of **3**/DNA were 5, 10, 20, 40, 60, 120, respectively.

The potential advantage of biodegradable gene vectors as compared to their non-degradable counterparts is their reduced cytotoxicity and the avoidance of accumulation of the polymer in the cells after repeated administration.[21] The degradability of the polymers can be helpful as a tool to release the plasmid DNA into the cytosol. Hence, the DNA release from the formed polyplexes by the degradation of these polymers was also studied by gel retardation analysis. **8a** was first studied via being pre-incubated in PBS (pH 7.4, 150 mM NaCl) solution at 37 °C for 10 h. As shown in Figure 2, **8a** showed much better DNA-binding ability than **3a**, and full DNA retardation was observed at the weight ratio of 0.8 (Lane 5). Slightly more amount of DNA was detected after 10 h of incubation (Lanes 8–10), indicating that the ester bonds could be hydrolyzed and some of the condensed DNA was released from the polyplex. For disulfide-containing cross-linked polymers **8b–d**, complete DNA condensation was achieved at the weight ratio of 1.2 (Fig. 3A, Lanes 6, 12, and 18). Figure 3B shows the reductive DTT-promoted polymer degradation, which led to weakened DNA retardation abilities, and DNA release could be observed at the weight ratio of 1.2 (Fig. 3B, Lanes 7, 13, and 19). Further, the M_ws of **8a–d** were measured by GPC after being treated with PBS (**8a**) or DTT (**8b–d**), and the values were given as 9654 (79%), 3426 (20%), 5092 (90%) and 3969 (28%), respectively. Remarkable M_w decreases were found for **8b** and **8d**. We hope that the reductive degradation property may decrease the cytotoxicity of the polymers and facilitate the release of DNA in the reductive intracellular environment, leading to better gene transfection.

The properties of polymer/DNA nanoparticles are of critical important for polyamines being used as gene vectors. An appropriate particle size would facilitate the endocytosis and subsequent gene transfer. The particle sizes of the lipoplexes formed from title polymers **3** and **8** were measured by DLS at various weight ratios. Figure 4A shows the particle sizes of **8**/DNA complexes. It was shown that polymer **8** could efficiently compact pDNA into small nanoparticles with the sizes of around 400 nm in diameter at the weight ratio around 2–4. Further increase of weight ratio might result in the aggregation and increase of particle sizes, especially for **8b**. On the other hand, polymers **3** were unfortunately found to have much less ability to condense DNA into nanoparticles with proper sizes, and no reasonable particle size was given by DLS. This might be attributed to the low polymerization degree of **3**, leading to their weak DNA binding ability.

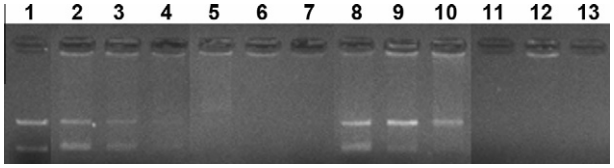


Figure 2. Electrophoretic mobility of pEGFP-N1 plasmid after pre-incubation for 0 h (Lanes 2–7) and 10 h in PBS (pH 7.4, lanes 8–13) with **8a** (ethidium bromide staining). Lane 1: DNA control; **8a**/pDNA weight ratios for lanes 2–7 and 8–13 were 0.4, 0.5, 0.6, 0.8, 1.6, 2.4, respectively.

Zeta-potential is an indicator of surface charges on the polymer/pDNA nanoparticles. A positively charged surface allows electrostatic interaction with anionic cell surfaces and facilitates cellular uptake.[22] As shown in Figure 4B, the zeta-potential values of **8**/DNA polyplexes increased along with the increase of weight ratios. In the lower weight ratio range of 0.5–4, the zeta-potentials increased sharply with charge reverse to positive, indicating the DNA condensation by the cationic polymers. Further increase of weight ratio (>4) resulted in only slightly enhancement of zeta potential, suggesting that full DNA condensation might be achieved at the weight ratio of 4. The potential values of **8**/DNA appeared to reach a maximum plateau of 25–35 mV, which is similar to that of the most reported PEI/DNA complexes (~25–30 mV).[23] Polyplex **8d**/DNA gave the lowest zeta potentials, which might be attributed to the oxygen-rich linking group in the structure of **8d**. The longer linking group with more oxygen atoms would screen and decrease its cationic charge density. Additionally, the polyplexes formed from cross-linked polymers **8** have higher zeta-potentials than those of linear polymers (about 5–20 mV),[17a] which might be the results of higher M_ws of **8**.

2.3. Cytotoxicity

The cytotoxicity of cationic polymers are thought to be caused by damage from the interaction with plasma membrane or other cellular compartments, and researches have found a rough correlation between toxicity and TE.[24] The in vitro cytotoxicity of **8** was evaluated in A549 and 293 cells by MTT assay, and the 25 kDa PEI was used as the control. As shown in Figure 5 and 25 kDa PEI displayed serious cytotoxicity in two cell-lines and the relative cell viability of PEI were less than 25% when its concentration was over 20 μg/mL. The ester bond-containing polymer **8a** showed higher cytotoxicity than the disulfide-containing polymers **8b–d**, indicating that the disulfide-containing materials are more prone to biodegradation due to the high intracellular glutathione (GSH) level.[25] In both cell lines, **8c** showed the lowest cytotoxicity, which might be attributed to its lower molecular weight. Above 60% of 293 cells survived even under the treatment of 100 μg/mL of **8c**. Compared with **8b**, the relatively lower cytotoxicity of **8c** and **8d** also demonstrated that the mono- or multi-ether group in the polymer structure would benefit the biocompatibility of the materials. Cell-dependent cytotoxicities were found for the cyclen-based cross-linked polymers, and A549 cells showed a relatively severe weakness against the cytotoxicity of **8**.

2.4. In vitro transfection

The gene transfection efficiencies of cyclen polymer complexes were assessed by in vitro delivery experiments of luciferase reporter gene (plasmid pGL-3) into A549 and 293 cells. PEI 25 kDa was used for comparison because of its high TE and easy availability. PEI/DNA polyplexes were prepared at an N/P ratio of 10 (weight ratio of 1.39), and polymer **8**/DNA complexes were prepared at various weight ratios. Firstly, we used **8a** to optimize the transfection

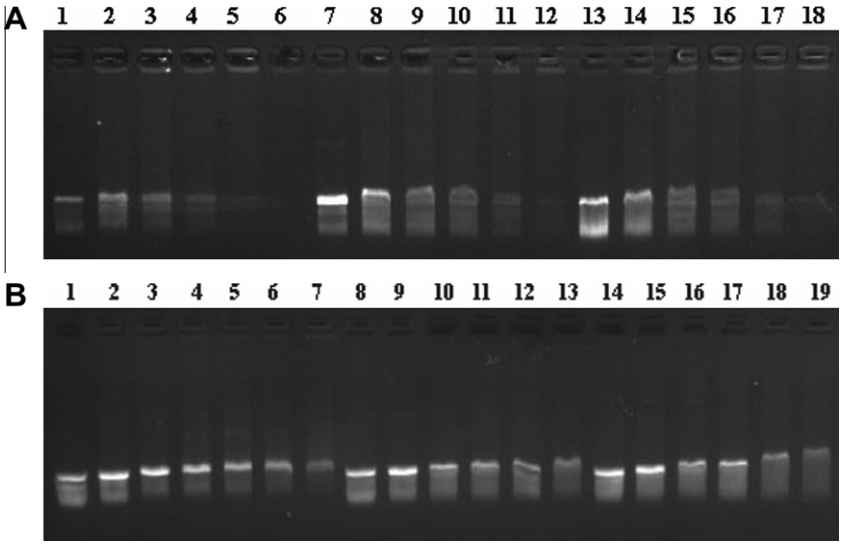


Figure 3. Electrophoretic mobility of pEGFP-N1 plasmid after pre-incubation for 0 h (A: Lanes 1–6: **8b**; Lanes 7–12: **8c**; Lanes 13–18: **8d**) and 1 h in the presence of DTT (B: Lane 1: DNA control; Lanes 2–7: **8b**; Lanes 8–13: **8c**; Lanes 14–19: **8d**) (ethidium bromide staining). For each band group including six lanes, **8b**–**d**/pDNA weight ratios were 0.3, 0.5, 0.7, 0.8, 1.0, 1.2, respectively.

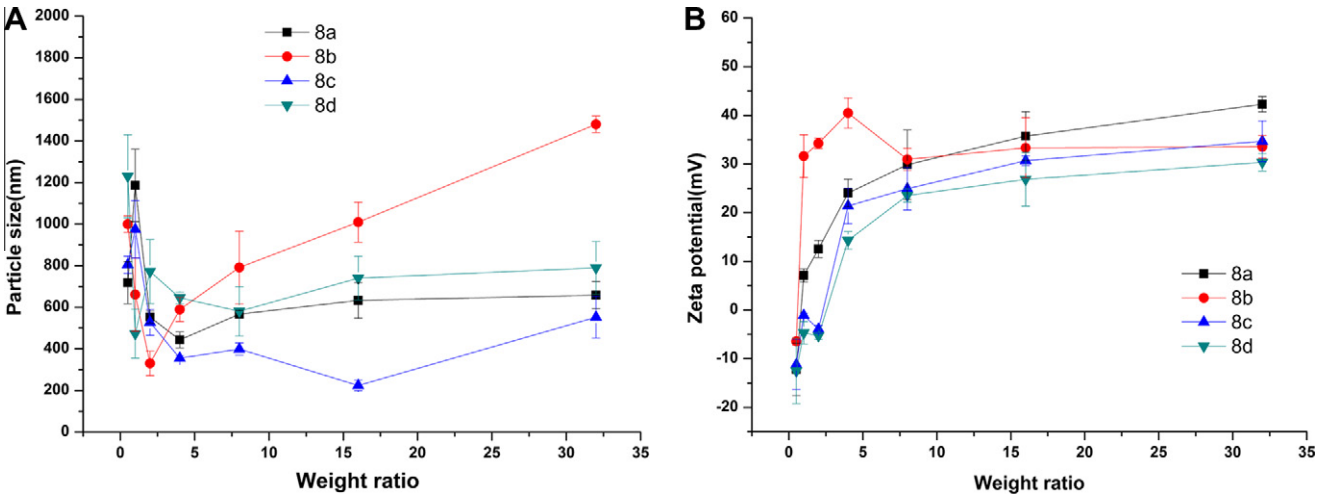


Figure 4. Average particle sizes (A) and zeta-potentials (B) of **8**/DNA at various weight ratios (mean ± SD, $n = 3$).

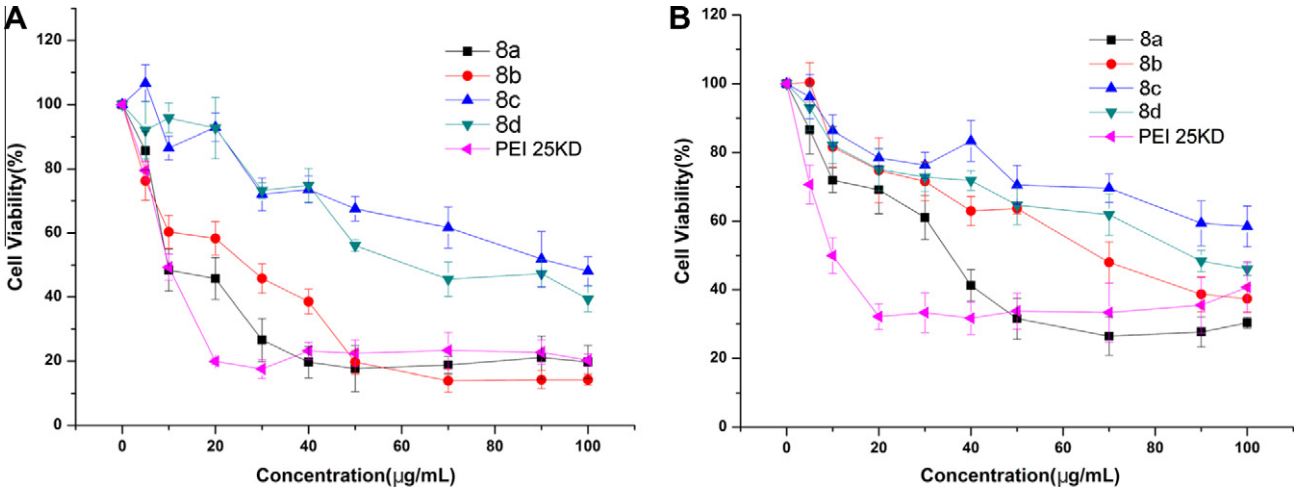


Figure 5. Relative cell viabilities of **8** and 25 kDa PEI in A549 (A) and 293 (B) cells.

conditions in the luciferase assay and green fluorescent protein (GFP) assay. In 293 cells (Fig. 6A), the best result was obtained under weight ratio of 4:1 (concentration of 12.0 μg/mL), and both lower (2:1, Fig. 6A) or higher (data not shown) weight ratios led to obviously decreased TE. The GFP assays observed by microscopy also demonstrated the optimal weight ratio of 4:1. To test the biocompatibility of the cross-linked polymer, transfection assays were also carried out in the presence of 10% serum. Furthermore, as commonly used transfection promoted reagents, chloroquine and Ca^{2+} cation were used for the purpose of TE improvement. As shown in Figure 6A, the TEs decreased with the presence of serum under both weight ratios. However, by addition of chloroquine, the TE increased dramatically to equal that obtained in serum-free experiment. And this result was much better than that obtained by using the polymer before cross-linking (**6a**). In A549 cells, the weight ratio of 2: 1 was found to be optimal for **8a**/DNA (Fig. 6B). Although lower luciferase expression was obtained in the transfection experiments using A549 cells, an interesting fact was that under both weight ratios, Ca^{2+} could largely improved the TE of the transfection with serum, and the TEs with Ca^{2+} were even much higher than those obtained in serum-free experiments. For other polymers **8b–d**, GFP assays gave similar optimal transfection conditions.

Figure 7 shows the TEs of **8a–d**/DNA toward 293 and A549 cells. Polymer **8c** displayed the best TE in 293 cells whether with serum or not (Fig. 7A). In A549 cells, **8a** was found to be the best choice in the serum-free transfections. However, in the presence of serum, the TE of **8a** was harshly decreased, and **8d** was found to give the best result (Fig. 7B). In both cell lines, serum had less effect on the disulfide-containing materials **8b–d** than ester bond-containing polymer **8a**, indicating that disulfide bond might be more compatible in vivo due to its higher liability to biodegradation. For example, in 293 cells, **8b** showed no TE loss by the participation of serum. In addition, chloroquine was found to have positive effect only in the transfection using **8c** in A549 cells. The transfection results exhibited that the slight differences on the bridge structure might have large influence on the TE in both cell lines.

To directly visualize the infected cells expressing pEGFP-Nl, enhanced green fluorescent protein expression in A549 and 293 cells was observed by an inverted fluorescent microscope. According to the results of luciferase assays, **3a**/DNA, **8a**/DNA, **8b**/DNA and PEI 25 kDa/DNA (as control) complexes were used at the optimal weight ratio, respectively. According to Figure 8, similar to the luciferase assays, the GFP expression in A549 cells was much lower than those in 293 cells. The linear polymer **3a** with low molecular weight only showed poor transfection, and only few green fluorescent points could be observed. Polymers **8** gave much better GFP expression, nevertheless the TE were still lower than 25 kDa PEI. The disulfide-containing polymer **8b** gave higher transfection efficiency than the ester bond-containing one **8a**. This might be attributed to its liability for biodegradation, as comparing to the GPC results.

3. Conclusions

In summary, several cyclen-based linear and cross-linked polymers containing biodegradable ester or disulfide bonds were prepared. Their interactions with plasmid DNA were investigated by agarose gel retardation, which showed that the cross-linked polymers have good DNA-binding ability, while the low molecular weight linear polymer **3** showed weak DNA affinity. Polymers **8** could condense DNA into nanoparticles with the diameters around 400 nm and zeta-potential values about 15–40 mV. The cytotoxicity and transfection efficiency of **8** toward A549 and 293 cells were studied. Results showed that these cross-linked polymers have relatively lower cytotoxicity and TE than 25 KDa PEI. Some additives such as chloroquine and Ca^{2+} were found to have the ability to improve the TE in the presence of serum. Further optimization of the polymer structure and purposeful modifications on such kind of polymer are now in progress.

4. Experimental

4.1. Materials and methods

All chemicals and reagents were obtained commercially and were used as received. Anhydrous ethanol and dichloromethane were dried and purified under nitrogen by using standard methods and were distilled immediately before use. 25 kDa PEI (branched, average molecular weight 25 kDa), chloroquine diphosphate ($C_{18}H_{26}ClN_3{\cdot}2H_3PO_4$, M_w = 515.87), and MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Disulfide compound **7** was synthesized according to literature.[26] The plasmids used in the study were pGL-3 (Promega, Madison, WI, USA, coding for luciferase DNA) and pEGFP-N1 (Clontech, Palo Alto, CA, USA, coding

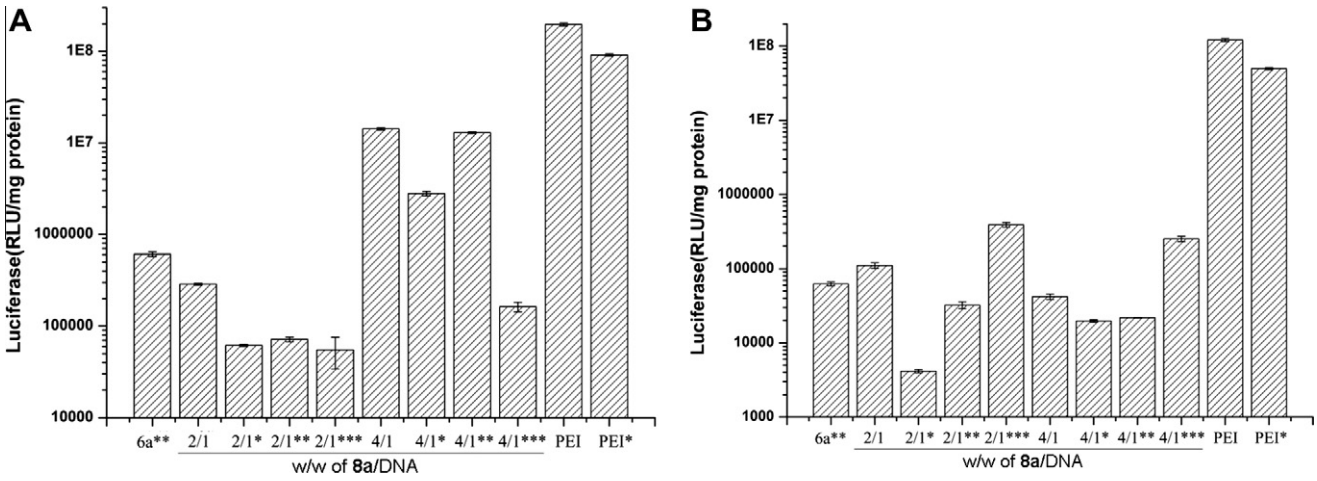


Figure 6. Luciferase expression mediated by **8a**/DNA polyplex in 293 cells (A) and A549 cells (B) (mean ± SD, n = 3). For comparison with Figure 5, the concentrations of compound **8a** in the transfection experiments were 6, 12 μg/mL for the weight ratio of 2 and 4, respectively, ∗: cultured with 10% serum, ∗∗: cultured with chloroquine and 10% serum, ∗∗∗: cultured with $CaCl_2$ and 10% serum.

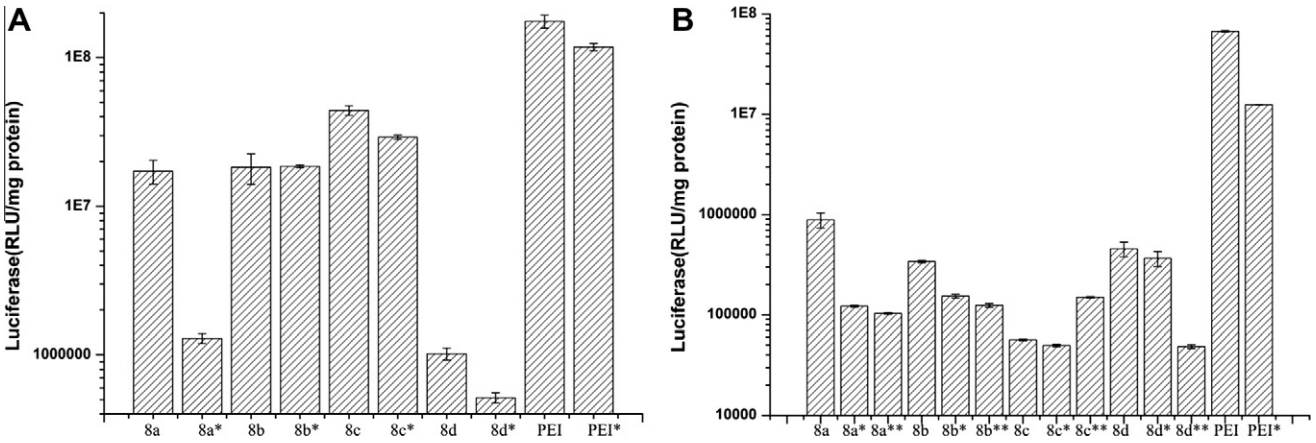


Figure 7. Luciferase expression in 293(A) and A549 (B) cells transfected by **8**/DNA polyplexes at optimal weight ratios (4 for 293 and 2 for A549 cells) (mean ± SD, $n = 3$), ∗: cultured with 10% serum, ∗∗: cultured with chloroquine and 10% serum. 25 KDa PEI/DNA was used as control under N/P of 10.

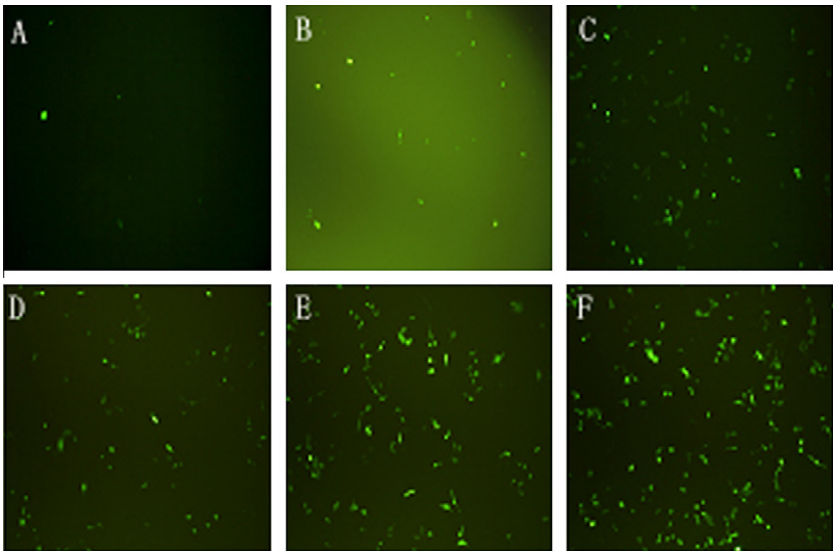


Figure 8. Fluorescent microscope images of pEGFP-transfected in A549 cells or 293 cells. (A) **3a**/DNA (w/w = 80) in A549 cells; (B) **8a**/DNA (w/w = 2) in A549 cells, treated with chloroquine and 10% serum; (C) PEI/DNA ($N/P = 10$, w/w = 1.39) in A549 cells, with 10% serum. (D) **8a**/DNA (w/w = 4) in 293 cells, with 10% serum; (E) **8b**/DNA (w/w = 4) in 293 cells, with 10% serum; (F) PEI/DNA ($N/P = 10$, w/w = 1.39) in 293 cells, with 10% serum.

for EGFP DNA). The Dulbecco's Modified Eagle's Medium (DMEM), 1640 Medium and fetal bovine serum were purchased from Invitrogen Corp. MicroBCA protein assay kit was obtained from Pierce (Rockford, IL, USA). Luciferase assay kit was purchased from Promega (Madison, WI, USA). Endotoxin free plasmid purification kit was purchased from TIANGEN (Beijing, China). The dialysis film (MWCO: Nominal: 1000 Methyl alcohol Recommended) was purchased from Shanghai green Bird Science & Technology Development Co., LTD. MS-ESI spectra data were recorded on a Finnigan LCQ^{DECA}. IR spectra were measured with a Shimadzu FTIR-4200 spectrometer. ^1H NMR spectra were obtained on a Bruker AV400 spectrometer. $CDCl_3$ or D_2O was used as solvent and TMS as the internal reference. The molecular weight of polyamine was determined by gel permeation chromatography (GPC) (Waters 515 pump, Waters 2410 Refractive Index Detector (25 °C), incorporating Shodex columns OHPAK KB-803). Filtered a mixture of 0.2 mol/L HAc/NaAc buffer which content 20% CH_3CN (volume ratio) was used as mobile phase with a flow rate of 0.5 mL min^{-1}. Molecular weight analysis were calculated against poly(ethylene glycol) standards of number average molecular weights ranging from 200 to 80,000.

4.2. Preparation of linear polymer 3

Diacid glycidyl diesters **1**[27] and 1,7-dibenzyloxycarbonyl-1,4,7,10-tetraazacyclodocane **2**[28] was prepared according to the references. Compounds **1**, **2** (1.50 mmol each) and 2 mL of anhydrous ethanol or DMF were mixed in a flask with magnetic stirring and refluxed for 120 h in oil bath. After the reaction, the mixture was diluted with 3 mL of anhydrous methanol, and the crude product was precipitated by the addition of anhydrous dichoromethane/cyclohexane (v/v = 2:1). The precipitation was collected and dried in vacuum to get the product as pale oil.

Removal of the Cbz group was accomplished by using Pd/C according to the described method.[29] In a 100 mL round-bottom flask the polymer (300 mg) was dissolved in 50 mL of CH_3OH. To this solution under a hydrogen atmosphere was added 0.4 g of Pd/C (10%). The reaction was stirred at room temperature for

14 h and filtered to remove the Pd/C. The combined filtrates were dried and evaporated under reduced pressure to give the product as oil. The crude product was then purified by dialysis (MWCO = 1000 Da) against CH_3OH for 24 h.

***3a**:* Yield 10.1%. ^{1}H NMR (400 MHz, D_2O): δ = 1.471–1.488 (4H, m), 2.244–2.307 (4H, m), 2.404–2.910 (20H, m), 3.403–3.538 (4H, m), 3.993–4.029 (2H, m). IR (KBr, cm^{-1}): 3338.98, 2950.36, 1735.31, 1666.17, 1371.12, 1260.00, 1163.45, 1089.93, 1033.40, 824.72, 765.73, 700.19.

***3b**:* Yield 7.1%. ^{1}H NMR (400 MHz, D_2O): δ = 2.361–2.521 (4H, m), 2.539–3.081 (20H, m), 3.382–3.513 (4H, m), 4.028–4.087 (2H, m). IR (KBr, cm^{-1}): 3327.05, 2950.29, 1730.59, 1659.11, 1457.32, 1221.01, 1084.58, 914.69, 815.11, 766.92.

***3c**:* Yield 22.1%. ^{1}H NMR (400 MHz, D_2O): δ = 2.479–2.550 (4H, m), 2.776–3.097 (16H, m), 3.405–3.595 (4H, m), 3.832–3.856 (2H, m). IR (KBr, cm^{-1}): 3342.37, 2933.79, 1665.94, 1446.48, 1045.44, 921.00, 768.06, 682.92.

4.3. Preparation of cross-linked polymers 8

Polymer **6a–d** were prepared by the method we described previously.[17] The cross-linked polymers **8a** was prepared from **6a** and ester-containing linker **1b**, while **8b–d** were prepared from **6b–d** and disulfide-containing linker **7**. The general procedure for the preparation of **8** is depicted as follows: Compound **6** (1 mmol, based on their MW measured by GPC) was dissolved in 1 mL of C_2H_5OH, then compound **1b** or **7** (1 mmol) was added to the solution. Under the protection of N_2, the reaction mixture was stirred at 80 °C for 80 h (for **8a**) or at 50 °C for 4 h (for **8b–d**). The solvent was removed under reduced pressure. The residue was recrystallized twice by $CH_3OH/CHCl_3$/hexane to give polymers **8**, which were characterized by ^{1}H NMR, IR and GPC.

***8a**:* Yield 65.0%. ^{1}H NMR (400 MHz, D_2O): δ = 1.615 (4H, br), 2.508–2.769 (22H, m), 3.392–3.699 (6H, m), 3.851 (2H, br). IR (KBr, cm^{-1}): 3389.17, 2936.25, 2865.30, 1663.21, 1437.28, 1258.63, 1114.97, 865.72, 761.61, 582.14.

***8b**:* Yield 70.5%. ^{1}H NMR (400 MHz, D_2O): δ = 2.368–2.709 (22H, m), 2.886–2.965 (2H, m), 3.386–3.545 (8H, m), 3.748 (2H, br). IR (KBr, cm^{-1}): 3397.06, 2851.37, 1653.43, 1560.51, 1460.26, 1357.95, 1300.03, 1120.15, 947.49, 762.50, 600.98.

***8c**:* Yield 54.1%. ^{1}H NMR (400 MHz, D_2O): δ = 2.479–2.810 (18H, br), 3.340–3.688 (14H, m), 3.920 (2H, br). IR (KBr, cm^{-1}): 3397.77, 2859.78, 1659.09, 1460.68, 1355.29, 1295.91, 1120.11, 969.82, 810.84, 762.97, 597.97.

***8d**: Yield* 50.1%. ^{1}H NMR (400 MHz, D_2O): δ = 2.460–2.726 (18H, br), 3.452–3.667 (18H, m), 3.804 (2H, br). IR (KBr, cm^{-1}): 3414.39, 2864.55, 1638.93, 1460.77, 1355.55, 1296.75, 1114.28, 953.38, 809.47, 763.02, 597.53.

4.4. Cell culture

HEK (human embryonic kidney) 293 cells and human nonsmall-cell lung carcinoma A549 cells were incubated respectively in Dulbecco's Modified Eagle's Medium (DMEM) and 1640 Medium containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin, 10,000 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2.

4.5. Amplification and purification of plasmid DNA

pGL-3 and pEGFP plasmids were used. The former one as the luciferase reporter gene was transformed in *Escherichia coli* JM109 and the latter one as the green fluorescent protein gene was transformed in *E. coli* DH5α. Both plasmids were amplified in terrific broth media at 37 °C overnight. The plasmids were purified by an EndoFree Tiangen™ Plasmid Kit. Then the purified plasmids were dissolved in TE buffer solution and stored at −20 °C. The integrity of plasmids was confirmed by agarose gel electrophoresis. The purity and concentration of plasmids were determined by ultraviolet (UV) absorbance at 260 and 280 nm.

4.6. Agarose gel electrophoresis

Polymer/DNA complexes at different weight ratios ranging from 0.5 to 32 were prepared by adding an appropriate volume of compounds(in 150 mM NaCl solution) to 0.8 mL of pEGFP-N1 DNA (120 ng/μL in 40 mM Tris–HCl buffer solution). The complexes were diluted by 150 mM NaCl solution to a total volume of 6 μL, and then the complexes were incubated at 37 °C for 30 min. After that the complexes were electrophoresed on the 0.7% (W/V) agarose gel containing EB and with Tris-acetate (TAE) running buffer at 110 V for 30 min. DNA was visualized with a UV lamp using a Bio-Rad Universal Hood II. The property of DNA release associated with the degradation of compounds **3** and **8** was further investigated by agarose gel electrophoresis. Polymer/DNA complexes at different weight ratios ranging from 0.5 to 80 for compound **3**, and 0.3 to 1.2 for compound **8** were prepared by adding an appropriate volume of polymers (in 150 mM NaCl solution) to 0.8 mL of pEGFP-N1 DNA (120 ng/μL in 40 mM Tris–HCl buffer solution). In the subsequent polymer degradation and DNA release studies, compound **8a–d** or their DNA polyplexes were incubated with PBS (pH 7.4), 10 mM of DL-dithiothreitol (DTT) or A549 cell extracts at 37 °C for 10 h.[30] The A549 cell extracts were prepared by ultrasonic cell disruptor with power of 120 w. 4×10^5 cells were disrupted in 1 mL of distilled water and stored in −20 °C. In the DNA release studies, 12.5 μL of cell extracts was added to 125 ng of plasmid DNA.

4.7. Particle size and zeta potential measurements

Particle size and zeta potential measurements of polyplexes were carried out using a Nano-ZS 3600 (Malvern Instruments, USA) with a He–Ne Laser beam (633 nm, fixed scattering angle of 90°) at 25 °C. Polymer/DNA polyplexes at weight ratios ranging from 0.5 to 32 were prepared by the same method with above-mentioned agarose gel electrophoresis. After 30 min incubation in 100 mL ultrapure water, polyplex solutions were diluted to final volume of 1 mL before measurements.

4.8. Polymer degradation study

For the polymer degradation study, compounds **8** dissolved in PBS or DTT solution was constantly shaken in a 37 °C incubator at 100 rpm for 10 h. The relative molecular weights of degraded products were determined by GPC using a Waters 515 pump and a Waters 2410 refractive index detector (25 °C).

4.9. Cell viability assay

Toxicity of compounds **8** toward 293 cells and A549 cells was determined by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay following literature procedures. The 293 cells (6000 cells/well) and A549 cells (9000 cells/well) were seeded into 96-well plates. The cells were then incubated in a culture medium containing compounds **8** with a particular concentration for 24 h. After that, the medium was replaced with 200 μL of fresh medium, and 20 μL of sterile filtered MTT (5 mg/mL) stock solution in PBS was added to each well. After 4 h, unreacted dye was removed by aspiration. The formazan crystals were dissolved in 150 μL DMSO per well and measured spectrophotometrically in an ELISA plate reader (model 550, Bio-Rad) at a wavelength of 490 nm. The cell survival was expressed as follows: Cell viability = $(OD_{treated}/OD_{control}) \times 100\%$.

4.10. In vitro transfection

4.10.1. Luciferase assay

The 25 kDa PEI was used as the positive control due to its high TE in vitro and in vivo. The plasmid pGL-3 was used as a reporter gene. Transfections of pGL-3 plasmid mediated by D-PEIs in 293 cells and A549 cells were studied as compared with 25 kDa PEI. 293 cells or A549 cells were seeded at a density of 6×10^4 cells/well in the 24-well plate with 0.5 mL of medium containing 10% FBS and incubated at 37 °C for 24 h. Then the complexes were prepared at different weight ratios by adding 2 μg plasmid DNA to an appropriate volume of compounds solution. Before transfection, the cells were washed by serum-free medium, and then the Polymer/DNA polyplexes were added with serum-free medium for 4 h at 37 °C. Then the serum-free medium was replaced by flash medium containing 10% FBS, and the cells were further incubated for 24 h. After that, the medium was removed. The luciferase assay was performed according to manufacture's protocols (Promega). Relative light units (RLUs) were measured with chemiluminometer (FLOROSKAN ASCENT FL). The total protein was measured according to a BCA protein assay kit (Pierce). Luciferase activity was expressed as RLU/mg protein. Data are shown as mean ± standard deviation (SD) based on 3 independent measurements. The statistical significance between two sets of data was calculated using Student's *t*-test. AP value <0.05 was considered statistically significant.

4.10.2. Green fluorescent protein assay

Transfections of pEGFP-N1 plasmid mediated by D-PEIs in 293 cells and A549 cells were also evaluated. The best weight ratios in 293 cells and A549 cells determined from the luciferase assay were used. Two hundred and ninety three cells and A549 cells were inoculated at a density of 5×10^4 cells/well in 24-well plates respectively, 24 h prior to transfection. Polymer/DNA polyplexes were prepared by adding an appropriate volume of polymers solution (in 150 mM NaCl solution) to 50 μL pEGFP-N1 DNA solution (30 μg/mL in 40 mM Tris–HCl buffer solution) with the final volume of 100 μL. Then the complexes were incubated at room temperature for 30 min. The plates were washed by PBS twice, and 100 μL Polymer/DNA polyplexes were then added to a well with an additional 150 μL of medium with or without FBS. The final concentration of DNA in the complexes was calculated to be 8 μg/mL. After 4 h of incubation, the Polymer/DNA polyplexes containing medium was replaced with 0.5 mL of fresh medium containing 10% FBS, and the cells were further incubated for 24 h at 37 °C. The cells were directly observed by an inverted microscope (Olympus IX 71). The microscopy images were obtained at the magnification of 100× and recorded using Viewfinder Lite (1.0) software.

4.10.3. In vitro transfection in serum and treatment with chloroquine or $CaCl_2$

The process of transfection experiments was the same as described above, except that the medium at the time of transfection was replaced with DMEM containing 10% FBS (fetal bovine serum).[31] For the treatment with chloroquine, after cells were incubated for 10 min with serum-free DMEM of 0.5 mL with 100 μM chloroquine, gene transfection was carried out; particle vectors with DNA were incubated with cells for 4 h at 37 °C in DMEM containing 10% FBS with 100 μM chloroquine, the medium with chloroquine was replaced with 0.5 mL of DMEM containing 10% FBS, and then the cells were incubated for further 20 h. Twenty four hours following the gene transfection, luciferase expression was measured as described above. For Ca^{2+} supplementation studies, vector/DNA complexes were immediately added to tubes containing an aqueous solution of 1 M $CaCl_2$ at the desired concentration and left at room temperature for 30 min before diluting to the final volume with DMEM containing 10% FBS. After incubation for 3 h in DMEM containing 10% FBS (250 μL), the cells were further incubated for 21 h at 37 °C in DMEM (0.5 mL) containing 10% FBS. Ca^{2+} was in medium throughout the incubations.[32]

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