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Metal-free multicomponent polymerization toward cationic polyamidines

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ABSTRACT

Cationic polymers, also known as polycations, are considered to be the most potential non-viral gene carriers due to their unique advantages such as the ability to bind the negative charge of nucleic acid molecules. Multicomponent polymerization (MCP) is a one-step, tandem strategy to construct complex structures based on multicomponent reactions. Herein, we developed a metal-free MCP method based on three monomers of *p*-dinitrovinylbenzene (*p*-DNVB), 1,1-dimethylethyl *N,N*-dibromocarbamate ($BocNBr_2$), and bis-secondary-amines with a ratio of 1:2:1, to access a library of Boc-substituted polyamidines with well-defined structures and suitable molecular weights (M_w ranging from 4400 Da to 11,000 Da) in high yields (up to 85%) under mild conditions. Upon the removal of Boc groups, a series of water-soluble polymers with cationic property were prepared and their gene binding capability was further evaluated.

Multicomponent reaction (MCR), in which three or more starting materials participated into a reaction to afford a complex molecule in a one-pot and time-saving manner, shows great potential and strongly fascinates the chemists [1,2]. MCR is a cascade strategy for quickly obtaining versatile and important motifs with highly functional structures [3], such as amides [4,5], propargylamine [6], *N*-sulfonylamidines [7] and dihydropyrimidin-*2H*-ones [8]. Multicomponent polymerization (MCP), derived from MCR, is a burgeoning area in polymer chemistry with unique advantages including structural diversity and operational simplicity. Compared with the traditional polycondensation methods using two monomers, MCP dramatically broadens the versatility of the polymers through the involvement of different combinations of three or more monomers [9]. Till now, numerous types of MCRs, including the Passerini, Ugi, Biginelli and Cu-catalyzed azide-alkyne cycloaddition (CuAAC) reactions have been successfully reassessed and disclosed by polymer chemists to prepare novel and functional polymers [3,7,10-13]. However, further development and application of MCPs are always hindered by the limited MCRs suitable for MCPs, narrow scope of monomers, low solubility of the obtained polymers as well as the undesired side reactions caused by the introduction of incompatible monomers. Therefore, it is of great significance to develop new MCP methods to prepare functional polymers with well-defined structures and desired molecular weights (M_ws).

Polyamidine is a kind of cationic polymer containing amidine groups as the repeating unit. Amidine, also called iminoamide, is not only a useful structure existing in numerous bioactive molecules, but also widely used in agrochemicals, drugs, as well as the pharmaceutical and fine chemical industries [14-19]. Even though various strategies have been reported to prepare amidines, most of the methods were based on toxic reagents such as isonitriles, nitriles, azides, or transition-metals like Pd, all of which were environmental-unfriendly [20-24]. Moreover, few of them have been utilized in constructing polyamidine, let alone the efficient preparation of polyamidine in a relatively green manner. Some MCP methods have been reported using Mannich reaction starting from formaldehyde or formalin to prepare cationic polymers, such as polyamines [25,26]. As a derivative of amine-containing polymer like polyvinylamine or polyethyleneimine, polyamidine features the advantages of good thermal stability, low toxicity, and high positive charge density [27]. Besides that, polyamidine as a typical cationic polymer, also exhibits the potential as the ideal non-viral gene delivery vector to condense and delivery negatively charged nucleic acids [28,29].

In 2019, Li and An's groups reported a metal-free MCR starting from *trans*-β-nitrostyrene derivatives, dibromo amides, and various amines to rapidly construct amidine frameworks with high diversity and complexity [30]. Inspired by such method, in this work, a metal-free MCP was developed using *p*-dinitrovinylbenzene (*p*-DNVB), *N,N*-dibromocarbamate ($BocNBr_2$), and bis-secondary

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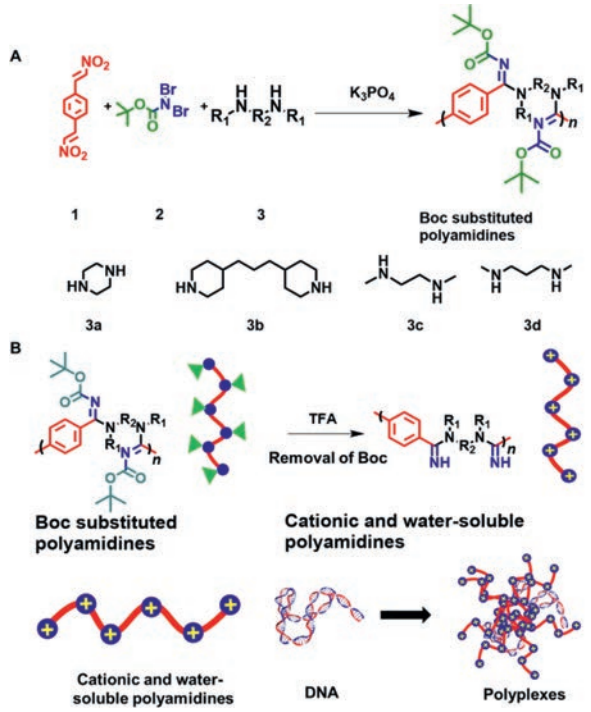
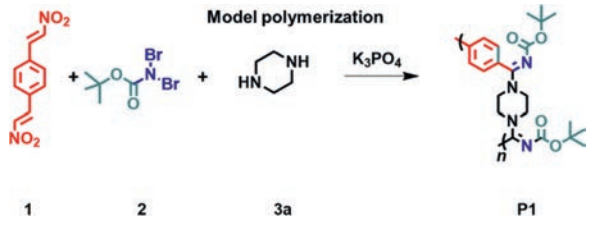


Scheme 1. (A) Synthetic route of the polyamidine library using MCP from *p*-DNVB, $BocNBr_2$ and bis-secondary-amines. (B) Preparation of cationic polyamidines and formation of polyplexes with plasmid DNA.



Scheme 2. Model MCP using **1**, **2** and **3a** as monomers.

amines to access a library of *tert*–butyloxycarbonyl (Boc)-substituted polyamidines (Scheme 1A). Since Boc could be facilely cleaved by trifluoroacetic acid (TFA), a library of cationic polyamidines with non-fully substituted amidines and water-soluble property could be afforded upon the removal of Boc groups. Such cationic polyamidines could be potentially utilized in the gene delivery area as the non-viral vectors due to the high density of positive charges on the main chain (Scheme 1B).

Prior to the optimization of polymerization condition, *p*-DNVB **1** and $BocNBr_2$ **2** were initially synthesized and purified following the methods reported before [31,32] (Schemes S1 and S2, Figs. S1-S4 in Supporting information). The model MCP between **1**, **2** and commercially available piperazine **3a** was firstly performed to afford the targeting polymer with moderate M_w of 3600 g/mol and yield of 92.5% at the optimized conditions reported by Li [30]. To further improve the polymer's M_w, effect of temperature (10–60 °C), monomer concentration (0.05–0.3 mol/L), polymerization time (12–48 h), and solvent (DMF, MeCN, MeOH, THF, DCE, $CHCl_3$ and toluene) were systemically evaluated to optimize the polymerization conditions (Scheme 2).

The effect of temperature was firstly studied by performing the polymerization in $CHCl_3$ at the feeding ratio of [**1**]:[**2**]:[**3a**] = 1:2:1 under various temperatures. As shown in Table 1, high temperature could notably increase the M_w of polymers and promote such MCP. It was mainly because of the more improved reactivity and better

Table 1
Effect of temperature on the MCP of **1**, **2** and **3a**.[a]

Entry	Temperature (°C)	Yield (%)	M_n[b]	M_w[b]	$Đ$[b]
1	10	48.8	2000	2500	1.23
2	25	92.5	2500	3600	1.48
3	40	92.2	2700	3900	1.47
4	60	69.4	3000	4700	1.59

[a] Conditions: experiments carried out in $CHCl_3$ for 48 h. [**1**] = [**3a**] = 0.05 mol/L, [**2**] = 0.1 mol/L, K_3PO_4 = 0.2 mol/L.
[b] M_n, M_w and $Đ$ were determined by GPC in DMF with polymethyl methacrylate as standards.

Table 2
Effect of monomer concentration on the MCP of **1**, **2** and **3a**.[a]

Entry	Concentration (mol/L)	Yield (%)	M_n[b]	M_w[b]	$Đ$[b]
1	0.05	69.4	3000	4700	1.59
2	0.1	85.5	3300	5600	1.66
3	0.15	77.8	2900	4300	1.47
4	0.2	57.4	3100	4500	1.48
5	0.3	49.0	2800	4100	1.46

[a] Conditions: experiments carried out at 60 °C for 48 h in $CHCl_3$.
[b] M_n, M_w and $Đ$ were determined by GPC in DMF with polymethyl methacrylate as standards.

Table 3
Effect of polymerization time on the MCP of **1**, **2** and **3a**.[a]

Entry	MCP time (h)	Yield (%)	M_n[b]	M_w[b]	$Đ$[b]
1	12	16.6	3000	4400	1.47
2	24	39.0	2900	4900	1.66
3	36	41.3	2900	4700	1.64
4	48	85.5	3300	5600	1.66

[a] Conditions: experiments carried out at 60 °C in $CHCl_3$. [**1**] = [**3a**] = 0.1 mol/L, [**2**] = 0.2 mol/L, K_3PO_4 = 0.4 mol/L.
[b] M_n, M_w and $Đ$ were determined by GPC in DMF with polymethyl methacrylate as standards.

solubility of the monomers at higher temperature. As a result, 60 °C was screened to be the optimal temperature and used in the further experiments. The monomer concentration played a crucial role in most of MCP. As shown in Table 2, the M_w of the polymers peaked (5600 g/mol) at the monomer concentration of 0.1 mol/L. Both low monomer concentration (0.05 mol/L) and high monomer concentration (> 0.15 mol/L) led to the decrease of M_w. When the monomer concentration was as low as 0.05 mol/L, the M_w was only 4700 g/mol although the yield of the polymer could reach 70%. The possible reason was that diluted concentration would hinder the molecular collision between monomers. When the concentration of the monomer was higher than 0.1 mol/L, the yield and M_w would also decrease, because the high concentration would lead to a fast initial reaction rate to quickly form a large number of oligomers. The solubility of such oligomers would severely decrease at high concentrations to affect the final yield and M_w. Therefore, all the following MCP experiments were performed with a concentration of 0.1 mol/L at 60 °C. The polymerization time was subsequently investigated and the results in Table 3 clearly indicated that the M_w of the polymer quickly reached 4400 g/mol at the first 12 h and further increased to 5600 g/mol when elongating the polymerization time to 48 h. Considering that continuously extending the reaction time would not remarkably improve the M_w, 48 h was screened to be the optimal polymerization time and used in the further experiments.

Solvent effect was finally evaluated since the polarity of the solvents would also affect the polymerization results. As shown in Table 4, *N*,*N*-dimethylformamide (DMF), dichloroethane (DCE), chloroform ($CHCl_3$), acetonitrile (MeCN), methanol (MeOH), tetrahydrofuran (THF) and toluene with different polarities were selected to optimize the solvent and the results indicated that such

Table 4
Effect of solvent on the MCP of **1**, **2** and **3a**.[a]

Entry	Solvent	Yield (%)	M_n[b]	M_w[b]	$Đ$[b]
1	DMF	88.0	3900	4700	1.23
2	MeCN	42.7	4800	5100	1.06
3	MeOH	23.4	3900	5000	1.28
4	THF	36.7	4200	5400	1.30
5	DCE	77.3	4200	5200	1.23
6	$CHCl_3$	85.5	3300	5600	1.66
7	Toluene	83.1	3800	4900	1.28

[a] Conditions: experiments carried out at 60 °C for 48 h in different solvents. [**1**] = [**3a**] = 0.1 mol/L, [**2**] = 0.2 mol/L, K_3PO_4 = 0.4 mol/L.
[b] M_n, M_w and $Đ$ were determined by GPC in DMF with polymethyl methacrylate as standards.

Table 5
Effect of $BocNBr_2$ concentration on the MCP of **1**, **2** and **3a**.[a]

Entry	[**2**]/[**1**]	Yield (%)	M_n[b]	M_w[b]	$Đ$[b]
1	2	86	3300	5600	1.66
2	3	81	3100	5000	1.30
3	4	77	2900	4600	1.15
4	5	69	2800	4300	1.10

[a] Conditions: experiments carried out at 60 °C in $CHCl_3$. [**1**] = [**3a**] = 0.1 mol/L, K_3PO_4 = 0.4 mol/L.
[b] M_n, M_w and $Đ$ were determined by GPC in DMF with polymethyl methacrylate as standards.

MCP could well tolerate most of the organic solvents and polymers with moderate M_w (around 5000 g/mol) could be afforded. $CHCl_3$ was screened as the optimal solvent for the further experiments because of the relatively higher yield (85.5%) and M_w (5600 g/mol) compared with other solvents.

Finally, the concentration of $BocNBr_2$ was optimized to evaluate whether increasing the amount of $BocNBr_2$ would further improve the M_w. Unfortunately, the results in Table 5 indicated that the optimized ratio of *p*-DNVB to *p*-DNVB was 2. Further increasing the amount of *p*-DNVB would lead to severe solubility issue and decrease the M_w.

To gain exact structure information of the products, both the ^1H NMR and ^{13}C NMR spectra of **P1** and the corresponding monomers **1**, **2**, **3a** were shown in Fig. 1 as a model example. Comparing the ^1H NMR spectra of **P1** and **1** (Fig. 1A), the peaks of -CH=CH- for nitroalkene at δ 8.17 and δ 8.35 corresponding to **1** completely vanished in the ^1H NMR spectrum of **P1**, demonstrating the completed consumption of the **1** monomer and the high conversion rate in MCP. The peaks at δ 1.50 associated with the *tert*–butyl protons of **2** notably appeared in **P1**, indicating that **2** successfully participated into the MCP. The peak at δ 2.77 representing the protons adjacent to the secondary amine in **3a** slightly moved to low fields, indicating that the electron-withdrawing ability of the amidine group was stronger than the secondary amine, which reduced the electron cloud density around the methylene group, causing the chemical shift of the methylene hydrogen to move to low field. Comparing the ^{13}C NMR spectra of **P1** and **1** (Fig. 1B), the peak of -CH=CH- at δ 137.9 and δ 139.3 corresponding to **1** completely vanished in the ^{13}C NMR spectrum of **P1**, which was in consistent with the ^1H NMR result. The peaks at δ 156.6 associated with the -C=O- on *tert*–butyl of **2** appeared on the spectrum of **P1** at the same position without obvious shifts, indicating that **2** participated into the MCP and the final polymer had the Boc functional group. In the same way, the peaks at δ 47.0 associated with the $-CH_2-$ of **3a** also appeared on the spectrum of P1 at the similar position. More importantly, the typical carbon peak of -C=N on the amidine group emerged at around δ 161.8, demonstrating that the MCP was successful. ^1H NMR spectra and ^{13}C NMR spectra data proved that all the three components, **1, 2**, and **3a** totally participated into the MCP to form the targeting (Boc)-substituted polyamidine. In order

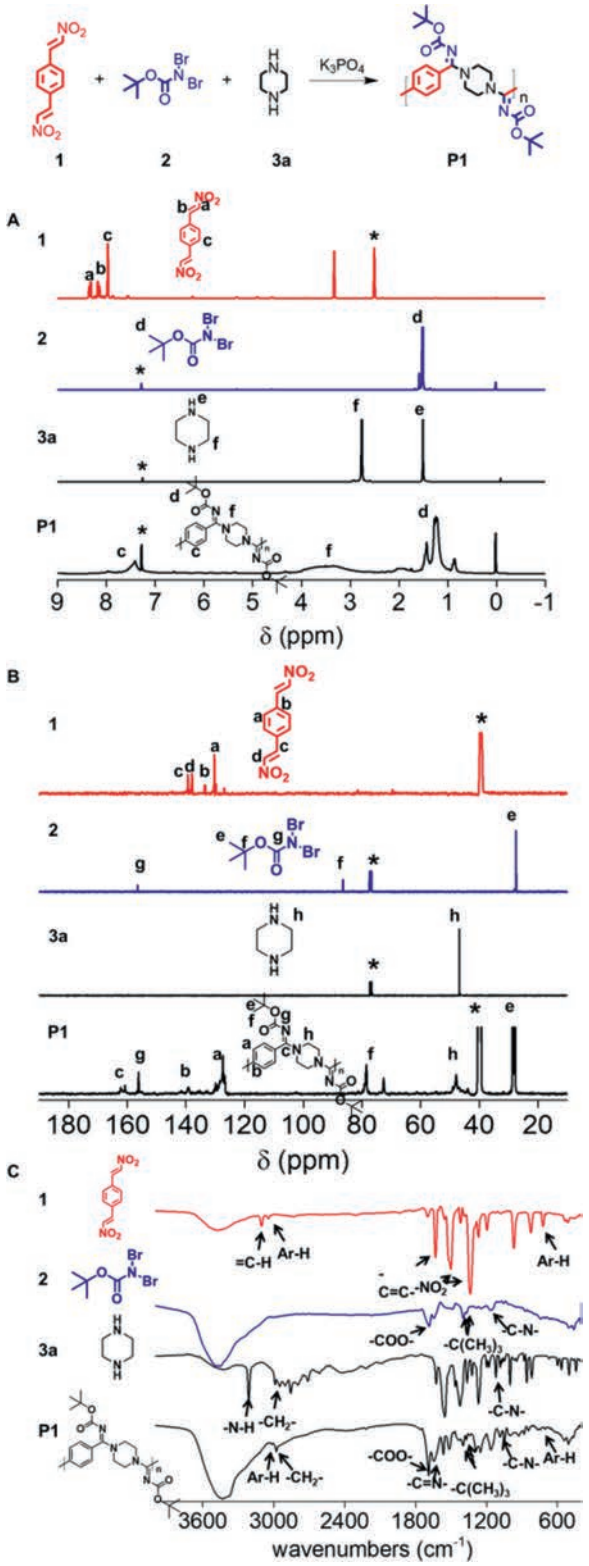


Fig. 1. Structural characterization of model MCP product **P1** using **1**, **2**, and **3a** as monomers. (A) ^1H NMR spectra of **1**, **2**, **3a** and polymer **P1** in DMSO–d_6 (1) and $CDCl_3$ (**2, 3a, P1**). The peaks of related solvents are marked with asterisks. (B) ^{13}C NMR spectra of **1, 2, 3a**, and polymer P1 in DMSO–d_6 (**1, P1**) and $CDCl_3$ (**2, 3a**). The peaks of related solvents are marked with asterisks. (C) FT-IR spectra of monomer **1, 2, 3a** and **P1**.

to further confirmed the structure, FT-IR spectra of **1, 2, 3a** and polymer **P1** were performed (Fig. 1C). Comparing the spectra of **P1** and **1**, the peaks of =C-H at 3100 cm^{-1}, -C=C- at 1633 cm^{-1}, $-NO_2$ at 1500 cm^{-1} and 1335 cm^{-1} corresponding to **1** disappeared

Table 6
MCP results of *p*-DNVB, $BocNBr_2$ and various bis-secondary-amines.[a]

Entry	Polymer	Monomer **3**	Yield (%)	M_n[b]	M_w[b]	$Đ$[b]
1	**P1**	**3a**	86	3300	5600	1.66
2	**P2**	**3b**	83	7600	11,000	1.50
3	**P3**	**3c**	70	3700	4500	1.22
4	**P4**	**3d**	50	3400	4100	1.19

[a] Conditions: experiments carried out at 60 °C for 48 h in $CHCl_3$. **[1]** = **[3a]** = 0.1 mol/L, **[2]** = 0.2 mol/L, K_3PO_4 = 0.4 mol/L.
[b] M_n, M_w and $Đ$ were determined by GPC in DMF with polymethyl methacrylate as standards.

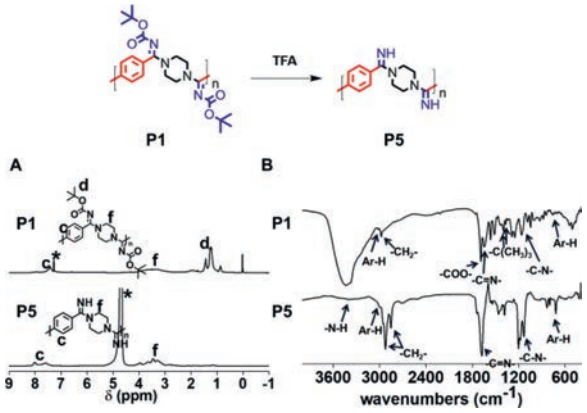
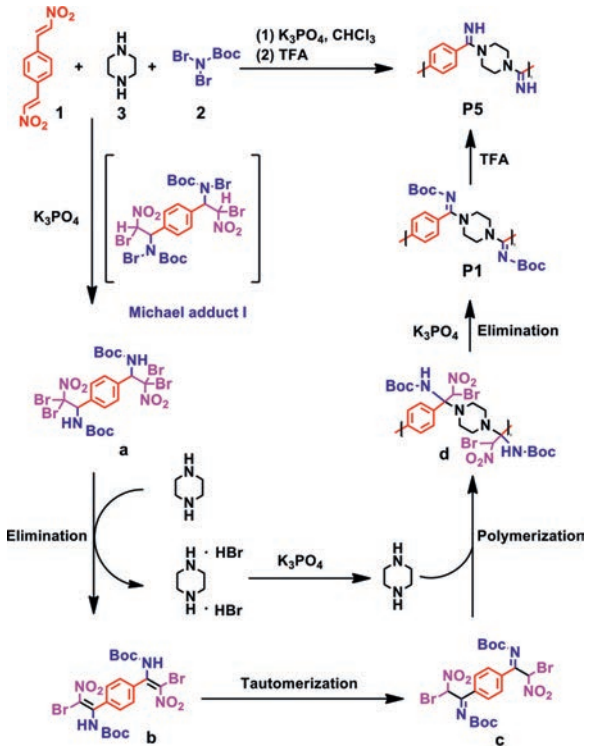


Fig. 2. Preparation of cationic polyamidine upon the removal of Boc group. (A) ^1H NMR spectra of **P1** and **P5** in $CDCl_3$ **(P1)** and D_2O **(P5)**. The peaks of related solvents are marked with asterisks. (B) FR-IR spectra of **P1** and **P5**.



Scheme 3. Mechanism of MCP to afford cationic polyamidine.

in the FT-IR spectrum of **P1**. In addition, the peaks of Ar-H at 3040 cm^{-1} and 720 cm^{-1} were obviously found in **P1**, demonstrating **1** participated in MCP. The peaks of -COO- at 1700 cm^{-1}, $-C(CH_3)_3$ at 1395 cm^{-1} and 1365 cm^{-1} corresponding to **2** perfectly existed in the FT-IR spectrum of **P1**, showing that **2** participated in MCP and the final product containing the Boc group. Comparing the spectra of **P1** and **3a**, the peak of -N-H at 3210 cm^{-1} was vanished while the peaks of $-CH_2-$ at 2930 cm^{-1} and -C-N- at 1150 cm^{-1} still existed in the polymer, showing that **3a** was also involved in the polymerization reaction.

With the optimized conditions at hand, four kinds of commercially available bis-secondary amines with different steric hindrance, 1,4-diazacyclohexane (**3a**), 1,3-di-4-piperidylpropane (**3b**), *N,N*-dimethyl-1,2-ethanediamine (**3c**) and *N,N*-dimethyl-1,3-propanediamine (**3d**) were explored to further expand the substrate scope and demonstrate the robustness of such MCP (Scheme 1A). A library of Boc-substituted polyamidines with similar pendants but various backbone structures were facilely prepared and fully characterized (Figs. S5-S9 in Supporting information, Table 5). As shown in Table 6, we found that cyclic bis-secondary amines (**3a** and **3b**) could promote such MCP to afford the polymer with relatively high yields (>80%) and M_w (> 5000 g/mol) compared to linear monomers. **3b** with less steric hinderance effect exhibited highest M_w (11,000 g/mol), meaning that cyclic secondary amines with flexible chains favored such amidination polymerization.

To explore whether the (Boc)-substituted polyamidine could be furthermore modified to prepare the cationic and water-soluble polyamidine, TFA was used to remove Boc in **P1** and form the non-fully substituted polyamidine, **P5** (Fig. 2). After **P1** was thoroughly deprotected by TFA, **P5** exhibited excellent water solubility at the pH values lower than 7.4. The chemical structure of **P5** was evaluated using ^1H NMR and FT-IR spectra. As shown in Fig. 2A, the peaks at the range of δ 1.2 associated with the *tert*–butyl protons of **P1** totally disappeared, which verified the successful deprotection of Boc group from **P1**. In addition, as shown in Fig. 2B, the peaks of -COO- at 1700 cm^{-1} and $-C(CH_3)_3$ at 1395 cm^{-1} and 1365 cm^{-1} corresponding to Boc group in **P1** vanished in **P5**, and the broad peak at 3400 cm^{-1} indicating -N-H appeared. All the data showed that a kind of water-soluble and cationic polyamidine could be successfully prepared using such MCP method, followed by a step of Boc removal.

Based on the mechanism proposed by Li and An's groups [30], a possible mechanism for the MCP to prepare the cationic polyamidine was shown in Scheme 3. Firstly, N—Br bond in $BocNBr_2$ **2** was dissociated and adduct I was afforded by Michael addition. I was then transformed into **a**, followed by an elimination to afford intermediate **b** bearing bis-enamine, which could be tautomerized to form bis-imine **c**. Piperazine **3** with bis-secondary amines underwent the nucleophilic addition to bis-imine **c**, giving the intermediate **d**, followed by the elimination to provide the polymer **P1**. Finally, the cationic polyamidine **P5** was obtained by the removal of Boc groups using TFA.

Owning to the universal of such MCP and the successful modification mentioned above, four cationic non-fully substituted polyamidine, **P5-P8**, were subsequently prepared and all the chemical structures were characterized by ^1H NMR in D_2O (Fig. 3A and Figs. S10-S13 in Supporting information). Taking advantages of the water-soluble and cationic properties of the obtained polymers, their potentials in gene condensation were evaluated using gel retardation assay. As shown in Fig. 3B, only **P7** could successfully bind plasmid DNA while **P5, P6** and **P8** could not facilitate gene condensation. That was mainly because that **P7** had the less hindrance around the positive charges of amidine groups compared to **P5** and **P6** using cyclic amine monomers, which would make the interaction and condensation *via* electrostatic effect more favorable. It was interesting that **P7** exhibited excellent DNA binding ability while **P8** with similar structures failed, which was possibly due to the odd-even effect [33]. Nanocomplex formed by **P7** and plasmid DNA at the weight ratio of 20 was further evaluated by DLS, and the result indicated that such complex had the particle size below 200 nm, which was suitable for the further gene deliv-

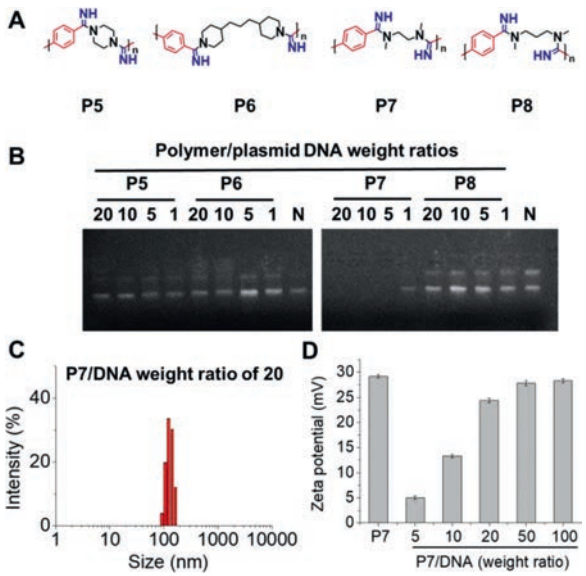


Fig. 3. (A) Structures of cationic polyamidines (**P5-P8**). (B) Gene binding ability of the cationic polyamidines evaluated by gel retardation assay. N represented naked plasmid DNA. (C) DLS results of the **P7**/DNA complexes at the weight ratio of 20. (D) Zeta potentials of **P7** and **P7**/DNA complexes at various weight ratios.

ery application (Fig. 3C). Zeta potentials of **P7** and the complexes formed by **P7** and plasmid DNA were also evaluated by DLS as shown in Fig. 3D. It was clearly to find that all the complexes exhibited a positive surface charge when the weight ratios of **P7**/DNA higher than **5**. The zeta potential was around ~30 mV at the weight ratios higher than 20, indicating the successful condensation for the potential gene delivery application.

In summary, a mild and metal-free MCP method was developed to prepare a library of Boc-substituted polyamidines using bis-secondary-amine, *p*-DNVB and $BocNBr_2$. Upon the removal of Boc using TFA and the formation of non-fully substituted amidine groups, cationic polyamidines could be facilely obtained with water-soluble property. Potential gene binding capability was evaluated to afford the optimized structure P7. Further application of such polymer in gene delivery area and the relationship between the structure and gene delivery capability were undergoing in the lab.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2021.09.031.

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