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Short Communication

Molecular weight determination of a newly synthesized guanidinylated disulfide-containing poly(amido amine) by gel permeation chromatography

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ABSTRACT

A cationic gene delivery vector, guanidinylated disulfide-containing poly(amido amine) (CAR-CBA), was synthesized by Michael addition reaction between N,N′-cystaminebisacrylamide (CBA) and guanidine hydrochloride (CAR). Gel permeation chromatography (GPC) was used to evaluate the molecular weight of synthesized CAR-CBA. Polyethyleneimine (PEI) with molecular weight of 25 kDa was adopted as a reference, and polyethylene glycols (PEG) with different molecular weights were used to establish a standard curve for determining the molecular weight of CAR-CBA. The effects of two critical factors, namely columns and eluents, on the molecular weight measurement of CAR-CBA were investigated to optimize the GPC quantitative method. The results showed that Ultrahydrogel columns (120, 250) and HAc–NaAc (0.5 M, pH 4.5) buffer solution were the optimal column and GPC eluent, respectively. The molecular weight of the synthesized CAR-CBA was analyzed by the optimized GPC method and determined to be 24.66 kDa.

1. Introduction

The application of non-viral gene delivery vectors, especially cationic polymers, in gene delivery field has aroused extensive attention due to their various superiorities over viral vectors, such as non-immunogenicity, no integration of exogenous genes into host chromosomes, and convenience of manufacturing and handling [1]. However, the relatively high cytotoxicity and low transfection efficiency [2] are the main weaknesses of cationic polymers as gene delivery vectors.

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Many cationic polymers have been studied as gene delivery vectors, including HPAA [3], HPAMAM [4], PEG-PEI [5] etc. Recently, a series of biodegradable cationic polymers with disulfide linkages in the backbone have been reported [6–8]. These disulfide bonds are stable in extracellular matrices, and can be rapidly cleaved in the reductive environment of the cytosol through disulfide bond reduction [9], thus resulting in increased release of DNA from the complexes and decreased toxicity . In addition, polymers conjugated with arginine were reported to show substantial improvement of cell-penetrating ability [10]. It was proved that the guanidine groups in arginine functioned most importantly for improving gene transfection efficiency. And simple chemical modification of polymers with guanidine could lead to a significant enhancement in transfection efficiency [11]. Taken together, a novel cationic polymer introduced disulfide linkage and guanidine group is expected to provide great benefits in gene delivery systems.

The transfection efficiency and toxicity of cationic polymers have been reported to be closely related to their molecular weights (Mw) [12]. For example, polyethyleneimine (PEI), a widely used polycation gene vector, exhibited high transfection efficiency and high cytotoxicity with a higher molecular weight, e.g., 25 kDa, while PEI with lower molecular weight, e.g., 800 Da, showed much less transfection efficiency and negligible cytotoxicity [12]. Therefore, the molecular weight determination of cationic polymers is of great importance to their transfection efficiency and toxicity.

Gel permeation chromatography (GPC) is one of the most commonly used methods for determining the molecular weight of polymers, where macromolecules are separated according to their molecular sizes as sample solution flows through a packed bed of porous gels. However, in the case of molecular weight determination of cationic polymers, issues such as aggregation [13] and ion exclusion [14] often appear, leading to less accurate molecular weight results [15]. Thus, it is critical to investigate different factors affecting the molecular weight measurement of cationic polymers, so as to obtain more accurate molecular weight results.

In this study, we synthesized a novel water-soluble, cationic polymer with disulfide linkages and guanidine groups (CAR-CBA), aiming to obtain a gene delivery vector with favorable transfection activity and low toxicity. N,N′-cystaminebisacrylamide (CBA) and guanidine hydrochloride were selected, and Michael addition reaction was utilized in the synthetic processes. A quantitative GPC method was used to determine the molecular weight of CAR-CBA. Effects of different types of columns and eluents on the molecular weight measurement of CAR-CBA were investigated, and then the molecular weight of the synthesized CAR-CBA was determined.

2. Materials and methods

2.1. Materials

N,N′-cystaminebisacrylamide (CBA) was purchased from Alfa Aesar Co., Ltd., Shanghai, China. Branched PEI (bPEI, waterfree) with molecular weight of 25 kDa was purchased from Sigma Aldrich Co., Ltd., St. Louis, USA. Guanidine hydrochloride (CAR) was purchased from Sinopharm Chemical Reagent Co., Ltd., China. Trifluoroacetic acid (TFA), triisopropylsilane (TIS) and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl chloride (Pbf-Cl) were obtained from GL Biochem Ltd., Shanghai, China. The Polyethylene glycol (PEG) standards were purchased from ZZBIO Co., Ltd., Shanghai, China. Acetone and N,N-dimethylformamide (DMF) were purchased from Dikma Technologies Inc., China.

2.2. Synthesis of CAR-CBA

The synthetic route of CAR-CBA is illustrated in Fig. 1 The synthetic reactant selection and process optimization are described in details in another paper [16]. Firstly, an important intermediate CAR-Pbf was synthesized. Briefly, guanidine hydrochloride (1.00 g) was dissolved in water (10 ml) and added to a three necked flask. After the solution was cooled to 0–5 °C, Pbf-Cl (3.02 g, dissolved in 10 ml acetone) was added dropwise into the flask at 0–5 °C. After the addition was completed, the reaction mixture was stirred at room temperature for three hours. During the reaction process, the pH of the system was maintained at 11–12 with NaOH solution (4 M). Finally, white precipitation of CAR-Pbf was obtained after suction filtration. The reaction ratio of CAR and Pbf-Cl was analyzed by a mass spectrometer (micrOTOF_Q, Bruker Co., Ltd., USA).

The reducible CBA-CAR-Pbf-Cl was synthesized by Michael addition polymerization between CBA and Pbf-protected carbamidine (CAR-Pbf). In brief, CBA (0.10 g) and CAR-Pbf (0.24 g) were dissolved in DMF (5 ml) and added to a round-bottom flask. The reaction mixture was stirred in dark under nitrogen atmosphere at 60 °C for 7 days. Subsequently, 10% of excess CAR-Pbf (0.03 g) was added and stirring was continued for 2 days at 60 °C. Next, a mixture of TFA:H_2O:TIS (94:3:3, 10 ml) was added and stirred at room temperature for additional 3 hours. The reaction mixture was then diluted with water, alkalized to pH 7 with NaOH solution, and purified with an ultrafiltration membrane (MWCO 1000). Lastly, the solution was lyophilized to obtain the final product of CAR-CBA. The chemical structure of CAR-CBA was analyzed by proton nuclear magnetic resonance (Ascend 600, Bruker, USA).

2.3. Determination of molecular weight by GPC

The molecular weight and polydispersity index (PDI) of the synthesized CAR-CBA were determined by a GPC method using a Waters 1515 HPLC system (Waters Co., Ltd., USA) equipped with a Waters 2414 refractive index detector (RID). Three kinds of columns, namely Styragel columns (Waters Co., Ltd., USA), CLM 1031 column (Malvern Instruments Co., Ltd., UK), and Ultrahydrogel columns (Waters Co., Ltd., USA) were tested. The synthesized CAR-CBA and PEG were dissolved in three kinds of eluents, i.e. water, DMF and HAc–NaAc buffer solution, which correspond to the columns used. The prepared CAR-CBA and PEG solutions (2 mg/ml) were filtered with 0.22 μm syringe filters (Dikma Technologies Inc., China). The eluents were degassed prior to each analysis. The columns and detector were thermostated at 35 °C. The samples were analyzed at a flow rate of 0.7 ml/min and the injection volume was set at 20 μl. Both data collection and analysis were carried out using a Breeze 2 (Waters Co., Ltd., USA) software.

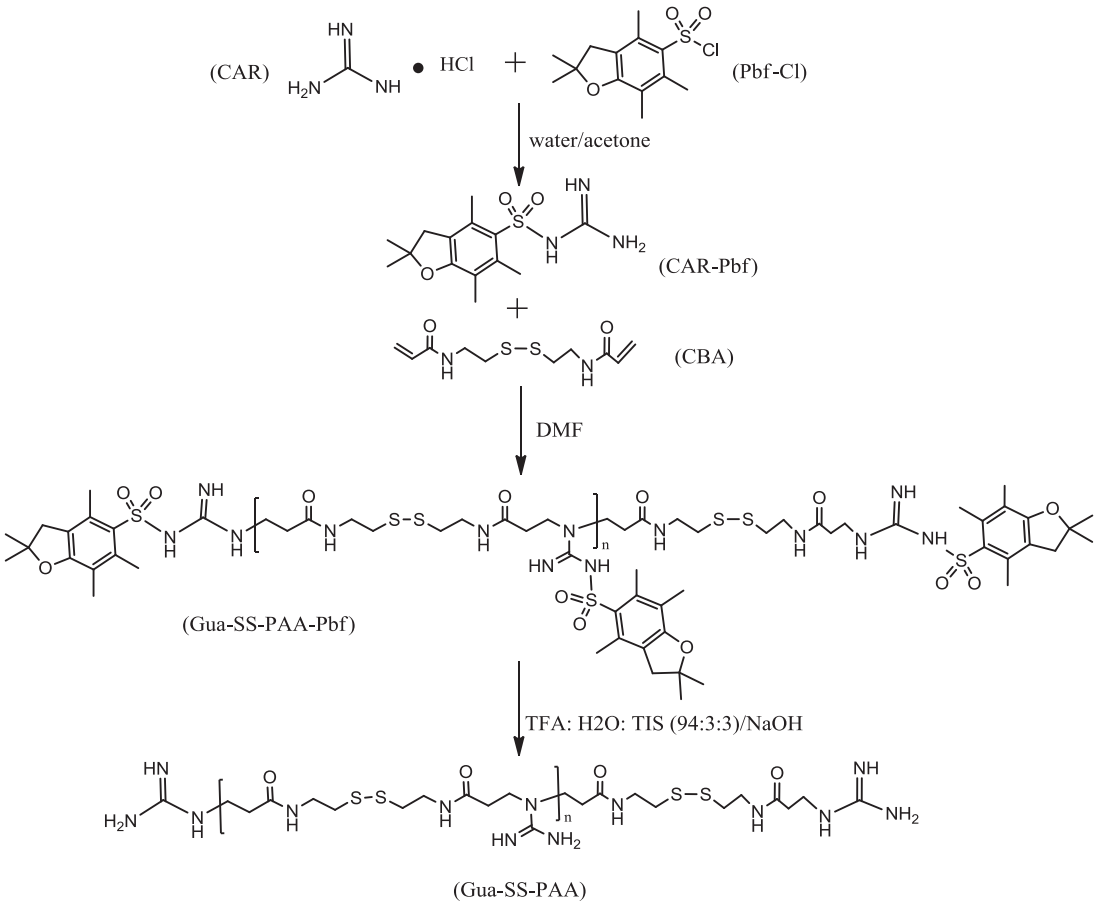

Fig. 1 – Synthesis route of CAR-CBA.

3. Results and discussion

3.1. *Synthesis of CAR-CBA*

A non-viral gene vector, CAR-CBA was successfully synthesized in this study. Due to the low reactivity of guanidine groups, Pbf-Cl was introduced to activate guanidine groups prior to the reaction of Michael addition polymerization.

During the course of the Michael addition reaction, an increase of viscosity was observed in the vessel. The termination of the reaction was conducted by adding 10% of excess CAR-Pbf to the solution to consume any unreacted acrylamide groups. Excessive amount of mixture of TFA:H_2O:TIS (94:3:3) was added to deprotect the guanidine groups. Thereafter, the synthesized polymer was in the form of TFA salt, thus NaOH was added to consume TFA. The polymer was then dissociative. The reaction mixture was purified by dialysis for 3 d. During this period of time, precipitation, which might be a mixture of removed Pbf from the chain of the CAR-CBA and unreacted reactants, appeared in the dialysis bag. Since CAR-CBA has good water solubility due to mass amino groups remaining in the main chain, the supernatant from the dialysis was kept and the precipitation was discarded. After 36 h of freeze-drying, a white, viscous powder of CAR-CBA was obtained.

As illustrated in Fig. 2, the mass spectrum of the synthesized CAR-Pbf showed a molecular ion peak at m/z = 312.14, which was close to the theoretical molecular weight of CAR-Pbf (312.33 g/mol). This indicated that guanidine hydrochloride was successfully connected with Pbf at a molar ratio of 1:1.

Fig. 3 shows the ^{1}H NMR spectrum of the final product. It is marked with a red box to illustrate that the characteristic olefin peak of CBA has disappeared, which suggested the disappearance of CBA in the synthetic product and the successful polymerization of CBA with CAR.

3.2. *Optimization of GPC quantitative method*

Even though GPC is a convenient and efficient tool to evaluate the molecular weight of macromolecular compounds, it is a difficult task to apply GPC in determining the molecular weight of cationic polymers. The reason lies in the adsorption of cationic polymers onto the surface of GPC stationary phase, which limits the reproducibility and separation efficiency [17]. These interactions can generally be suppressed with high salt eluents and co-solvents [18,19]. In order to obtain a more accurate measurement result, two critical factors, namely the types of columns and eluents, were investigated to optimize the GPC quantitative method.

3.2.1. *Selection of column*

As PEI-25 kDa and CAR-CBA are soluble in both water and DMF, several types of columns using aqueous solution or DMF as

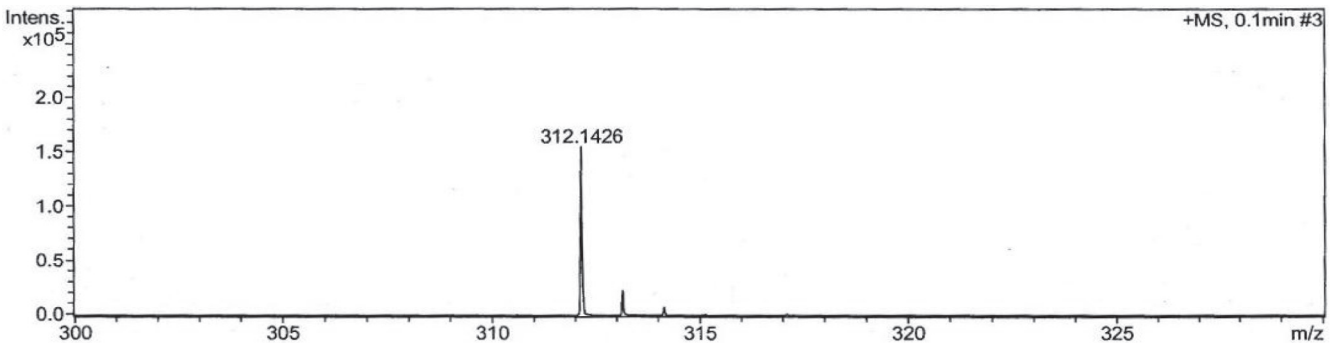

Fig. 2 – Mass spectrum results of CAR-Pbf.

Table 1 – t_R of PEI-25 kDa and PEG in Styragel columns (HT 3, HT 4, HT 5), CLM 1031 column and Ultrahydrogel columns (120, 250).

Samples	t_R (min)		
	Styragel columns (HT 3, HT 4, HT 5)	CLM 1031 column	Ultrahydrogel columns (120, 250)
PEG 1500	26.71	12.06	21.21
PEG 1600	26.41	11.89	20.99
PEG 4000	24.31	11.92	19.29
PEG 5000	23.90	11.83	18.91
PEG 6000	23.30	11.86	18.57
PEG 7290	22.33	11.79	18.09
PEG 21600	19.83	11.79	17.02
PEI-25 KDa	34.67	11.47	17.66

eluents were examined in the study. Styragel columns (HT 3, HT 4, HT 5) covering a molecular weight range of 0.5–4000 kDa, CLM 1031 column with an exclusion limit of more than 20 kDa, and Ultrahydrogel columns (120, 250) with a Mw range of 0.1–80 kDa were investigated to optimize the column for measuring the molecular weight of the synthesized CAR-CBA. The eluent for Styragel columns was DMF, while the eluent for both CLM 1031 column and Ultrahydrogel columns (120, 250) was HAc–NaAc buffer solution (0.3 M, pH 4.5). PEI-25 kDa was chosen as a reference. A series of PEGs with different molecular weights (PEG 1500, PEG 1600, PEG 4000, PEG 5000, PEG 6000, PEG 7290, PEG 21600) were applied to establish the standard curve. The retention time (t_R) of PEI-25 kDa and PEGs loaded in different GPC columns was shown in Table 1.

When Styragel columns (HT 3, HT 4, HT 5) were used as testing columns and DMF as an eluent, quantification of molecular weight was found to be difficult because of a prolonged retention time (e.g., 34.67 min for PEI-25 kDa). Moreover, the peak of PEI-25 kDa was even eluted after the solvent peak (DMF, 30.70 min), which made it impossible to determine the molecular weight of PEI-25 kDa by using the standard curve built up by PEGs. This might be attributed to the interactions between PEI-25 kDa and the packing materials of the column. Therefore, Styragel columns (HT 3, HT 4, HT 5) were deemed unsuitable in this study.

CLM 1031 column was designed specially to measure cationic polymers by aminating the surface of the packing material. However, in this testing condition (RID as the only detector), CLM1031 column showed low resolution value and failed to distinguish the retention time of PEGs as shown in Table 1. It seemed that all the retention times were consistently around 11.8 min, even when the molecular weights of PEG were changed (Table 1). Although different eluents, i.e. water, 5% HAc and 0.1 M $NaNO_3$ were tested, the retention time of PEG standards remained unchanged, which made it impossible to establish the standard curve. Consequently, CLM 1031 column was deemed unsuitable in this study.

When Ultrahydrogel columns (120, 250) coupled with an acidic eluent (0.3 M HAc–NaAc, pH 4.5) were utilized, the PEGs were well separated. Furthermore, PEI-25 kDa was also successfully analyzed and its molecular weight was calculated as 16.67 kDa using the standard curve built up by PEG standards. Therefore, the Ultrahydrogel columns (120, 250) were selected as the optimized column and applied in the molecular weight measurement of CAR-CBA.

3.2.2. *Selection of eluent*

As it was reported that high salt eluents and co-solvents [18,19] could reduce the interactions between polymers and the packing materials of the column, different concentrations of acidic ionic aqueous solutions, namely HAc–NaAc (0.25 M, pH 4.5) and HAc–NaAc (0.5 M, pH 4.5), were tested separately. Water was used as a blank eluent control. PEI-25 kDa was used as the molecular weight reference, and PEG molecular weight standards (PEG 1500, PEG 1600, PEG 4000, PEG 5000, PEG 6000, PEG 7290, PEG 21600) were used to establish the standard curve. The data were presented in Table 2, and the elution curves were shown in Fig. 4.

No peak was observed in the GPC elution curve of PEI-25 kDa when water was used as an eluent and RID as the single detector. (The marked peak in (A) is too small that we consider it as the noise of the detector). Thus, it was unsuitable

Table 2 – t_R, Mw and PDI of PEI-25 kDa and CAR-CBA with water, HAc–NaAc (0.25 M, pH 4.5) and HAc–NaAc (0.5 M, pH 4.5) as eluents.

Eluents	PEI-25 kDa			CAR-CBA		
	t_R (min)	Mw (kDa)	PDI	t_R (min)	Mw (kDa)	PDI
Water	–	No peak	–	–	–	–
HAc–NaAc (0.25 M, pH 4.5)	17.75	11.73	1.55	17.67	11.26	1.29
HAc–NaAc (0.5 M, pH 4.5)	17.29	23.61	1.96	17.24	24.66	1.83

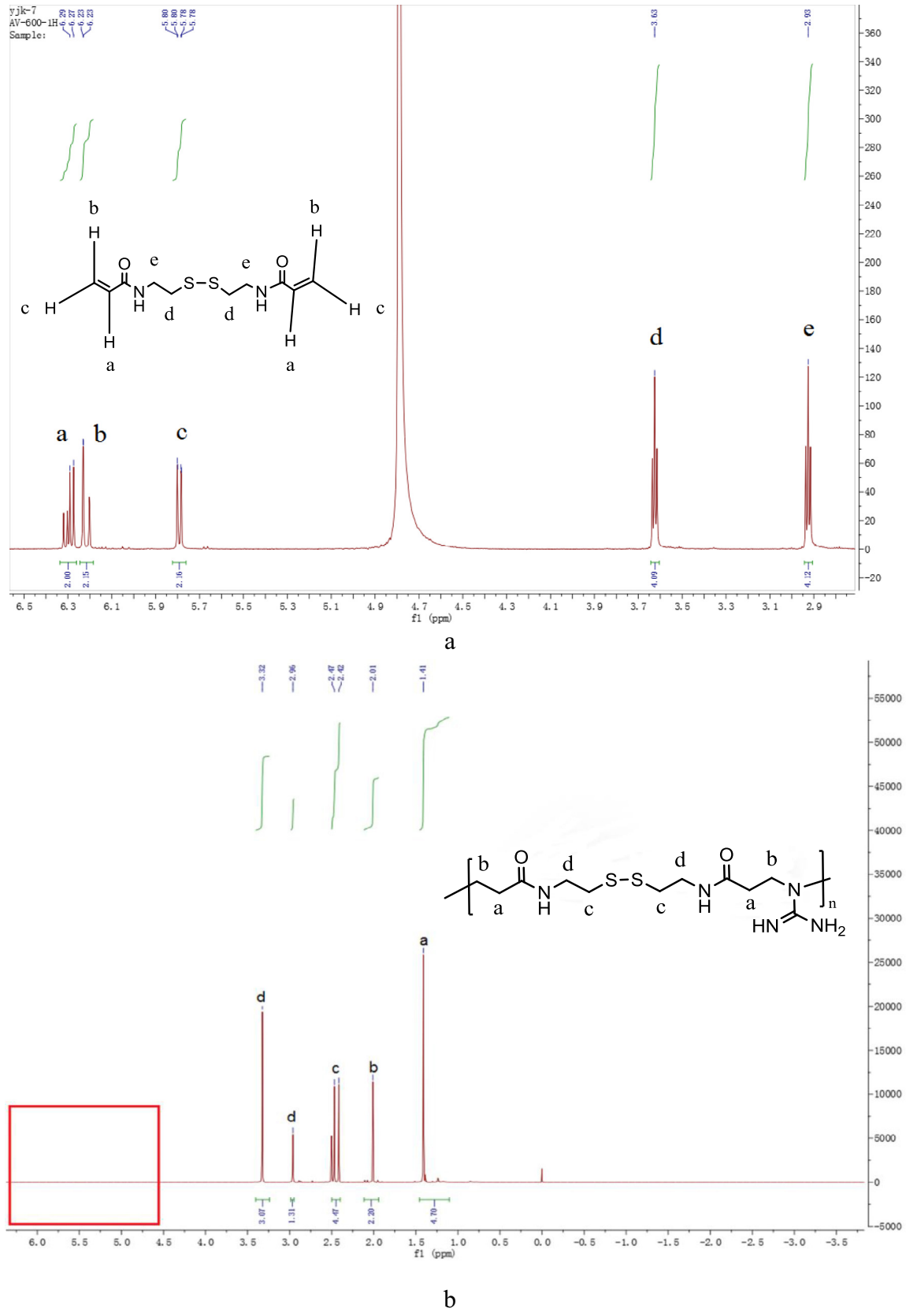

Fig. 3 – ^{1}H-NMR results of: (a) CBA, (b) CAR-CBA polymer.

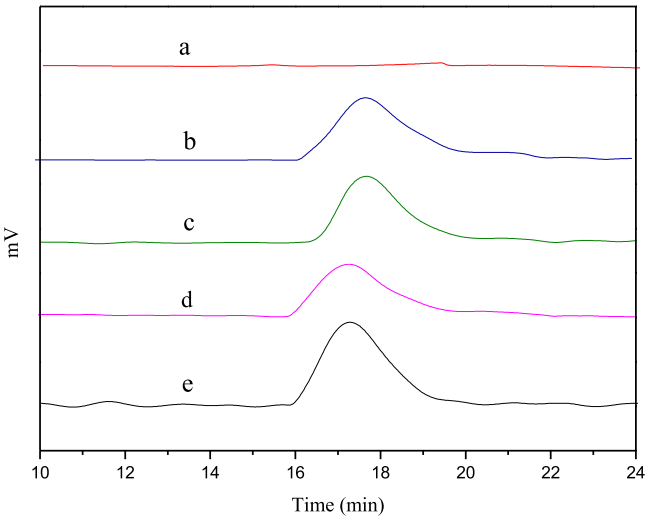

Fig. 4 – Elution curves of (a) PEI-25 kDa with water as eluent, (b) PEI-25 kDa with 0.25 M HAc–NaAc as eluent, (c) CAR-CBA with 0.25 M HAc–NaAc as eluent, (d) PEI-25 kDa with 0.5 M HAc–NaAc as eluent, (e) CAR-CBA with 0.5 M HAc–NaAc as eluent.

to use water as eluent in the molecular weight determination of PEI-25 kDa as well as the prepared CAR-CBA. When HAc–NaAc (0.25 M, pH 4.5) buffer solution was used as an eluent, the molecular weight of PEI-25 kDa was determined to be 11.73 kDa. However, when HAc–NaAc (0.5 M, pH 4.5) buffer solution was used as the eluent, the molecular weight of PEI-25 kDa was determined to be 23.61 kDa, which was much closer to the theoretical value (25 kDa). The results suggested that the HAc–NaAc (0.5 M, pH 4.5) buffer solution could better reduce the interactions between the cationic polymer and the packing materials of the column.

As displayed in Table 2 and Fig. 2, the synthesized CAR-CBA showed similar elution and retention profile as PEI-25 kDa did. Therefore, testing condition that was suitable for PEI-25 kDa might be applicable to the synthesized CAR-CBA. In this study, when lower concentration of HAc–NaAc buffer solution was used as an eluent, it led to an underestimated molecular weight of CAR-CBA (i.e., 11.26 kDa). However, when higher concentration of HAc–NaAc buffer solution was used as the eluent, it resulted in a higher and more accurate molecular weight determination of CAR-CBA (i.e., 24.66 kDa). Additionally, the relatively low PDI (1.83) showed that the synthesized CAR-CBA had a narrow molecular weight distribution. On the other hand, the relatively high molecular weight indicates that CAR-CBA has the potential to possess high transfection activity as a gene delivery vector. (Data were shown in another paper [16]).

4. Conclusions

A novel guanidinylated poly(amido amine) with disulfide linkages in the backbone was successfully synthesized. The result from mass spectrometry indicated the successful connection of CBA with guanidine hydrochloride, and the formation of CAR-CBA was further confirmed by ^{1}H NMR spectrometry. GPC was used to determine the molecular weight of CAR-CBA. The GPC quantitative method was optimized by studying the effects of two critical factors, i.e., columns and eluents on the molecular weight measurement of PEI-25 kDa. It was shown that Ultrahydrogel columns (120, 250) were the optimal GPC column and HAc–NaAc (0.5 M, pH 4.5) buffer solution was the optimal eluent for analyzing the molecular weight of CAR-CBA. The molecular weight of synthesized CAR-CBA was analyzed by the optimized GPC method and determined to be 24.66 kDa.

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