Indian Journal of Chemistry
Vol. 56A, July 2017, pp. 774-779

Notes

Geminally biotinylated cyclotriphosphazenes as molecular binding probe

Xuebin Ma[a], Tingting Xu[b], Wei Chen[b], Rui Wang[b], Zheng Xu[b], Bo Chi[b,*], Zhiwen Ye[a,*]

[a]Shoool of Chemical Engineering, Nanjing University of Science and Technology, Nanjing, China

[b]State Key Laboratory of Materials-Oriented Chemical Engineering, Nanjing Tech University, Nanjing, China

Email: yezw@njust.edu.cn (YE)/ chibo@njtech.edu.cn (BC)

Received 19 July 2017; revised and accepted 30 June 2017

Biotinylated cyclotriphosphazenes polymer (g-BTP) with germinal octopus-like molecular shape has been prepared and characterized by ^{31}P-NMR, ^{1}H-NMR and Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (Maldi-Tof-Ms). Analysis of the size distribution of the complexes showed that the aggregated g-BTP/avidin complexes were rather polydispersed and uniform as compared to complexes formed by random coil biotinylated linear polymer (BLP) and avidin. Binding kinetics studies show that both of g-BTP and BLP can bind avidin very quickly. The binding ability as evaluated by Scatchard plot indicates the binding ability of g-BTP to be about 5.5 times that of BLP.

Macrocycles have been widely investigated since the three pioneers of the field, Jean-Marie Lehn, Donald Cram and Charles Pedersen, won the 1987 Nobel Prize in chemistry for synthesizing molecules and compounds with cavities and cages within which metal ions and other molecules could be bound. Among these, cyclic systems based on cyclodextrin and calixarenes are mostly studied to build platforms for the design of probes for chemical sensing. The properties of cyclodextrins, comprising varying numbers of D-glucopyranoside units linked by 1, 4-glycosidic bonds, can be altered or modified by derivatizing them[1-4]. Modified β-cyclodextrins appended with seven naphthoate chromophores are reported to be much more efficient than the native β-cyclodextrin in binding DCM-OH, an elongated merocyanine[2]. The other cyclic system, calix[n]arene, is a class of vase-like molecules that can host neutral molecules and metal ions. The selectivity to the guest species is tunable by varying the shape and size of the cavity or by modifying the substituent on the upper and lower rims[5]. These small cyclic structural molecules with proper orientation of substituent can exhibit different properties and have been used in molecular recognition and sensing widely[6-8]. Most of the derivatives of cyclodxtrins and calix[n]arenes have the same characteristic, i. e., one type of function moiety conjugated on one side of the macrocycle while another function substituent is on the opposite side of the macrocycle. Many can self-assemble in aqueous solution and thus exhibit recognition properties different to that of the original host molecule.

A unique molecular structure of octopus-like shape, thermosensitive cyclotriphosphazenes has been first synthesized by substitution of hexachlorocyclotriphosphazene, $(NPCl_2)_3$, with alkoxy poly(ethylene glycol) (APEG) and amino acid esters (AAE)[9]. The three hydrophilic APEG groups were oriented in one direction opposite to the other three hydrophobic AAE groups with respect to the trimer ring plane. These trimeric derivatives exhibited remarkably different lower critical solution temperature (LCST) than the conventional thermosensitive polymers[9-11]. A cyclotriphosphazene bearing six naphthoyloxy chromophores has also been designed for mimicking the natural photosynthetic antennae[12]. In addition, from a molecular design point of view, one of the most useful benefits of cyclophosphazenes is their great versatility in substitution without changing their rigid backbone length. Cyclophosphazenes with appropriate appended groups including fluorophores and biotin will be a promising class of sensors due to their great potential in terms of molecular design.

The avidin-biotin systems, based on the high affinity between avidin and biotin or biotinylated molecules, have proved to be attractive models for detection, characterization, and isolation of various biological materials[13]. In particular, Hoffman, Stayton, and coworkers[14–19] have extensively studied "smart" polymer bioconjugates based on the biotin-(strep) avidin system and their potential applications.

Herein, a new type of cyclotriphosphazene has been prepared by stepwise substitutions of $(NPCl_2)_3$ with hydrophilic methoxy-poly(ethylene glycol) (MPEG350) on one side of the ring and biotin moieties at the other side of the ring. The properties of the complexes, including the binding ability of polymers to avidin, are investigated. The biotinylated

linear polymer has also been synthesized as a control to compare the binding ability of the polymers to avidin.

Experimental

2-Aminoethyl methacrylamide hydrochloride (AEMA), poly(ethylene glycol) methacrylate (PEGMA360), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and HABA/avidin reagent were purchased from Sigma-Aldrich. Hexachlorophosphazene trimer, avidin and D-biotin were purchased from Aladdin Industrial Corporation (Shanghai, China), while methoxy-poly(ethylene glycol) (MPEG350), sodium metal, toluene, tetrahydrofuran (THF), dimethyl formamide (DMF), trifluoroacetic acid (TFA), dichloromethane (DCM), N-α-Boc-lysine ethyl ester, chloroform and triethylamine were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China)

All the reactions were performed under inert atmosphere. A typical synthetic procedure for synthesis of g-TP is as follows: Sodium salt of methoxy-poly(ethylene glycol) (MPEG350) was prepared by reaction of MPEG350 (3.5 g, 10.0 mmol) with 1.5 equivalent of sodium metal in toluene (150 mL) at refluxing temperature for one day. After the resultant solution was filtered to remove excess sodium metal, the filtrate was dropped slowly in to the hexachlorophosphazene trimer (1.150 g, 3.33 mmol) dissolved in THF (10 mL). The reaction mixture was stirred for 4 h at -65 °C. Meanwhile, N-α-Boc-Lysine ethyl ester (3.06 g, 11.2 mmol) was dissolved in dry chloroform (100 mL) containing 6 equivalent of dry triethylamine. The N-α-Boc-lysine ethyl ester solution was added to the reaction mixture, which was stirred for 2 days at 50 °C. The reaction mixture was filtered to remove triethylammonium chloride precipitated. After the filtrate was evaporated, the resulting solid like mixture was dissolved in MeOH, 200 mL and then the solution was transferred to ultrafilration equipment (Millepore®, Solvent resistant stirred cell). In order to remove unreacted small molecules and inorganic salt, the solution was concentrated to 20 mL, which was repeated 5 times in the same solvent system. The dialyzed and desalting solution was evaporated to obtain the resulting phosphazene trimer. The protection group of amine, Boc, was removed with TFA/DCM (20%) for 10 hours and characterized by ^{1}H-NMR.

The g-BTP bearing biotin group was fabricated via a two-step synthesis. The D-biotin was first activated by the NHS to form biotin-NHS and then reacted with amine group of g-TP to obtain g-BTP as shown in Fig. 1. In order to minimize side reactions in the fabrication process, the DMF solution of Biotin-NHS was added dropwise into the flask containing g-TP to avoid any possible side reactions. The product was characterized by ^{1}H NMR, ^{31}P NMR and mass spectrometry to confirm the formation of the desired product. Briefly, D-Biotin (0.244 g, 1 mmol) in 5 mL DMF was added to 5 mL DMF solution of EDC (0.273 g, 1.4 mmol) and NHS (0.167 g, 1.4 mmol). The reaction mixture was stirred for 4 hours at 4 °C. Then the mixture was added dropwise into the flask containing g-TP (0.2001 g, 0.36 mmol) in 20 mL distilled water. After stirring the reaction mixture overnight at room temperature, the mixture was purified by dialysis against distilled water for 2 days using the dialysis membrane molecular weight cut-off (MWCO) of 1000 D. The desired g-BTP (0.1731 g) was freeze-dried under vacuum for 2 days.

BLP was prepared as follows: In a 10 mL flask, AEMA (1.66 g, 10 mmol) and PEGMA 360 (3.65 g, 10 mmol) were dissolved in distilled water (20 mL) at

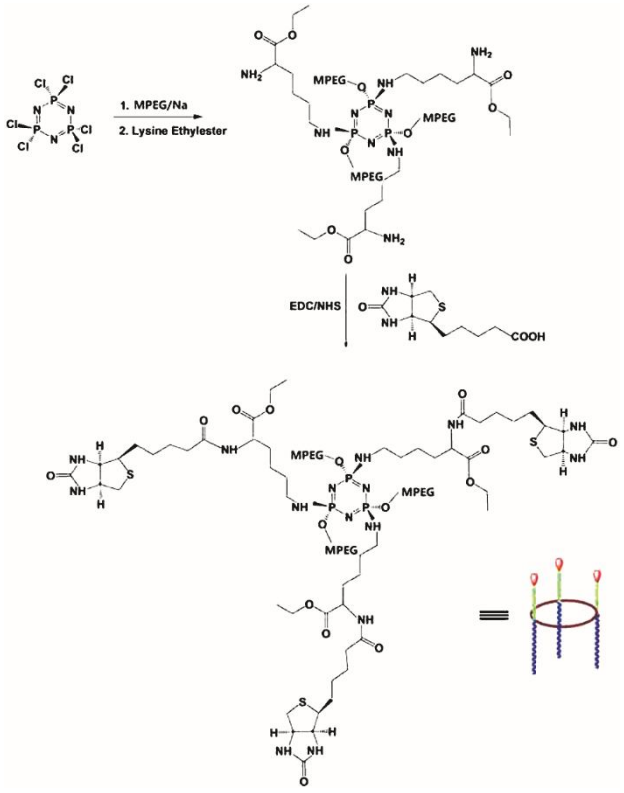

Fig. 1 — Synthetic scheme of geminally biotinylated cyclotriphosphazenes.

4 °C and then purged by N_2 bubbling for 30 min. APS (0.071 g, 0.28 mmol) and TEMED (0.030 g, 0.28 mmol) dissolved into 1 mL distilled water were added into the above solution. The reaction was kept at 4 °C for 24 h. The final solution was put into a dialysis tube (MWCO 3000D) against water for 2 days. The obtained product linear polymer (LP) was collected (4.52 g) by was freeze-dried under vacuum for 2 days.

D-biotin (0.248 g, 1mmol) in 5 mL DMF was added to 5 mL DMF solution of EDC (0.270 g, 1.4 mmol) and NHS (0.169 g, 1.4 mmol). The reaction mixture was stirred for 4 hours at 4 °C. Then the mixture was added dropwise into the flask containing LP (0.2331 g, 0.011mmol) in 20 mL distilled water. After stirring the reaction mixture overnight at room temperature, the mixture was purified by dialysis against distilled water for 2 days using the dialysis membrane (MWCO 1000D). The desired BLP (0.1453 g) was freeze-dried under vacuum for 2 days.

To study the complex size distribution as a function of ratio of biotin to avidin, dynamic light scattering studies (DLS) were performed using a Nano 2000 light scattering instrument. Avidin concentration was fixed at 1.47×10^{-6} M. g-BTP and BLP polymer in a range of 0.01~ 1 mg was dissolved in 1 mL distilled water respectively. The complex was formed between avidin and biotin in different molar ratios. Measurements were taken after avidin and biotin were mixed for 10 min at 25 °C.

To determine the binding ability of the polymers, the avidin/HABA assay was carried out by monitoring the absorbance values at 500 nm using a Scinco S-3100 UV-vis spectrophotometer for the HABA/avidin reagent as well as the solution after addition of biotinylated polymers. HABA is a dye that can bind to avidin and has maximum absorbance at 500 nm); the avidin-complex is the only species that absorbs at 500 nm. Uncomplexed HABA has negligible absorption at 500 nm, and its absorbance was removed by using the same concentration of HABA in PBS buffer as the reference during absorbance measurements. By adding biotin or biotinylated reagents to the HABA/avidin solution, HABA was displaced quantitatively by available biotin. This displacement was quantitatively monitored by the decrease in UV absorbance at 500 nm. A calibration curve was generated by measuring the change in UV absorbance upon the gradual addition of free biotin of known concentration sequentially into the HABA/avidin reagent. The equivalent amount of free biotin available on the biotinylated polymers was calculated from the calibration curve. Briefly, the avidin concentration increased in a range from 0.2 to 2.0 n*M*. After complexes formed by g-BTP/avidin and BLP/avidin were incubated for 10 min, 0.1 mL was taken out and the free biotin was obtained by avidin/HABA assay. Thus the dissociation constant (K_D) was obtained.

In order to investigate the binding kinetics between biotinylated polymers and avidin, the avidin concentration was fixed to 1.0 n*M* and the complex was formed between avidin and biotin with fixed molar ratio 1:4. The biotin bound to avidin was obtained by avidin/HABA assay as a function of time.

Results and discussion

The synthesis of geminally biotinylated cyclotriphosphazenes was carried out through the two following steps: (i) synthesis of geminally cyclotriphosphazenes bearing MPEG moieties; (ii) coupling of the biotin derivatives to the functionalized cyclotriphosphazenes. The synthesis of g-TP was according to the method reported[9, 10]. The biotinylated g-BTP was obtained by a condensation reaction between the carboxylic end groups of D-biotin and amino end groups of g-TP by EDC/NHS coupling agent. The detailed synthesis procedure is illustrated in Fig. 1. The structure was characterized by ^{31}P-NMR (Fig. 2a) and ^{1}H-NMR (Supplementary data, Fig. S1). The molecular weights were determined by Maldi-Tof-Ms as 1540 for g-TP and Mw 2285 for g-BTP (Fig. 2b). The synthesis scheme of BLP is presented in Fig. S2 (Supplementary data). The polymerization and coupling were confirmed by ^{1}H NMR spectrum and the molecular weight was determined by gel permeation chromatography with Mw 20000 for BLP (PEG as standard and DMF as mobile phase) (Fig. S2).

When g-BTP was dissolved in distilled water, the biotin moiety was exposed on one side of the rigid ring of cyclotriphosphazenes, with the hydrophilic MPEG chains stretching at the another side of the ring. Avidin possesses four subunits and can potentially bind biotinylated g-BTP with very high affinity. It is also one of the most widely used proteins in affinity separations, bioassays, and clinical diagnostics because of its high affinity to biotin. To investigate the interaction between g-BTP and avidin, the size of the g-BTP-avidin complex of varying

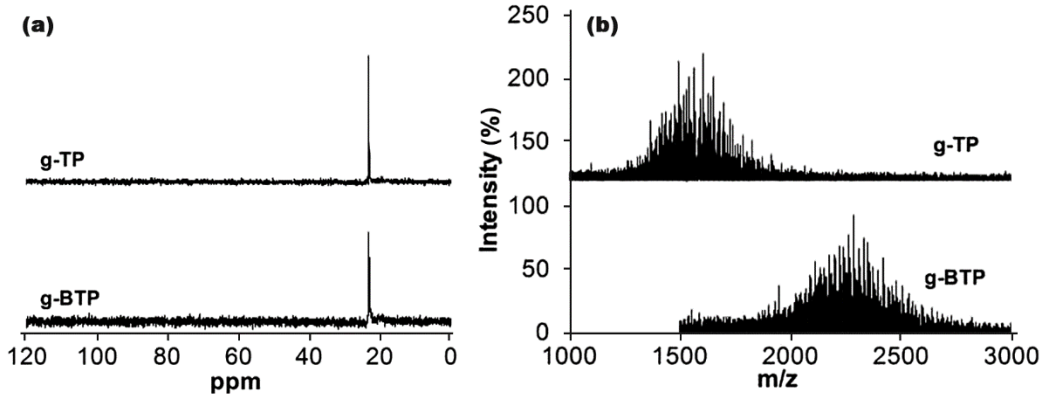

Fig. 2 — (a) ^{31}P-NMR [Solvent: DMSO-d_6] and (b) Maldi mass spectra of g-TP and g-BTP.

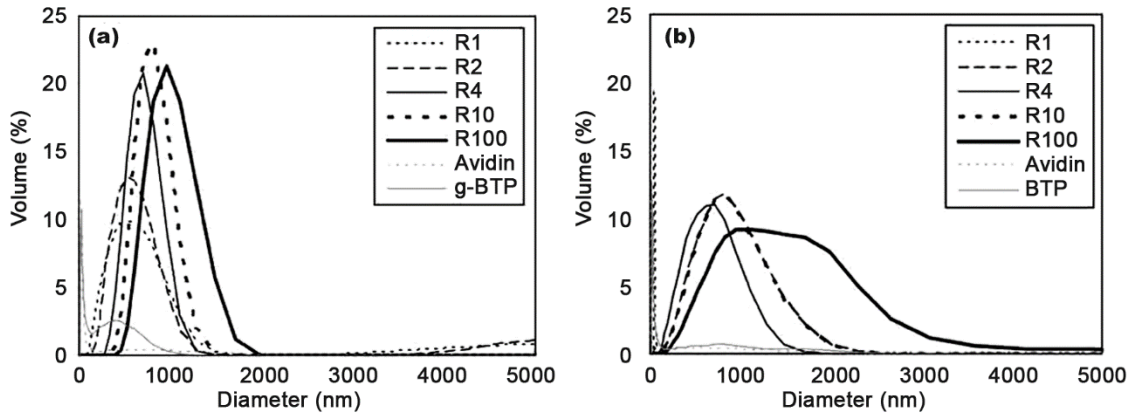

Fig. 3 — Complex size distribution of (a) g-BTP-avidin, and, (b) BLP-avidin as a control. [R1, R2, R4, R10 and R100 represent biotin (of g-BTP):avidin molar ratios of 1, 2, 4, 10, 100 respectively].

molar ratios of biotin (of g-BTP): avidin (1, 2, 4, 10, 100) was studied at 25 °C. In theory, the complex size will increase as avidin binds biotinylated g-BTP. The addition of avidin to g-BTP aqueous solution resulted in the formation of aggregates as revealed by DLS measurements (Fig. 3a). The average diameter of complexes increased from 458 nm to 955 nm and with the increase in the molar ratio the distribution was rather polydisperse and uniform, which suggests that there is high affinity between biotin of g-BTP and avidin. However, the complexes formed by BLP and avidin in aqueous solution tended to form larger aggregates (Fig. 3b). The average diameter of BLP/avidin complex increased from 531 to 1500 nm with increasing molar ratio, showing a broad distribution. These results indicate that g-BTP/avidin can form tighter structures than BLP/avidin complex.

The binding of g-BTP to avidin was investigated after mixing time of 20 minutes at 25 °C (Fig. 4). For comparison, BLP-avidin binding was investigated as a control experiment. After 8 min incubation, the g-BTP binding to avidin was almost constant. As

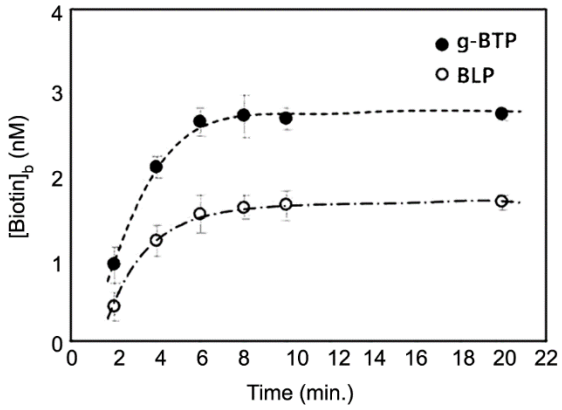

Fig. 4 — Binding kinetics of g-BTP and avidin. [BLP as a control experiment. Biotin:avidin is 4].

shown in Fig. 4, the maximum biotin bound to g-BTP was much higher than that of BLP bound to avidin, which indicates that g-BTP exhibits a higher affinity to avidin than BLP.

To compare the binding ability of g-BTP to avidin, the binding of g-BTP to avidin was investigated in an avidin concentration range from 0.2–2.0 n*M* at 25 °C

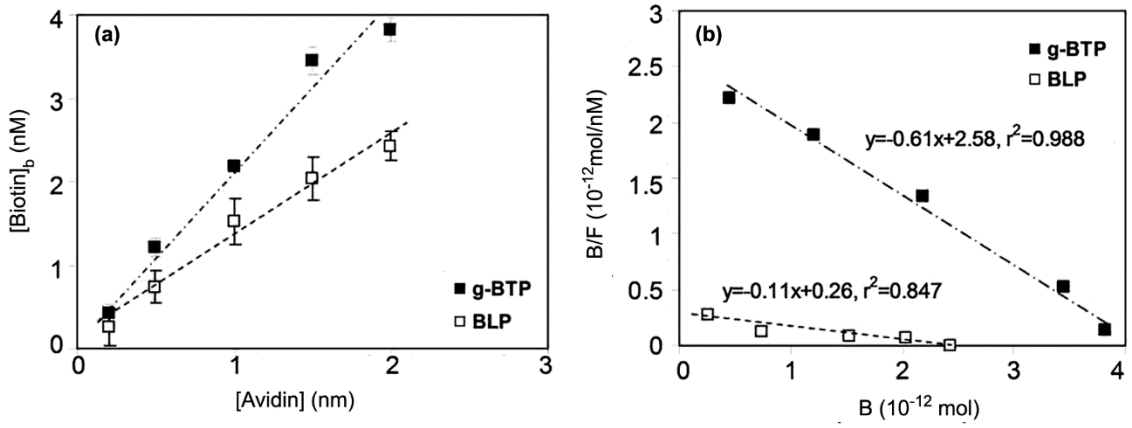

Fig. 5 – Binding isotherms of g-BTP-avidin. [(a) Binding isotherm; (b) Scatchard plot. BLP as control].

(Fig. 5a). Binding with BLP was compared as a control experiment. The binding curves of g-BTP to avidin were analyzed by Scatchard plot [23, 24] (Fig. 5b).

$$B/F = B_{max}/K_D - B/K_D$$

where B is the amount of biotin bound to avidin and F is the concentration of free biotin. From the slope of the Scatchard plot, the dissociation constant (K_D) was obtained. Results show that K_D is 1.64 nM for g-BLP and 9.09 nM for BLP at 25 °C, indicating g-BTP possesses a higher binding affinity to avidin than BLP. This may be due to the germinal structure which plays a key role in the high affinity to avidin. In the structure of g-BTP, biotin moiety is conjugated on one side of cyclotriphosphazene, while the hydrophilic MPEG chains extended on the opposite side of the macrocycle. The biotin moieties are exposed in aqueous solution and can bind avidin to form complexes. However, in the random coil biotinylated linear polymer BLP, the BLP chain may crosslink and some of the biotin moieties may not be exposed, resulting in the relatively low affinity to avidin.

In this study, a new type of biotinylated cyclotriphosphazenes with germinal octopus-like molecular shape was prepared by stepwise substitution of cyclotriphosphazenes with hydrophilic MPEG and biotin moieties. Germinal biotinylated cyclotriphosphazenes polymer g-BTP showed higher binding affinity to avidin and was polydispersed as compared to the random coil biotinylated linear polymer BLP. Cyclophosphazenes with appropriate appended groups including fluorophores and biotin may be a promising class of sensors due of their high potential in terms of molecular design.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (31401588 and51403103), the National Basic Research Program of China (973 Program) (2013CB733603), and the State Key Laboratory of Materials-Oriented Chemical Engineering (ZK201606 and ZK201403).

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