Carbohydrate Polymers 131 (2015) 80–89

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Quaternized chitosan particles as ion exchange supports for label-free DNA detection using PNA probe and MALDI-TOF mass spectrometry

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ARTICLE INFO

Article history:
Received 9 January 2015
Received in revised form 20 May 2015
Accepted 20 May 2015
Available online 29 May 2015

Keywords:
Chitosan
Pyrrolidinyl peptide nucleic acid
Quaternary ammonium group
MALDI-TOF mass spectrometry
DNA sequence analysis

ABSTRACT

Quaternized chitosan particles are introduced as anion-exchanged captures to be used with a conformationally constrained pyrrolidinyl peptide nucleic acid (*acpc*PNA) and MALDI-TOF mass spectrometry for DNA sequence analysis. Methylated chitosan (MC) and methylated *N*-benzyl chitosan (MBzC) particles were obtained by heterogeneous chemical modification of ionically cross-linked chitosan particles via direct methylation and reductive benzylation/methylation, respectively. *N,N,N*-trimethylchitosan (TMC) and *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC) particles were prepared by ionic cross-linking of quaternized chitosan derivatives, homogeneously modified from chitosan, namely TMC and HTACC, respectively. The particles formed had a size in a sub-micrometer range and possessed positive charge. Investigation by MALDI-TOF mass spectrometry suggested that some quaternized particles in combination with *acpc*PNA were capable of detecting a single mismatched base out of 9–14 base DNA sequences. Potential application of this technique for the detection of wild-type and mutant K-*ras* DNA, a gene that mutation is associated with certain cancers, has also been demonstrated.

1. Introduction

Chitosan is a natural polymer derived by partial deacetylation of chitin – a substance abundantly found in the exoskeletons of insects, the shells of crustaceans, and fungal cell walls. Due to the presence of ammonium groups in the structure, chitosan can form a stable complex with DNA in the solution having a pH range of 2–7 (Hayatsu, Kubo, Tanaka, & Negishi, 1997) and extract DNA with very high extraction efficiency (63–92%) even in complex DNA samples such as human genomic DNA from whole blood samples (Cao, Easley, Ferrance, & Landers, 2006). The hydrophilic sugar-based structure of chitosan ensures low non-specific binding–mainly through hydrophobic interactions – with other non-DNA components such as proteins and other interfering samples. These, together with the fact that the ammonium groups of chitosan can be converted into various quaternary ammonium entities prompts us to develop into an effective capture for negatively charged DNA that may be applicable for DNA sequence analysis.

Peptide nucleic acid (PNA), a neutral-peptide backbone DNA mimic firstly developed by Nielsen and co-workers (Egholm, Buchardt, Nielsen, & Berg, 1992; Nielsen, Egholm, Berg, & Buchardt, 1991), has a great potential to be used as a probe for DNA biosensor. The hybrid formed between PNA and its complementary DNA is more stable than the corresponding DNA·DNA hybrid due to the absence of electrostatic repulsion between negative charges along the phosphate backbone existing in the DNA structure (Sassolas, Leca-Bouvier, & Blum, 2007). Recently, a conformationally restricted pyrrolidinyl PNA based on D-prolyl-2-aminocyclopentane-carboxylic acid backbones (*acpc*PNA) was introduced by Vilaivan and co-workers. This PNA system possessed higher binding affinity and sequence specificity toward DNA than that of Nielsen's PNA, and with a much stronger preference for the antiparallel binding mode (Ananthanawat, Vilaivan, Hoven, & Su, 2010; Suparpprom, Srisuwannaket, Sangvanich, & Vilaivan, 2005; Vilaivan & Srisuwannaket, 2006). The powerful discrimination for single mismatched DNA makes *acpc*PNA a potential candidate for the development of highly effective DNA biosensors, which has

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http://dx.doi.org/10.1016/j.carbpol.2015.05.043
0144-8617/

been continuously proven for both labeling (Boonlua, Vilaivan, Wagenknecht, & Vilaivan, 2011; Maneelun & Vilaivan, 2013; Mansawat, Boonlua, Siriwong, & Vilaivan, 2012; Rashatasakhon, Vongnam, Siripornnoppakhun, Vilaivan, & Sukwattanasinitt, 2012; Reenabthue, Boonlua, Vilaivan, Vilaivan, & Suparpprom, 2011) and non-labeling techniques (Ananthanawat, Hoven, Vilaivan, & Su, 2011; Ananthanawat, Vilaivan, & Hoven, 2009; Ananthanawat, Vilaivan, Mekboonsonglarp, & Hoven, 2009; Jampasa et al., 2014; Sankoh et al., 2013; Theppaleak, Rutnakornpituk, Wichai, Vilaivan, & Rutnakornpituk, 2013a, 2013b; Thipmanee et al., 2012).

Recently, a simple label-free DNA sensing platform which relied on distinct electrostatic properties of *acpc*PNA and DNA was developed for analysis of single nucleotide polymorphism and food adultery detection (Boontha, Nakkuntod, Hirankarn, Chaumpluk, & Vilaivan, 2008). Because PNA is an electrostatically neutral molecule, it cannot be captured by a positively charged anion-exchanger unless hybridized with its complementary DNA target. The captured PNA can then be detected by MALDI-TOF MS (Boontha et al., 2008; Theppaleak et al., 2013a, 2013b) or alternatively by enzyme-mediated colorimetric detection (Laopa, Vilaivan, & Hoven, 2013). In this assay, the crude DNA products obtained after PCR can be used directly without prior purification and the analysis can be performed at room temperature under non-stringent conditions. Besides the satisfactory binding properties and specificity of the PNA probe, the success of this approach should also depend upon the physical properties of the anion exchanger, especially the ability to prevent non-specific adsorption of the unhybridized PNA. Q-sepharose, a commercially crosslinked agarose particles bearing quaternary ammonium groups (Boontha et al., 2008), and quaternized poly(dimethylamino)ethyl methacrylate -grafted cellulose paper (Laopa et al., 2013) or magnetic nanoparticles (Theppaleak et al., 2013a, 2013b) were previously used as solid support for the DNA capture. From material science perspective, the issue related to chemical functionality and hydrophobicity/hydrophilicity of the anion exchanger on the DNA capturing is also worth to be explored and has not yet been addressed in previous investigations.

Here in this research, we synthesized and compared the efficiency of modified-chitosan particles as anion exchangers for PNA-based detection of DNA by MALDI-TOF mass spectrometry. Two routes for the preparation of quaternized chitosan particles were performed. The first route is based on heterogeneous chemical modification of ionically cross-linked chitosan particles via direct methylation and reductive benzylation/methylation that yielded methylated chitosan (MC), methylated *N*-benzyl chitosan (MBzC) particles, respectively. The second route is based on ionic cross-linking of quaternized chitosan derivatives, homogeneously modified from chitosan, namely *N,N,N*-trimethylchitosan (TMC) and *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC) that gave TMC and HTACC particles, respectively. Synthetic methodology of both routes is outlined in Scheme 1. It is anticipated that this research should open up a new direction for chitosan applications in the area of biosensors in the format, to the best of our knowledge, that has never been reported.

2. Materials and methods

2.1. Materials

Chitosan flake (M_V = 45,000 Da) was purchased from Seafresh Chitosan (Lab) Co., Ltd (Thailand). Degree of deacetylation (%DD) of chitosan calculated based on ^{1}H NMR data (shown in Fig. S1 and Table S1 in Supplementary Data) was 96.8%. Acetaldehyde, methyl iodide (CH_3I), sodium borohydride ($NaBH_4$), sodium hydroxide (NaOH), sodium iodide (NaI), and sodium tripolyphosphate (TPP) were obtained from Fluka (Switzerland). Acetic acid, benzaldehyde, *N*-methylpyrrolidone (NMP), and methanol (MeOH) were purchased from Merck (Germany). Glycidyltrimethylammonium chloride (GTMAC) was obtained from Fluka (Switzerland). Genomic DNA from *Escherichia coli* strain B type VIII was obtained from Sigma (USA). PNAs were synthesized manually by solid phase peptide synthesis on TentaGel S RAM resin (Fluka) preloaded with Fmoc-L-Lys(Boc)-OPfp (Calbiochem Novabiochem Co., Ltd., USA) following the procedure described earlier (Vilaivan & Srisuwannaket, 2006). The identity of the PNAs was verified by MALDI-TOF mass spectrometry (Bruker Daltonik GmbH, Germany). Oligonucleotides were purchased from Bioservice Unit, National Science and Technology Development Agency (Thailand). All reagents and materials are analytical grade and used without further purification. Ultrapure distilled water was obtained after purification using a Millipore Milli-Q system (USA) that involves reverse osmosis, ion exchange, and a filtration step.

2.2. Preparation of quaternized chitosan particles by heterogeneous route

Chitosan (CS) particles were fabricated using TPP as a cross-linking agent according to the method reported by Qi, Xu, Jiang, Hu, and Zou (2004). Two types of quaternized chitosan particles, namely methylated *N*-benzyl chitosan (MBzC) and methylated chitosan (MC) particles were prepared following the method reported by Wiarachai, Thongchul, Kiatkamjornwong, and Hoven (2012). An anhydrous methanol solution of 1.0 M benzaldehyde (10 mL) was added into a flask containing the chitosan particles (0.03 g). After stirring for 4 h at room temperature, $NaBH_4$ (0.3 g, 8.0 mmol) was added into the reaction mixture and the solution was stirred for 24 h. The resulting *N*-benzyl chitosan particles were then isolated by centrifugation at 12,000 rpm for 30 min. The supernatant was discarded and the particles were centrifugally washed three times as above with methanol and then dried in vacuo. Methylation was then performed by an addition of anhydrous methanol solution (10 mL) of NaI (0.30 g, 2 mmol) and NaOH (0.13 g, 3.3 mmol) into the flask containing the *N*-benzyl chitosan particles (0.03 g) followed by CH_3I (2.0 M, 3.1 mL) via a syringe. The reaction mixture was stirred at 50 °C for 12 h and then the MBzC particles obtained were isolated and purified as above. MC particles, on the other hand, were prepared by direct methylation of the CS particles as follows. Anhydrous methanolic solution (10 mL) of NaOH (0.13 g, 3.3 mmol) and NaI (0.30 g, 2.0 mmol) was added into a flask containing chitosan particles (0.05 g), followed by an addition of CH_3I (0.4 mL, 6.4 mmol). The same portion of CH_3I was added after the mixture was stirred for 4 h at 50 °C. The reaction was allowed to proceed for another 4 h under the same condition. The synthesized MC particles were then isolated by the same procedure as described above.

2.3. Preparation of quaternized chitosan particles by homogeneous route

TMC was synthesized according to a modified method of Domard, Rinaudo, and Terrassin (1986). Chitosan (2.0 g) was dispersed in 80 mL of NMP followed by adding NaOH (0.81 g, 0.02 mol) and NaI (1.51 g, 0.01 mol). CH_3I (4.32 g, 0.03 mol) was added to the reaction mixture which was then stirred magnetically at 40 °C for 4 h. An additional portion of CH_3I was then added to the mixture and the reaction was allowed to proceed for another 4 h under the same condition. The final product was precipitated using acetone and isolated by centrifugation at 12,000 rpm for 30 min. The precipitate was dissolved in 15.0% (w/v) NaCl solution for 4 h in order to replace the iodide counterion with chloride. The suspension was dialyzed against deionized water in a dialysis bag (cut-off molecular weight of 14,000 g/mol) at ambient temperature for 5 days to