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COMMUNICATION

Affinity Purification of Multifunctional Oligomeric Ligands Synthesized via Controlled Radical Polymerization

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Abiotic oligomeric ligands with a strong affinity for a target peptide sequence were isolated by affinity purification from a pool of 30-mer acrylic random ter-oligomers that were synthesized via controlled radical polymerization process. Our results indicate that oligomer ligands with suitable sequence and/or stereo-chemical configurations for the target can be isolated from as-polymerized random co-polymer based on the affinity to the target. The process will be a powerful tool for the development of stable and inexpensive ligands that can be used to detect, neutralize and purify proteins with a target epitope sequence.

Abiotic polymer/oligomer ligands with a strong affinity for their target biomacromolecules are of significant interest as stable and inexpensive substitutes for biomacromolecular ligands such as antibodies and aptamers. In recent years, multifunctional polyacrylamides have been designed as robust ligands for protein and peptide recognition[1,2]. Schrader and co-workers reported that linear polymers that work as a protein-specific host can be prepared by free radical polymerization of carefully optimized combination of functional monomers that interact with surface of the target molecules.[3,4] Shea, Dawson and co-workers designed multifunctional polyacrylamide nanoparticles that interact with target peptide/protein via combination of weak interactions such as electrostatic, hydrophobic, aromatic and hydrogen bonding interaction[5-7]. Some of the ligands further showed biological functions such as inhibition of target activity[8,9], toxin neutralization[7,10], inhibition of fibrillation[6] and re-solubilization of target aggregates[11]. Polymer ligands with a greater affinity for their targets can be prepared using molecular imprinting polymerization[12,13]. However, in contrast to the biomacromolecular ligands, polymerized materials have a heterogeneous distribution of recognition sites.[10,14] These are typical characteristics for acrylic copolymers that are synthesized by one pot chain reaction under kinetic control, in contrast to other ligands such as dendrimers, peptides and nucleic acids that are prepared via a combination of multistep coupling, deprotection and purification processes[15-17] or synthetic hosts that are prepared by self-assembly under equilibrating conditions using reversible bond formation[18-20].

Controlled radical polymerization procedure opened a route to synthesize the acrylic polymers with well-defined size distribution, sequences and structures[21,22]. Taking advantage of sophisticated radical process, such as atom transfer radical polymerization (ATRP)[23] and reversible addition fragmentation chain transfer (RAFT) polymerization[24-27], multifunctional polymers with defined size, distribution and sequence of functional group have been prepared. Recently, controlled radical polymerization procedures have also been applied to synthesize polymer ligands, which have defined molecular weight distribution and localization of functional groups, that interact with target proteins and peptides[28-32]. It was reported that size, arrangement and topology of the well-defined functionalized polymers effect on the affinity to target proteins[33,34] and viruses[35]. However, controlled radical polymerization procedures still generate mixtures of polymers with varying degrees of polymerisation (DP) and stereo-chemical configurations.

Chromatography is a powerful tool to isolate acrylic polymers based on affinity to functional group on solid stationary phases[36-38]. Hawker, Xu, and co-workers has shown that multi-functional oligoacrylates with discrete DPs can be isolated in multigram scale using multi-step normal- and reverse-phase liquid chromatography from a pool of oligomers that were synthesized via controlled radical polymerization[36,37]. The isolated oligomers demonstrated a characteristic behavior, in contrast to the pre-purified mixtures[38,39]. Shea, Piletsky, Haupt, Bui and co-workers have shown that multifunctional polyacrylamide with strong affinity to target molecules can be isolated by single step affinity purification process using the solid phase on which the targets are immobilized[40-42]. However,

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affinity purification of polymers that were synthesized via controlled radical polymerization has not been reported.

In this study, we report multifunctional abiotic ligands with a strong affinity to their target peptide can be purified by affinity chromatography from a pool of 30-mer ter-oligomers synthesized via a RAFT polymerization using target peptide-immobilized beads.

Melittin and magainin 1 were selected as the model target peptide and control peptide, respectively. Fig. 1a shows the primary sequences of these peptides. Melittin is an α-helical hemolytic toxin from bee venom and has been studied as a model target molecule for the ligands[32,39,43-45]. The unique cationic sequence consisting of lysin and arginine (KRKR sequence) is known to function as a good tag sequence for protein purification using polymer nanoparticles consisting of combination of *N*-tert-butylacrylamide (TBAm) and negatively charged acrylic acid (AAc) as an affinity reagent[46]. Magainin 1 was selected as a control peptide because it is also a cell-lytic toxin and has similar characteristics to melittin in terms of molecular weight (2846 Da and 2409 Da, respectively), hydrophobicity (50% and 43%, respectively), and number of positively charged amino acids (six positive charges each including an *N*-terminal amine)[45].

(a)

Melittin	H_3N^+-GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2
Magainin 1	H_3N^+-GIGKFLHSAGKFGKAFVGEIMKS-coo^-
Melittin RRKKKK	Ac-RIGAVLRVLTTGLPALISWIKKKKQQ-NH_2

(b)

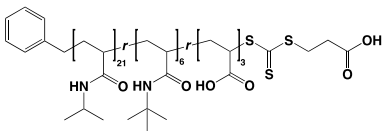

Figure 1. (a) Amino acid sequence of melittin, magainin 1 and melittin RRKKKK (a melittin analog peptide) with hydrophobic (green), positively charged (red), and negatively charged (blue) amino acids highlighted. (b) Structure of multifunctional polymer ligands composed of *N*-isopropylacrylamide (NIPAm), *N*-*tert*-butylacrylamide (TBAm) and acrylic acid (AAc) (DP = 30-mer, monomer composition: NIPAm/TBAm/AAc = 21/6/3).

Ter-oligomers were synthesized by RAFT polymerization using benzylsulfanylthiocarbonylsulfanyl propionic acid (BPA) as a chain transfer agent and *N*-isopropylacrylamide (NIPAm) as a main monomer (Fig. 1b)[32,39,44,47,48]. The polymerization reaction is described in Supporting Information (SI, Scheme S1, S2). As melittin contains both hydrophobic and cationic amino acids, hydrophobic *N*-*tert*-butylacrylamide (TBAm) and negatively charged acrylic acid (AAc) were selected as functional monomers to interact with melittin via hydrophobic and electrostatic interactions, respectively. The average numbers of incorporated TBAm and AAc functional monomers were controlled (6 and 3 respectively), and the DP was minimized (30-mer) to maximize the binding specificity for melittin[32]. Here, both density (feed ratio) and number of hydrophobic and negatively charged functional group on the oligomer (6 and 3 respectively) was controlled to be smaller than those on melittin (15 and 6 respectively) because the oligomers with greater hydrophobicity (larger number and density of TBAm) and larger amount of AAc showed non-specific interaction to the control peptide, magainin 1[32]. The composition, Mn (3500 g/mol) and PDI (1.26) of the oligomer were confirmed by ^{1}H-NMR and GPC (Fig. S1, S2).

We hypothesized that the synthesized oligomers contained a heterogeneous mixture of polymer chains, and it was highly likely that some chains had a higher affinity to melittin than others. To confirm this, we performed an affinity chromatography step of the oligomers using melittin-immobilized beads. Melittin was covalently immobilized onto hydrophilic porous methacrylic polymer beads functionalized with epoxides (average diameter: 56 μm, average pore diameter: 53 nm; Mitsubishi Chemicals Corp. JP) (SI, Scheme S3). The melittin-immobilized beads (4.3 mL, with 6 mg of immobilized melittin) were then packed into a glass column (Tri corn, GE healthcare UK Ltd.). Two control columns were also prepared using equivalent methods: one contained beads prepared in the absence of peptides and the other contained magainin 1-immobilized beads.

The buffer conditions and temperature for the affinity purification processes were based on previous reports[40]. Aqueous solution of oligomer samples (200 mg in 100 mL of phosphate buffered saline (PBS; 35 mM phosphate pH 7.3, 150 mM NaCl)) was loaded onto the melittin-immobilized column equilibrated with PBS at 37 °C (0.5 mL/min for 200 min from superloop using a low pressure liquid chromatography system (AKTAprime plus, GE healthcare UK Ltd) equipped with UV 280 nm and conductivity detectors). The column was then washed with PBS for 30 min at 37 °C, and weakly-bound oligomers were eluted using a linear pure water gradient (0.5 mL/min for 180 min at 37 °C). The temperature of column was then lowered to 4 °C using an ice bath to elute the tightly-bound oligomers.

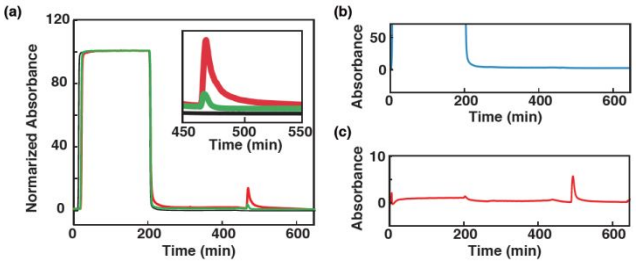

Figure 2. Chromatogram of affinity purification process monitored by UV detector (280nm). (a) Randomly copolymerized oligomers (2 mg/mL in PBS) were loaded onto melittin-immobilised beads (red), magainin 1-immobilized beads (green) and peptide-free control beads (black). (b) Detection of non-binding oligomers (flow through fraction) eluted by PBS washing following reloading of the flow through fraction onto melittin-immobilized beads. (c) Detection of cold-eluted oligomers (0.01 mg/mL in PBS) following reloading of the original cold elution fraction onto melittin-immobilized beads. In all experiments, 100 mL of sample solutions were loaded onto the columns (37 °C, 0.5 mL/min). The columns were washed by PBS for 30 min (37 °C), followed by a pure water linear gradient for 180 min (from 230–410 min; 37 °C, 0.5

mL/min). The temperature of the columns was lowered from 37 °C to 4 °C at 460 min.

Chromatograms from the affinity purification processes are shown in Fig. 2a. When the oligomers were loaded onto peptide-free control beads, most oligomers passed through the column (10–210 min) (Fig. 2a, black). Following washing of the peptide-free beads with PBS and a pure water linear gradient (230–410 min), no oligomers were eluted upon cooling of the column to 4 °C (460–550 min) (Fig. 2a insert, black). This results indicate that there were little interaction between oligomers and the porous polymer beads. On the other hand, when an identical sample was loaded onto the melittin column, some tailing was observed after the passage of loaded oligomers (210–230 min) (Fig. 2a, red), indicating there was an interaction between oligomers and melittin on the beads. Following identical washing steps as above, elution of oligomers was observed upon cooling of the melittin-immobilised beads using an ice bath (460–550 min) (Fig. 2a insert, red). When an identical sample was loaded to the magainin 1 column, tailing was not observed and the absorbance of the cold elution fraction was very small compared to the melittin-immobilized beads (Fig. 2a, green). These results suggest that the cold elution fraction from the melittin column contains oligomers that have a strong affinity to melittin. The the oligomer could distinguish sequence of immobilized peptides, which has similar molecular weight and number of negative charges, maybe because carboxylic acids on the oligomer has stronger affinity to arginines on melittin via combination of electrostatic and hydrogen binding than to cationic functional groups on magainin such as primary ammonium and imidazolium ions as reported[32,39]. The yield of the cold-eluted oligomers was 1.2 mg, which is 0.6% of the total sample of oligomers loaded onto the column.

To verify that there was a difference in melittin binding between the flow through and cold elution fractions obtained from the melittin-immobilized column, both fractions were separately loaded onto the melittin-immobilized column again under the same conditions as described in the previous experiments. The flow through fraction did not yield any eluate upon cooling following loading and washing of the flow through fraction and no oligomers were observed to bind to the column (Fig. 2b). In contrast, when 1.0 mg of the cold elution fraction (in 100 mL PBS) was reloaded onto the melittin column again, the majority of oligomers bound to the column and were re-eluted upon column cooling (Fig. 2c). These results suggest that the isolated fraction (cold elution) has a stronger affinity to melittin in PBS and pure water at 37 °C compared to the oligomers in the flow through fraction. The interaction of bound oligomers to melittin can be reversibly weakened by lowering the temperature to 4 °C, presumably because the hydrophobic interactions between oligomers and melittin are weaker at lower temperatures[40]. Our results determined that the copolymerized oligomers are comprised of a heterogeneous mixture, some of which have stronger melittin affinity under the binding conditions than others. The yield of the oligomers from the second cold elution was 0.23 mg which is 23% of the reloaded sample. An explanation for the low recovery may be a result of bleeding of high affinity oligomers from melittin-immobilized beads during the purification process because the concentration in the second binding experiment (0.01 mg/mL, 3 μM) was much lower than that in the first experiment (2 mg/mL, 600 μM).

The composition of isolated oligomers from the cold elution fraction was characterized and compared with the pre-purified oligomers using ^{1}H-NMR spectra in methanol-*d*4 (Fig. 3). The spectrum of the ligands from the cold elution fraction was similar to that of the oligomers before purification. However, integration of the chemical shifts observed at 1.3–1.4 ppm corresponding to the –CH_3 groups of the *tert*-butyl group was larger for the oligomers in the cold elution fraction compared to before affinity purification, indicating that the purified fraction contains a higher proportion of TBAm monomers. Further analysis of the ^{1}H-NMR spectra revealed that the average numbers of each monomer unit (NIPAm/TBAm/AAc) in the purified oligomer fraction was 18/9/3, compared to 21/6/3 before affinity purification.

The interaction between melittin and oligomers before and after affinity purification was analysed using a 27-MHz quartz-crystal microbalance (QCM) as previously reported (Fig. 4)[40]. When as-synthesized oligomers (0.7-2.5 mM) were injected into the QCM sensor cells on which melittin was immobilized, little frequency change was observed (Fig. 4a blue). On the other hand, significant frequency change was observed when the oligomers isolated in the cold-elution was injected into the cells (Fig. 4a red). Apparent dissociation constants of 4.5 μM was obtained by fitting the QCM results by Langmuir isotherm (Fig. 4b).

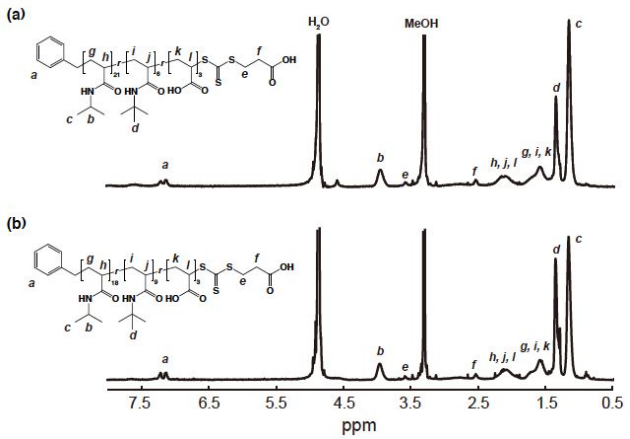

Figure 3. ^{1}H NMR spectra (400 MHz) of oligomers before (a) and after (b) affinity purification (cold elution) in methanol-d_4.

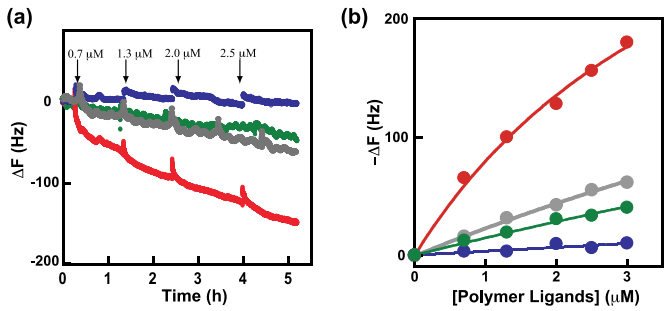

Figure 4. Interaction between oligomeric ligands and melittin observed by a 27-MHz QCM in water at 25 °C. Randomly copolymerized oligomers (NIPAm/TBAm/AAc = 21/6/3) before (blue) and after purified by Melittin (red) and Melittin RRKKKK (gray) beads, and randomly copolymerized oligomers (NIPAm/TBAm/AAc = 18/9/3) before purification were injected onto melittin-immobilised QCM sensor cells. (a) Time course of the frequency change (ΔF) of the QCM sensors after the injection of oligomers solutions (at the time points indicated by the black arrows). (b) Binding isotherms of interaction between oligomers and melittin.

To gain a further insight into the composition of the affinity purified fraction, oligomers with the same composition as the isolated ones (NIPAm/TBAm/AAc = 18/9/3) were prepared by RAFT polymerization and their affinity to melittin was investigated using a QCM[40]. As shown in Fig 4 green plots, the polymerized oligomers showed a much weaker interaction with melittin compared to the isolated ones, despite the average monomer composition being almost identical. This data indicate that the isolated oligomers have the similar monomer composition but have a different functional group sequence and/or stereo-chemistry compared to the polymerized oligomer sample.

In order to test target specificity, a melittin analog peptide (melittin RRKKKK, Fig1a), in which the position of the primary ammonium group and guanidinium group is exchanged, was immobilized on the beads for the affinity purification of the oligomers (DP = 30-mer, monomer composition: NIPAm/TBAm/AAc = 21/6/3). When the oligomer was purified using the solid state via same procedure, significant amount of oligomer was eluted at the cold elution process (Fig. S3). This result indicate that the as-synthesized random ter-oligomer contains fraction of oligomers that interact strongly with the melittin analog. However, the purified fraction showed little interaction with the melittin immobilized QCM sensors (Fig. 4 gray plots). This results indicate that the fraction purified by melittin RRKKKK-immobilized beads has specific affinity to the sequence of peptides and has completely different target specificity from the ones purified by Melittin-immobilized beads.

Based on these results, we concluded that synthetic polymer ligands with a strong affinity for their target biomacromolecules can be purified from a pool of multi-functional polymers, that are synthesized via controlled radical polymerization process, using affinity purification process. The purified ligands have an improved affinity for target peptide compared to an un-purified mixture ones. The ligands purified by affinity purification showed a reversible, temperature-dependent binding to their target peptide, and have a much stronger and narrower affinity distribution compared to un-purified mixtures. It is likely that the purified ligands contain suitable sequence and/or stereo-chemical configurations to bind strongly to their target peptide.

It should be noted that the purified fractions theoretically still contain oligomers with a vast variety of sequence and configurations. However, a combination of this affinity purification strategy with other purification process[36,39], including normal- and reverse-phase, ion-exchange, and gel-filtration chromatography technique, and with more sophisticated controlled polymerization procedures[37], including block polymerization[30,36,39], sequence-controlled polymerization and stereo controlled polymerization, will enable production of near homogeneous, inexpensive and physico-chemically stable substitutes for biomacromolecular ligands such as RNA, DNA, and peptide aptamers.

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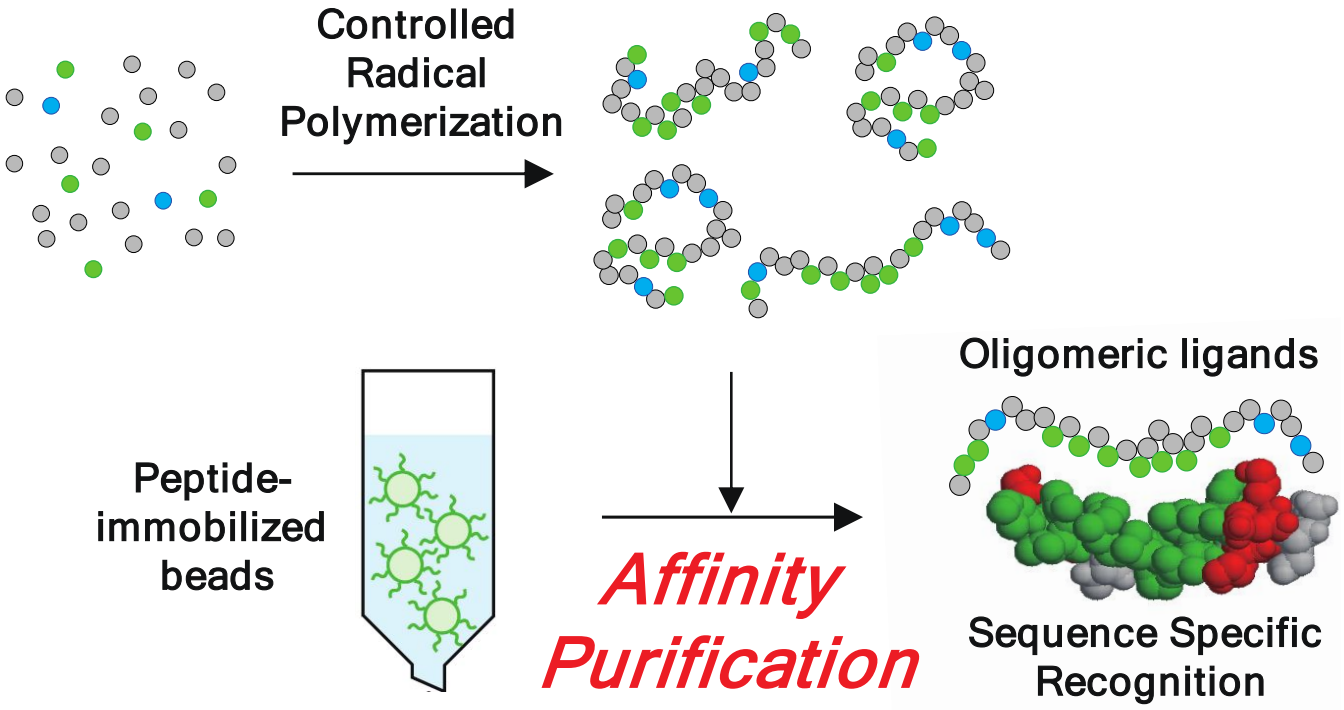
Abiotic oligomeric ligands with a strong affinity for a target peptide sequence were isolated by affinity purification from a pool of 30-mer acrylic random ter-oligomers that were synthesized via controlled radical polymerization process.



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