



## Improvement of monolithic solid material by utilization of spacer for identification of the target using affinity resins

Emiko Iwaoka<sup>a</sup>, Tomoko Mori<sup>b</sup>, Tadashi Shimizu<sup>a</sup>, Ken Hosoya<sup>b</sup>, Akito Tanaka<sup>a,\*</sup>

<sup>a</sup> Department of Pharmacy, Hyogo University of Health Sciences, 1-3-6 Minatogima, Chuo-ku, Kobe 650-8530, Japan

<sup>b</sup> Graduate School of Environmental Sciences, Tohoku University, Aoba 6-6-20, Aramaki, Aoba-ku, Sendai 980-8579, Japan

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### ABSTRACT

Affinity chromatography is an important strategy for target identifications. However, commercial available solid materials have limitations while selection of that is sometimes vital for the purpose. We have reported a synthetic resin with a monolithic structure in previous papers. In this paper, introduction effects of spacer to the monolithic material on identification of specific binding protein was quantitatively analyzed using benzensulfonamide as a bait, which exhibited introduction of  $\omega$ -substituted heptanoic acid as spacer enabled affinity resins to capture the target proteins effectively. Utilization of the optimized spacer enable the monolithic material bearing FK506 to identify not only FKBP12 but FKBP52, calcineurin A and calcineurin B at silver stained level, while that without spacer had failed.

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Affinity resins bearing bioactive compounds such as natural products, drugs, and toxins play an important role in the identification of specific protein targets for these small molecules.<sup>1,2</sup> These findings are essential to modern pharmaceutical research since they can often facilitate the development of novel drugs with greater selectivity and/or potency through effective screening systems and structure-based design. The successful isolation of target proteins by affinity matrices often depends on the synthesis of polymeric resins that can capture the cellular target with maximum selectivity and efficiency. Affigel™, an agarose derivative,<sup>3</sup> is one of the most popular matrices for this purpose, and its hydrophilic character helps reduce the non-specific protein absorption.<sup>4</sup> However, Affigel™ becomes easily denatured under organic synthesis conditions, and its chemical approaches are often restricted.<sup>4</sup> Toyopearl™, a poly(methacrylate) derivative,<sup>5</sup> is stable under most synthetic conditions, which allows greater versatility for the preparation of effective affinity resins. But, these methacrylate polymers bearing bioactive compounds often show high levels of non-specific binding protein with the target protein because of their hydrophobic property.<sup>4</sup> New solid phases for affinity resins that are chemically stable and hydrophilic enough for elimination of non-specific absorption are necessary.

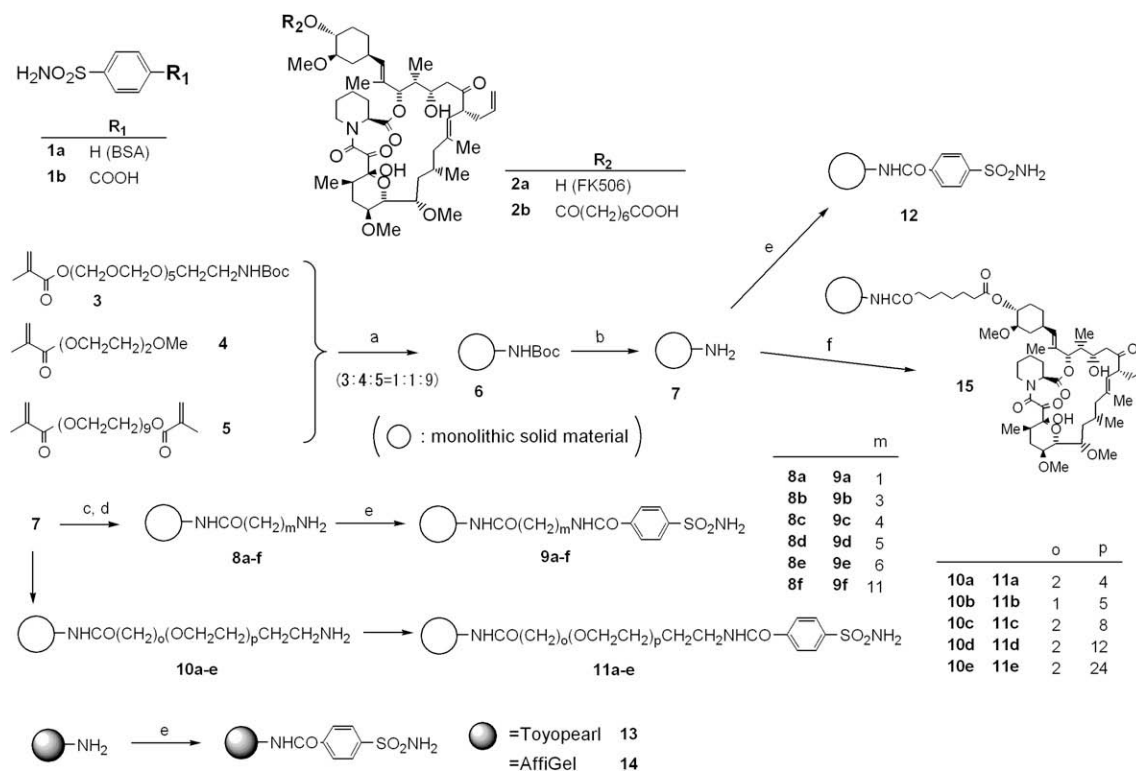
In previous papers,<sup>6,7</sup> we have reported the development of a novel poly(methacrylate) solid material for affinity resins, which is hydrophilic enough to reduce non-specific protein absorptions.

This material has a monolithic structure and is suitable for identification of specific binding proteins. For example, this solid material bearing FK506 can capture of its specific binding protein, FKBP12, with little non-specific protein absorption. However, the effect of introducing spacers between the poly(methacrylate) material and immobilized compound on target protein binding has not been clarified. Thus we carried out structure–property relationship study using various spacers for selection of the optimized spacer for identification of target. We adopted benzensulfonamide (**1a**) as bait in this work, since it is a specific carbonic anhydrase II (CA2) inhibitor with a  $K_d$  of 0.32–1.25  $\mu$ M.<sup>8,9</sup> For evaluation of spacers used in this work, we assessed “selectivity” between capture of specific binding protein, CA2, and that of non-specific protein absorptions such as tubulin after mixed with lysate from rat brain. Furthermore, we investigated the “capacity” of effective ligands on the affinity resins, since the density of effectively immobilized ligand on affinity resins is often critical for identification of target protein.<sup>10</sup>

The monolithic material consists of three components as shown Scheme 1. Methacrylate monomer (**3**), which contains a hydrophilic poly(ethyleneglycol) spacer and protected amine was polymerized with molar equivalent of **4** and nine equivalents of **5** at 60 °C to afford the monolithic solid material (**6**, Fig. S1). As we have demonstrated previously, this poly(ethyleneglycol) spacer reduces non-specific protein binding.<sup>4</sup> After de-protection of Boc group in 95% trifluoroacetic acid, linkers of differing structure were introduced (**8a–f**, **10a–e**). In particular, we selected  $\omega$ -amino-alkanoic acid (alkyl type) and poly(ethyleneglycol) derivative (PEG type)

\* Corresponding author. Tel.: +81 78 304 3067; fax: +81 78 304 2767.

E-mail address: [tanaka-a@huhs.ac.jp](mailto:tanaka-a@huhs.ac.jp) (A. Tanaka).



**Scheme 1.** Synthesis of affinity resins using the monolithic solid materials bearing *p*-carboxyl-benzotriazole (1b) with spacers (9a–f, 11a–e), without a spacer (12), and bearing FK506 with optimized spacer (15). Preparation of affinity resins using commercial available solid material, Toyopearl™ (13) and Affigel™ (14), bearing 1b is also shown. Reagents and conditions: azobisisobutyronitrile, 60 °C overnight; (b) 95% trifluoroacetic acid, 2 h; (c) HOOC-(spacer)-NHfmoc, EDC HCL, HOBT, NET<sub>3</sub>/NMP; (d) 20% piperidine/DMF; (e) 1b EDC HCL, HOBT, NET<sub>3</sub>/NMP; (f) 2b EDC HCL, HOBT, NET<sub>3</sub>/NMP.

spacers of various lengths in this study. We thought that the alkanic acid would be suitable for evaluating the distance between the ligand and resin surface required to reduce of steric interactions with the protein targets. PEG type spacers were selected because they were suitable to maintain the hydrophilic property while alkyl type spacers often afford hydrophobic one. A benzotriazole derivative (1b) was immobilized on the solid materials by *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC HCL) in the presence of 1-hydroxybenzotriazole (HOBT) to afford the desired affinity resins 9a–f and 11a–e. Affinity resins bearing 1b without spacer (12), Toyopearl™ (13), and Affigel™ (14) were also prepared.

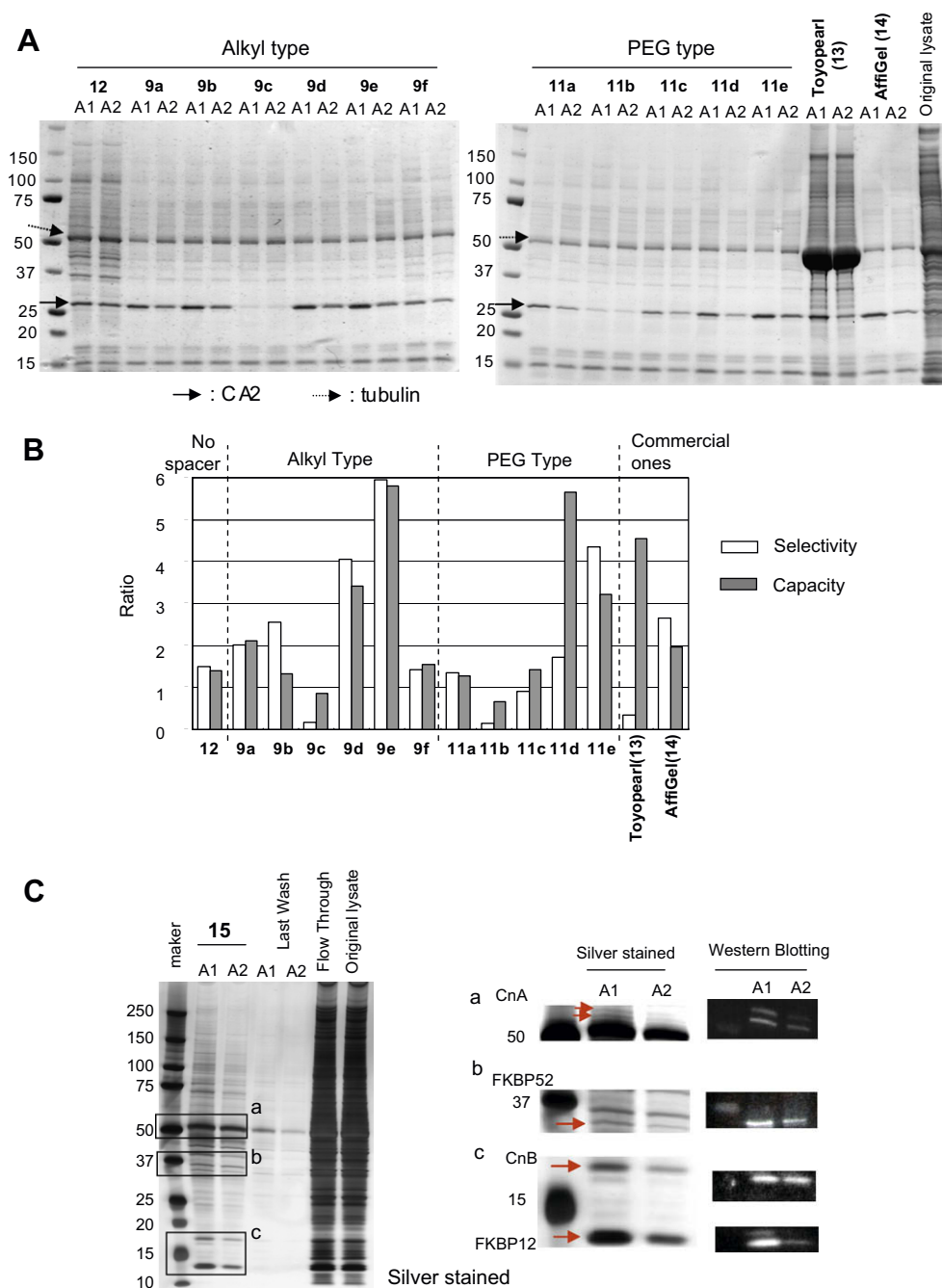
Solid materials bearing benzotriazole (9a–f, 11a–e, 12–14) were mixed with lysate obtained from rat brain, washed with lysate buffer, and then analyzed for specific and non-specific binding proteins (Fig. 1A).<sup>11</sup> We utilized serial affinity chromatography (SAC) method<sup>12</sup> to distinguish between specific and non-specific binding proteins instead of the competition method since 1a is not sufficiently soluble to perform this traditional method. In the SAC method, lysates are sequentially treated at least twice with affinity resins bearing the bioactive ligand, and the binding proteins on each resin are compared. Most of the specific binding proteins should be captured by the first resin (A1) due to its high affinity for the ligand, and little remains in the lysate. Therefore the amount of specific binding protein on second resin (A2) should be much lower than that on A1. In contrast, the amount of non-specific binding proteins on the two resins should be similar, because most of them remain in the lysate due to their low affinity for the resin. Therefore, 'selectivity' can be estimated by the ratio of the CA2 and tubulin levels, and evaluation of the "capacity" can be determined by comparing the selectivities of the A1 and A2 steps.<sup>13</sup>

The monolithic solid material bearing 1b without spacer (12) successfully captured CA2 as a specific binding protein as shown

in Figure 1A. There was, however, a variety of non-specific protein absorbed onto the resin including tubulin. On the other hand, the commercial available synthetic resin, Toyopearl™, bearing the same compound (13) captured CA2 with large amount of tubulin and other non-specific binding proteins, illustrating its poor selectivity for capture of specific binding protein. The amount of non-specific protein absorption for the monolithic solid material was reduced by the introduction of spacers (9a–f, 11a–e, Fig. 1A), and those results were similar to that of Affigel™ (14). The amount of captured CA2 was increased in proportion to increment in the methylene of spacer moiety up to *m* = 6 (9e), except for 9c (*m* = 4). This result indicated that steric hindrance between the protein and solid surface can be improved by the introduction of spacer. Introduction of spacer with long chain (9f, *m* = 11) resulted in decrease in the amount of CA2, comparing with that on 9e. These results showed the existence of an optimum length of the spacer, *m* = 6 in this study. The reason for the nonlinear anomalies on 9c is not clear, but it is unlikely to be due to a failure to immobilize 1b on 9c since the amount of non-specific binding proteins was similar to those on other resins.

The structure–property relationship on affinity resins bearing PEG spacer (11a–e) was not clear, that is, only small amount of CA2 was found on 11b (*p* = 5) while other resins (11a, 11c–e) successfully captured this protein. The amount of affinity resin bearing the longest PEG spacer (11e, *p* = 24) was the maximum in this PEG series, which did not coincide with results on 9a–f since the length of PEG spacer is much longer than that of alkyl spacer. We thought the reason for this discordance could be due to the structural complexity of long PEG spacer.

In order to assess effectiveness of the 'selectivity' and 'capacity' quantitatively, we measured the amount of tubulin and CA2 on the gel with a densitometer (Fig. 1B and Table S1). The selectivity between amount of tubulin and CA2 on each affinity resins was repre-



**Figure 1.** Binding proteins on affinity matrices studied in this study after mixed with lysate from rat brain. (A) Ten microliters of each resin was mixed with 1 mL of lysate obtained from rat brain at 4 °C for 1 h. After separation of the resins by centrifugation and extensive washes with lysate buffer, binding proteins were completely eluted by SDS sample buffer solution and analyzed by SDS–polyacrylamide gel electrophoresis. Identifications of proteins mentioned in this study were performed by MS/MS ions search method using MASCOT database (ver. 2.1.0, Matrix Science) an ESI ion trap mass spectrometry (Thermoelectron, LTQ) after in-gel digestion, respectively. The terms, A1 and A2, represent binding proteins on the first and second affinity resins in the SAC method, respectively. (B) Selectivity of each affinity resins between specific binding and non-specific one were estimated by ratio of (area of CA2)/(area of tubulin) (open bar), and their capacity were calculated by ratio of (selectivity in A1)/(selectivity in A2) (closed bar). Measurement of amount of CA2 and tubulin on the gel were carried out by a KODAK 1D system (scientific imaging system). The raw data were tabulated in Table S1. (C) Ten microliters of **15** was mixed with 1 mL of lysate obtained from rat brain at 4 °C for 45 min. The identification of specific binding proteins was carried out by the SAC method.<sup>13</sup> After separation of the resins by centrifugation and extensive washes with the lysate buffer, binding proteins were completely eluted by SDS sample buffer solution and analyzed by SDS–polyacrylamide gel electrophoresis. Close-up view of the bands in rectangles a–c are shown to the right. Western blot studies were carried out using anti-calceineurin A (CnA), FKBP52, calcineurin B (CnB), and FKBP12 antibodies.

sented as open bars, and the capacity was stood out as closed bars.<sup>13</sup> **9b** bearing  $\omega$ -amino-butylic acid as spacer showed good selectivity (ca. 2.5) compared to **12** while its capacity (ca. 1.3) was not enough. These results indicated that introduction of this spacer is effective for reduction of the non-specific protein absorptions, but not enough to increase the capacity. Among affinity resins bearing  $\omega$ -amino-alkanoic acid as spacer (**9a–f**), **9e** bearing  $\omega$ -amino-heptanoic acid as

spacer exhibited excellent selectivity (ca. 5.9) and capacity (ca. 5.8), which were almost four times improved compared to those of affinity resins without spacer (**12**). Both selectivity and capacity of **9f**, bearing longest alkyl chain in this study, were much reduced. These results indicated the optimum length of the alkyl chain was  $m = 6$  (**9e**) in this study. Among affinity resins bearing PEG type spacer (**11a–e**), affinity resins with comparatively longer PEG spac-

ers, such as **11d** and **11e**, showed good results, that is, **11d** exhibited the excellent ratio on the capacity (ca. 5.6, solid bar), but showed weak selectivity (ca. 1.7). **11e** showed excellent selectivity (ca. 4.4) but its capacity was not so good (ca. 3.2). In conclusion,  $\omega$ -amino-heptanoic acid as spacer (**9e**,  $m = 6$ ) is the best selection among spacers studied in this paper.

We next synthesized FK506 affinity resins using the monolithic solid material with the selected spacer,  $m = 6$ , as spacer, to assess the efficacy of the spacer. We choose suberic acid instead of  $\omega$ -amino-heptanoic acid for FK506 affinity resins (**15**, Scheme 1),<sup>14</sup> because FK506 is unstable in the presence of basic amines.<sup>4</sup> We analyzed specific binding proteins using SAC method after mixed with the lysate from rat brain (Fig. 1C), which demonstrated that not only the abundant FK506 binding protein, FKBP12, but also other specific binding one, FKBP52, with the target complex proteins such as calcineurin A and B, were identified as specific binding proteins at silver stain level, while the same monolithic solid material without spacer missed to capture them level in the previous paper.<sup>7</sup> These proteins were identified by the western blotting experimental using their specific antibodies.<sup>15</sup> FKBP52, whose calculated molecular weight was 52 kDa, was thought to be degraded in treatments while it was found near at 37 kDa.

In conclusion, the effects of introducing spacers to a monolithic solid material for affinity chromatography were quantitatively analyzed using a benzensulfonamide derivative (**1b**) as bait, demonstrating that a  $\omega$ -substituted heptanoic acid spacer enables affinity resins to capture the target proteins effectively with low levels of non-specific protein absorption. Utilization of the optimized spacer enables the monolithic material bearing FK506 to identify not only FKBP12 but FKBP52, calcineurin A and calcineurin B at silver stained level.

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#### Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.050.

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13. The area of tubulin and CA2 were estimated by a KODAK 1D system (scientific imaging system, Table S1). The selectivity values were obtained by ratio of (area of CA2)/(area of tubulin), and the capacity were calculated by ratio of (selectivity in A1)/(selectivity in A2) (see Table S1 in details). These values was thought to be not precisely since the amount of binding proteins were not generally constant, that is, those amounts often had lot-dependency.
14. Synthesis of **2b** from **2a** is shown in Ref. 4.
15. Western blot analysis on each protein was carried out as standard method using goat polyclonal antibody IgG against FKBP52 (Santa Cruz Biotechnology, Inc., cat. sc-1803), rabbit polyclonal IgG against calcineurin A (Abcam Ltd, cat. ab12233), rabbit polyclonal IgG against calcineurin B (Serologicals Corporation, cat. 07-069), horseradish peroxidase (HRP) conjugated anti-goat antibodies (Santa Cruz Biotechnology, Inc., cat. sc-2033), and HRP conjugated anti-rabbit antibodies (Amersham Biosciences Corp., cat. NA934V) were purchased and used without pre-treatments, respectively. The specificities of used antibodies were assessed by Western blotting of the rat brain lysate (Fig. S2).