Dual specificity antibodies using a double-stranded oligonucleotide bridge

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Abstract The covalent conjugation of oligonucleotides to antibody Fab' fragments was optimized by using oligonucleotides modified with a hexaethylene linker arm bearing three amino groups. One oligonucleotide was coupled to antibody of one specificity and a complementary oligonucleotide to antibody of a second specificity. The antibodies were then allowed to hybridize by base pairing of the complementary nucleotide sequences and the generation of bispecific antibody was analyzed on SDS-PAGE and confirmed using BIAcore analysis. The strategy of complementary oligonucleotide-linked bispecific molecules is not limited to antibodies but is applicable to linking any two molecules of different characteristics.

Key words: Bispecific; Antibody; Oligonucleotide; BIAcore; Epitope; Linker

1. Introduction

An oligonucleotide-conjugated molecule has the potential to spontaneously associate, via DNA base pairing, with any other molecule to which the complementary oligonucleotide sequence has been attached. Whilst oligonucleotides have previously been attached to both IgG and IgM antibody molecules [1], the potential to link two antibodies via complementary base pairing has not previously been explored. Bispecific antibodies have potential uses in many areas including tumor imaging and therapy, ligand immobilization, immunohistochemistry and immunodiagnostics [2–5]. Three principal strategies have been employed for their production: chemical crosslinking, hybrid hybridomas and genetic engineering. Chemical crosslinking can result in heterogeneous products with batch to batch variation. The hybrid hybridoma approach generates various chain assortments requiring purification to isolate the desired bispecific molecules. More recently, recombinant bispecific molecules have been genetically engineered in various forms. For example, Neri and colleagues [6] produced chelating recombinant antibodies (CRAbs) utilizing a sequence encoding a polypeptide linker to join two scFv molecules. Bispecific molecules have also been formed by crosslinking two different genetically engineered Fab's at their hinge sulfhydryls by using fos and jun leucine zippers to facilitate heterodimerization [7]. The recombinant DNA approach has the potential disadvantage that some immunoglobulin variable region sequences may be difficult to express as soluble recombinant proteins.

We have devized a novel method of making bispecific antibodies in which oligonucleotides are covalently linked to Fab' fragments, the bispecific antibodies then being formed by allowing hybridization of complementary oligonucleotide-Fab' conjugates. This approach is illustrated in a model system in which two different anti-immunoglobulin reagents have been linked together.

2. Materials and methods

2.1. Antibodies

Goat anti-human IgG (γ-chain-specific) $F(ab')_2$ fragment (Sigma I-9885) and sheep anti-mouse IgG (whole molecule) $F(ab')_2$ fragment (Sigma M-1522) were purchased from Sigma (Poole, Dorset, UK). The antigens for these anti-immunoglobulins were human IgG, obtained as a 40% saturated ammonium sulfate precipitate from normal human serum and dialyzed into 0.1 M phosphate-buffered saline pH 7.4 (PBS), and mouse IgG, isolated from normal mouse serum by protein G (Pharmacia Biotech, Uppsala, Sweden) purification.

2.2. Oligonucleotides

The complementary 15-mer oligonucleotides TCGATGCAAATCTAT and TAGATTTGCATCGAT were used (Oswel, Southampton, UK), the latter synthesized with a 5′-terminal biotin. NH_2-oligonucleotides with one amino group at the 3′-end and a hexaethylene linker-modified oligonucleotide with three amino groups attached at the 3′-end [8] were investigated.

2.3. Production of Fab'-oligonucleotide conjugates

200 μl of 955 μM amine-derivatized oligonucleotide was reacted in the dark for 30 min at 20°C with a 100-fold molar excess of the crosslinking agent *N*-hydroxysuccinimidyl(4-iodoacetyl)aminobenzoate (SIAB, Pierce and Warriner, Chester, UK) in 0.1 M bicarbonate buffer, pH 9.0. Activated oligonucleotide was separated from unreacted crosslinker by gel filtration on a Sephadex G-25 PD10 column (Pharmacia Biotech), equilibrated with 0.1 M phosphate buffer, pH 7.5, containing 10 mM ethylenediamine tetraacetic acid (EDTA).

500 μl of 1.5 mg $F(ab')_2$ sheep anti-mouse IgG or $F(ab')_2$ goat anti-human IgG was incubated with an equal volume of 0.1 M phosphate buffer, pH 7.0, containing 10 mM EDTA and 20 mM 2-mercaptoethylamine at 37°C for 30 min to cleave the inter-heavy chain disulfide bonds of the hinge region. Under these mild reducing conditions, the disulfide bonds between the heavy and light chains are preserved [9]. The resulting Fab' fragments were concentrated and the 2-mercaptoethylamine removed, using a Centricon 10 spin column and washing with PBS containing 10 mM EDTA.

A 7-fold molar excess of the relevant activated oligonucleotide in 0.1 M phosphate buffer, pH 7.5, 10 mM EDTA was incubated with the concentrated Fab' overnight at 4°C. Fab'-oligonucleotide conjugate was purified by HPLC size exclusion chromatography using a BioCad Sprint apparatus (PerSeptives, Hertford, UK) with a Bio-Sil

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SEC-125 column (Bio-Rad Laboratories, Hemel Hempstead, UK), equilibrated with 25 mM Tris, 250 mM NaCl, pH 7.4 (Tris-buffered saline, TBS).

2.4. SDS-PAGE and immunoblotting

Conjugation reaction products were analyzed by both isoelectric focusing using the PhastSystem with pre-formed IEF gels, pH range 3–9 (Pharmacia Biotech), and by SDS-PAGE in a Mini Protean System (Bio-Rad Laboratories). Transfer onto Hybond C nitrocellulose membranes (Amersham International, UK) from SDS-PAGE was carried out in 25 mM Tris, 192 mM glycine, pH 8.2, at 4°C using 200 mA overnight. The blots were blocked using 0.1% Tween 20 and 3% dried milk powder in PBS and probed using horseradish peroxidase (HRP)-conjugated donkey antibodies to either sheep Fab' or goat Fab' (The Binding Site, Birmingham, UK) followed by detection using enhanced chemiluminescence (ECL kit, Amersham). Membranes were stripped using 50 mM glycine-HCl pH 2.7, washed in PBS and re-probed with either streptavidin-HRP (DAKO, Denmark) in PBS 0.05% Tween 20 (PBS-T) to detect biotinylated oligonucleotides or with complementary biotinylated oligonucleotides in hybridization buffer (5×SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.4), 0.05% SDS, 10% w/v dextran sulfate, 5×Denhardts, 100 μg/ml salmon sperm DNA) to detect non-biotinylated oligonucleotide. In the latter case, the membrane was pre-hybridized for 30 min in hybridization buffer without oligonucleotide and then hybridized overnight at 20°C. Following hybridization with biotinylated oligonucleotide, the membrane was washed three times at 20°C, 37°C or 55°C with 0.1% SDS in 2×SSC, then with PBS and probed with streptavidin-HRP.

For SDS-PAGE analysis of oligonucleotide-linked bispecific antibody, 2 μl of each of the two complementary Fab'-oligonucleotide conjugates in TBS was allowed to hybridize at 20°C for 15 min prior to loading.

2.5. BIAcore analysis

The basic procedure for BIAcore (Biacore AB, Uppsala, Sweden) analysis has been described before [10]. Mouse IgG was immobilized on a CM5 BIAcore chip using the BIAcore AB amine coupling kit. All reactions were carried out at 25°C at a constant flow rate of 10 μl/min. The matrix was activated by injecting 80 μl of a 1:1 mixture of 11.5 mg/ml *N*-hydroxysuccinimide and 75 mg/ml *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide to enable it to covalently bind free amino groups of proteins. This was followed by a 60 μl injection of a 30 μg/ml solution of mouse IgG in 10 mM acetate buffer, pH 4.5. Any remaining unreacted sites were blocked with an injection of 40 μl of 1 M ethanolamine. Approximately 6500 resonance units (RU) were immobilized. 5 μl of each of the two Fab'-oligonucleotide conjugates was mixed with 5 μl TBS and left on ice for 15 min to form the bispecific antibody and then injected over the immobilized mouse IgG (antigen 1) in running buffer (0.005% Tween 20 in PBS) at 10 μl/min. This was followed by injection of human IgG (antigen 2) in running buffer. To correct the drift due to dissociating conjugate, a control injection of buffer alone was subtracted using the BIAevaluation v3.0 software.

3. Results

3.1. Oligonucleotide conjugation to Fab'

The 3′-amino-linked oligonucleotides were reacted with the heterobifunctional crosslinking agent SIAB, the NHS-ester end of which reacts with the primary amine to produce a iodoacetyl-activated oligonucleotide. The subsequent reaction of the iodoacetyl group with protein containing free sulfhydryls proceeds by nucleophilic substitution of iodine with a thiol group, resulting in a stable thioether linkage. Optimum reaction conditions for conjugation (pH 7.0–7.5) were initially established using free cysteine. Separation on 10% acrylamide sequencing gels of the oligonucleotide-cysteine conjugate indicated that oligonucleotide with one 3′-linked primary amine group gave a maximum of 50% conjugation with cysteine whereas oligonucleotide modified with a hexaethylene linker arm containing three amine groups showed almost 100% conjugation efficiency. When the activated hexaethylene linker arm-modified oligonucleotide was reacted with Fab', the conjugation between Fab' and oligonucleotide was specific for the hinge region at pH 7.0–7.5 when only cysteine groups are reactive and non-specific at pH 8.5 when lysine residues are also reactive. An average of one oligonucleotide was attached per Fab' as calculated from the 260:280 nm absorbance ratio of the conjugate following removal of free oligonucleotide by gel filtration and using the Beer's equations according to Kuijpers and colleagues [11]. Following conjugation with biotinylated oligonucleotide, the Fab' increased in size to 60 kDa. The 60 kDa band was confirmed as being Fab'-oligonucleotide conjugate by probing a Western blot with anti-sheep Fab'-HRP, stripping with glycine-HCl buffer and re-probing with streptavidin-HRP to detect the biotinylated oligonucleotide (Fig. 1a, lanes 3 and 4). Covalent linkage of the oligonucleotide to the Fab' was confirmed using reducing SDS-PAGE (Fig. 1b) in which bands of equal intensity were observed at 29 kDa and 38 kDa (intact light chain and oligonucleotide-conjugated heavy chain fragment, respectively), whereas the original $F(ab')_2$ gave only the 29 kDa band (unresolved intact light chain and heavy chain fragment). The addition of a highly negatively-charged DNA molecule should shift the isoelectric point of the Fab'. Isoelectric focusing showed that the pI of oligonucleotide alone was 3.7 and of unreacted $F(ab')_2$ was 5.2–9, whereas the Fab'-oligonucleotide conjugate gave two broad bands with pI values of 4.5–5 and 5.3–6.5 (data not shown).

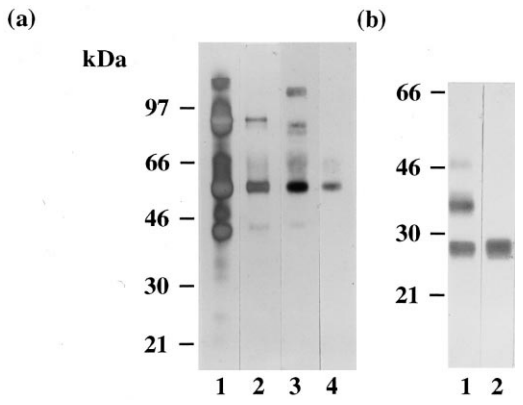

Fig. 1. (a) Sheep anti-mouse IgG Fab'-oligonucleotide-biotin conjugates separated on non-reducing 10% SDS-PAGE gels. Lane 1, silver stain of the reaction mixture; lane 2, silver stain following purification of the conjugate by size exclusion chromatography; lane 3, Western blot from a non-reducing 10% SDS-PAGE gel of purified conjugate probed with donkey anti-sheep Fab'-HRP; lane 4, Western blot of purified conjugate probed with streptavidin-HRP. (b) Reducing 12% SDS-PAGE. Silver stain. lane 1, sheep anti-mouse IgG Fab'-oligonucleotide-biotin conjugate; lane 2, sheep anti-mouse IgG $F(ab')_2$.

3.2. Oligonucleotide-conjugated Fab' is able to bind the complementary oligonucleotide

A Western blot of the goat anti-human IgG Fab'-oligonucleotide (non-biotinylated) was first probed with HRP-labelled donkey anti-goat Fab', stripped and then hybridized with biotinylated complementary oligonucleotide and re-probed with streptavidin-HRP. The calculated thermal stability of the hybridized complex measured as the melting temperature, *T*m,

was 40°C. Washing the hybridized blots at 20°C or 37°C showed that the oligonucleotide remained hybridized. However, washing at 55°C led to dissociation of the complementary oligonucleotide (results not shown).

3.3. Generation of bispecific antibody

Hybridized Fab'-oligonucleotide conjugate pairs form a stable complex which is of a higher molecular weight than unmodified F(ab')$_2$ (Fig. 2). The bispecific antibody is able to bind mouse IgG immobilized on a BIAcore chip (Fig. 3a) and subsequently bind fluid phase human IgG (Fig. 3b). Binding to the immobilized mouse IgG precludes the bispecific antibody binding fluid phase mouse IgG. Dual specificity was also observed using enzyme-linked immunosorbent assays (data not shown).

4. Discussion

Conjugation of oligonucleotides to antibodies has previously been aimed at generating reagents with a therapeutic potential such as the delivery of antisense sequences [11]. Oligonucleotide can be conjugated to antibody Fab' through the reduced disulfides of the hinge region, thus excluding the possibility of steric hindrance affecting antigen binding. We now show that antibodies may be linked together following conjugation of complementary oligonucleotides to the desired Fab' molecules and that the resulting hybridized complex is able to bind to antigen in a bispecific manner.

Oligonucleotide possessing a single available amine gave a maximum of 50% conjugation, whereas the use of a hexaethylene linker arm with three amino groups led to an almost 100% conjugation efficiency with an average of one oligonucleotide molecule coupled to each Fab' molecule. The majority of the Fab'-oligonucleotide molecules produced in this way were able to hybridize to complementary Fab'-oligonucleotide to generate bispecific antibody.

The *T*m of the oligonucleotides was 40°C and the bispecific antibodies remain hybridized at 37°C. Thus, it might be feasible to design bispecific antibodies which would not normally dissociate in vivo but would do so at sites of pyrexia, as during an inflammatory response during which temperatures of 39–40°C are reached [12]. This would then make the exposed oligonucleotide available for hydrogen binding to a more closely matched complementary oligonucleotide, if the design of the original oligonucleotide pair contained slight mismatches, or to a drug or other ligand able to target single-stranded nucleic acid.

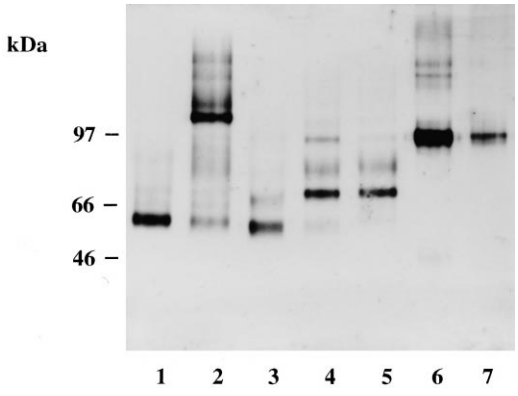

Fig. 2. Non-reducing 8% SDS-PAGE gel illustrating hybridization of the two oligonucleotide-Fab' conjugates to generate the bispecific antibody. Silver stain. Lane 1, sheep anti-mouse IgG Fab'-oligonucleotide-biotin conjugate; lane 2, oligonucleotide-linked bispecific antibody; lane 3, goat anti-human IgG Fab'-oligonucleotide conjugate; lane 4, goat anti-human IgG Fab'-oligonucleotide conjugate plus complementary oligonucleotide; lane 5, sheep anti-mouse IgG Fab'-oligonucleotide-biotin conjugate plus complementary oligonucleotide; lane 6, sheep anti-mouse IgG F(ab')$_2$; lane 7, goat anti-human IgG F(ab')$_2$.

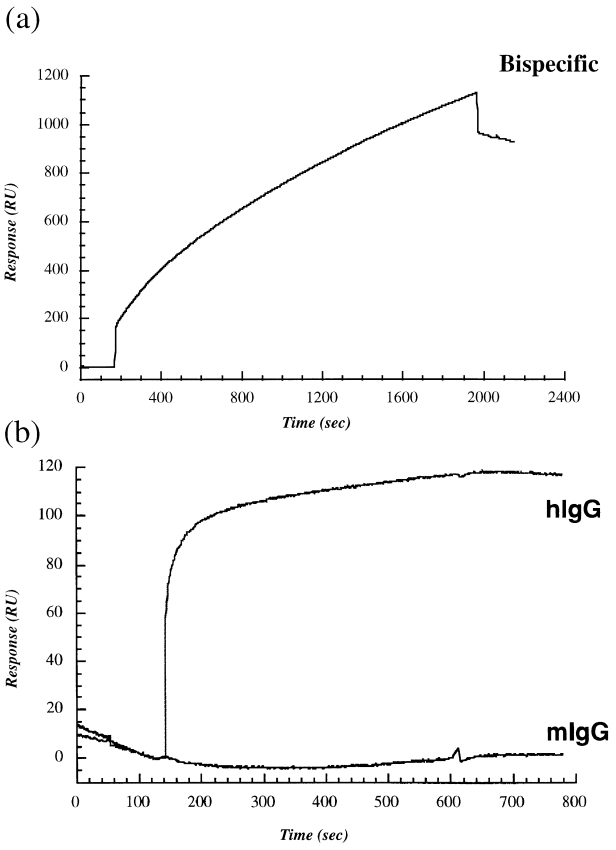

Fig. 3. (a) Passage of pre-formed oligonucleotide-linked bispecific antibody over mouse IgG immobilized on a BIAcore chip and (b) subsequent passage of fluid phase human IgG or mouse IgG.

The utility of bispecific antibodies against two different antigens has already been demonstrated in a number of situations including the targeting of FcγR1 on cytotoxic myeloid cells to the HER-2/neu proto-oncogene on tumor cells [13] and of the T-cell receptor on cytotoxic T-cells to vesicular stomatitis virus G protein on virus-infected cells [14]. Alternatively, dual epitope bispecific antibodies recognizing two non-overlapping epitopes on the same antigen would have an increased avidity for the antigen [6,15]. The use of oligonucleotides of a different chain length would introduce different spacing between the Fab's, so permitting optimization of the binding kinetics. For in vivo use especially, it may be advantageous to use peptide nucleic acids [16] in place of DNA in order to minimize the susceptibility to nucleases and to reduce the highly negative charge of DNA.

One novel feature of an antibody conjugated to an oligonucleotide is that it contains two types of biological information, namely, the antigen binding specificity of the antibody and the nucleic acid sequence of the oligonucleotide. The use

of such dual information has previously been exploited in antibody-directed PCR approaches [17]. In the current application, the sequence information encoded in an oligonucleotide-linked bispecific antibody could potentially be utilized to target a DNA binding protein. This could be generated as a fusion protein with, for example, an enzyme catalyzing the conversion of a pro-drug to a pharmacologically active compound. Oligonucleotide linkage to create dual or multispecific molecules is not limited to antibodies but can be utilized to connect any two molecules such as lectins, hormones, cytokines or adhesion molecules.

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