



Protein tag-mediated conjugation of oligonucleotides to recombinant affinity binders for proximity ligation

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While antibodies currently play a dominant role as affinity reagents in biological research and for diagnostics, a broad range of recombinant proteins are emerging as promising alternative affinity reagents in detection assays and quantification. DNA-mediated affinity-based assays, such as immuno-PCR and proximity ligation assays (PLA), use oligonucleotides attached to affinity reagents as reporter molecules. Conjugation of oligonucleotides to affinity reagents generally employs chemistries that target primary amines or cysteines. Because of the random nature of these processes neither the number of oligonucleotides conjugated per molecule nor their sites of attachment can be accurately controlled for affinity reagents with several available amines and cysteines. Here, we present a straightforward and convenient approach to functionalize recombinant affinity reagents for PLA by expressing the reagents as fusion partners with SNAP protein tags. This allowed us to conjugate oligonucleotides in a site-specific fashion, yielding precisely one oligonucleotide per affinity reagent. We demonstrate this method using designed ankyrin repeat proteins (DARPs) recognizing the tumor antigen HER2 and we apply the conjugates in different assay formats. We also show that SNAP or CLIP tags, expressed as fusion partners of transfected genes, allow oligonucleotide conjugations to be performed in fixed cells, with no need for specific affinity reagents. The approach is used to demonstrate induced interactions between the fusion proteins FKBP and FRB by allowing the *in situ* conjugated oligonucleotides to direct the production of templates for localized rolling circle amplification reactions.

Introduction

For biomedical research and diagnostics, there is a need for standardized high-performance assays to analyze proteins and their interactions and modifications *in situ*, in cell lysates, or in body fluids. Such assays are likely to play increasing roles in medical practice by revealing diagnostic protein patterns, and supporting the selection of optimal therapy. These applications

will require high-performance protein assays and broadly available repertoires of well-characterized affinity reagents for proteins of interest [1].

Currently, polyclonal or monoclonal antibodies are used in most affinity-based protein analyses, but several classes of recombinant affinity reagents are under development such as nanobodies [2], affibody [3], DARPs and others [4–6]. Such reagents can offer important advantages in terms of engineered specificity and affinity, and also in production. Because of properties such as excellent stability, small size and the possibility to engineer them

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for convenient application in specific assay formats, they can extend the range of applications of affinity reactions beyond those of antibodies. Examples of recombinant affinity reagents include molecules that represent fragments of antibodies and a range of scaffold proteins designed to exhibit contact surfaces that can be varied for binding to putative target proteins [4,5]. A scaffold protein with attractive properties for high-affinity protein binding is the designed ankyrin repeat proteins (DARPin) [6]. These are approximately 15–20 kDa in size (compared to 150 kDa for intact IgG antibodies) and they exhibit impressive stability, surviving for months at 37°C. Because the DARPins have no cysteines and fold well in the cytoplasm, they can also be expressed within the cell. A repertoire of DARPins directed against extracellular and intracellular target proteins is now available [6], and a particular effort has been devoted to generating DARPins against the ErbB receptor family, with affinities in the low nM to pM range [7–9].

Proximity ligation assay (PLA) can provide improved opportunities for measuring proteins [10] or more complex targets such as interacting or aggregated proteins [11] and exosomes [12] in blood samples and cell lysates [13]. The technique can also be applied to visualize endogenous protein interactions [14–16] and post-translational modifications [17] in cells and tissues. Detection of proteins or protein complexes using PLA requires binding by two or more

affinity reagents that are equipped with DNA oligonucleotides, and referred to as PLA probes. Upon proximal binding by a set of PLA probes amplifiable DNA strands, representative of proximal binding events, are formed by DNA ligation. The reaction provides high selectivity due to the requirement for multiple recognitions and good sensitivity of detection is ensured via amplified detection. For localized detection using *in situ* PLA, the DNA strands (*priming* and *non-priming*) coupled to affinity reagents serve first as templates for ligation, creating circular DNA molecules and then the *priming* acts as primer for localized amplification via rolling circle amplification (RCA) to form RCA products, containing hundreds of copies of the same oligonucleotide sequence. The RCA products are easily detected as bright fluorescent spots using fluorophore-labeled hybridization probes (Fig. 1A). These spots can then be digitally recorded using dedicated software [18]. Real-time PCR is generally used to measure ligation products representing proteins detected in solution phase such as in cell lysates (Fig. 1B).

The first demonstration of PLA reactions made use of DNA aptamers as affinity reagents for the targeted proteins [10], but in subsequent work poly- or monoclonal antibodies have generally been used. Several different chemistries are available to conjugate DNA strands to proteins. For antibodies, random coupling of oligonucleotides to primary amines is most commonly used

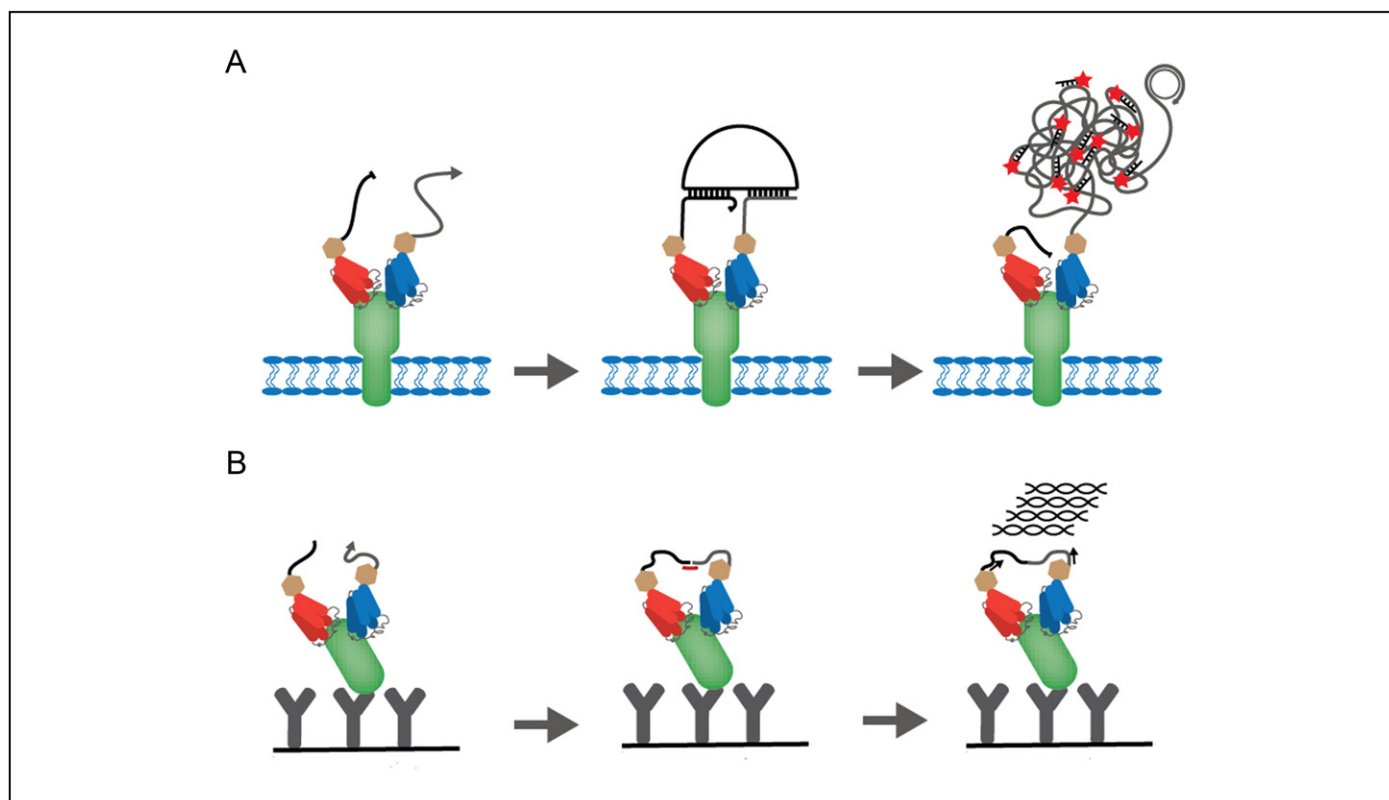


FIGURE 1

Schematic illustration of *in situ* PLA and solid-phase PLA using DARPin PLA probes. **(A)** In *in situ* PLA, targeted proteins or protein pairs are simultaneously bound by two DARPin PLA probes to which oligonucleotides (*priming* (with arrow) and *non-priming* (without arrow)) have been attached; circularization oligonucleotides are then hybridized to PLA probes in close proximity and enzymatically ligated to form amplifiable DNA circles. The circularized DNA is replicated with one of the PLA probes serving as a primer, forming an RCA product. Fluorophore-labeled detection oligonucleotides are then hybridized to the repeated sequence of the rolling circle products, resulting in bright sub-micrometer-sized DNA bundles. **(B)** In solid-phase PLA a sample (in this case cell lysate) is incubated with a target-specific antibody-coated solid phase. Upon simultaneous recognition of the target protein by a pair of DARPin PLA probes, the attached oligonucleotides are brought in close proximity and can be joined by enzymatic ligation in the presence of a connector oligonucleotide complementary to the free ends of the oligonucleotides. A DNA template is generated that can be exponentially amplified and quantified by quantitative PCR.

[11,13,19]. Conjugation of oligonucleotides to larger affinity reagents is relatively straightforward, but the process is poorly defined and target binding may be affected. It is therefore desirable to conjugate oligonucleotides in a site-directed fashion and preferably in a one-to-one ratio. For small proteins, where a significant fraction of the surface is potentially involved in binding to the target, random coupling of oligonucleotides is unsuitable. For DARPins, protocols have been reported for stoichiometric site-specific coupling of ligands such as polyethylene glycol (PEG) and fluorophore at the N-terminal amino group, at engineered cysteines or at modified amino acids capable of undergoing click chemistry [20]. These reactions require purification of the ligand (here an oligonucleotide) and the protein, as well as some considerations about buffer and reaction conditions. For high-throughput protein–oligonucleotide couplings, alternative strategies with lower demands on reagent purity are thus of interest. A mutant form of the human DNA repair protein O⁶-alkylguanine-DNA alkyltransferase reacts rapidly and specifically with O⁶-benzylguanine (BG) and also with derivatives that carry a large moiety linked to the benzyl group [21]. With guanine as the leaving group, the benzyl moiety becomes covalently attached to a cysteine in the active site of the enzyme. The enzyme has also been mutagenized to become specific for O⁶-benzylcytosine (BC) in a similar manner [22]. Both enzyme domains (about 20 kDa) are commercially available as SNAP and CLIP tags, respectively [22–24].

Here we have investigated the coupling of BG-modified DNA oligonucleotides to DARPins-SNAP fusion proteins. Using anti-HER2 DARPins [7–9] as a test case the resulting PLA probes were validated by visualizing expression and interactions of endogenous HER2 proteins in cells, and by measuring HER2 levels in cell lysates. Furthermore, we illustrated the possibility to demonstrate protein interactions *in situ* using BG- and BC-modified DNA oligonucleotides as probes for *in situ* PLA detection of SNAP or CLIP fusion proteins, expressed in transfected cells. The transfected cells were fixed and permeabilized and interactions were analyzed by adding BG- and BC-modified DNA oligonucleotides as PLA probes. The oligonucleotides coupled *in situ* guided the circularization of

two other DNA strands that were then amplified by RCA and visualized via hybridization of fluorophore-labeled oligonucleotides. We show that this form of *in situ* PLA could successfully demonstrate induced interactions between FKBP and FRB proteins fused to SNAP- and CLIP-domains, respectively, with no need for specific affinity reagents.

Materials and methods

Cloning, expression and purification of DARPins-SNAP fusion proteins

The ORFs for the anti-HER2 DARPins 9.01 [8] and G3 [7] were digested with *Bam*HI and *Hind*III (New England Biolabs, Ipswich, MA) and ligated into the compatible expression vectors pQiBi_11-SNAP or pQiBi_SNAP_11, which had been constructed by inserting the SNAP domain into vectors made for creating DARPins fusion proteins [9] (C. Gehring *et al.*, unpublished). This encodes DARPins N- or C-terminally fused to the SNAP-tag via an (G₄S)₂-linker. Fusion proteins were overexpressed in *Escherichia coli* XL1-Blue and purified via their N-terminal MRGSH₆ tag with nickel-nitrilotriacetic acid superflow resin (Qiagen, Hilden, Germany) giving yields of 25–40 mg of purified protein per liter expression culture [7,8].

Plasmid construction of FKBP-SNAP and FRB-CLIP gene fusion constructs

Gene construct encoding a fusion protein of FKBP (FK506-binding protein) and the SNAP-tag, or construct encoding a fusion protein of FRB (FKBP12/rapamycin-binding domain) and the CLIP-tag were each cloned in a pNuc vectors as previously described [25], hereafter, referred to as FKBP-SNAP and FRB-CLIP. To identify transfected cells, a GFP-encoding vector, the pLPS-AcGFP1-N vector (Cat. No. 632472; Clontech), was co-transfected with the FKBP-SNAP and FRB-CLIP gene fusion constructs.

Preparation of PLA probes

50 μL of 100 μM aldehyde-modified oligonucleotides (*priming*, *non-priming*, *biovic 1* and *biovic 2*, Table 1) were incubated for 3 hours at

TABLE 1
Oligonucleotide sequences

Name	Sequence	Modification	Company
Priming	AAAAAAAAAAGACGCTAATAGTTAAGACGCTT	5'-Aldehyde, or 5'-thiol	Trilink Biotechnology
Non-priming	AAAAAAAAAATATGACAGAACTAGACACTCTT	5'-Aldehyde	Trilink Biotechnology
Circularization oligonucleotide 1	CTATTAGCGTCCAGTGAATGCGAGTCCGTCTAA-GAGAGTAGTACAGCAGCCGTCAAGAGTGTCTA	5'-Phosphate	Integrated DNA Technology
Circularization oligonucleotide 2	GTTCTGTGTCATTTAAGCGTCTTAA	5'-Phosphate	Integrated DNA Technology
Detection oligonucleotide	CAGTGAATGCGAGTCCGTCT	5'-Alexa fluor 555	Trilink Biotechnology
Biovic 1	CGCATCGCCCTTGGACTACGACTGACGAACCG-CTTTGCTGACTGATCGCTAAATCGTG	5'-Aldehyde	Eurogentec
Biovic 2	TCGTGTCTAAAGTCCGTACCTTGATTCCTCA-ACCCTCTTGAAAAATTCGGCATCGGTGA	5'-Phosphate, 3'-aldehyde	Eurogentec
Fwdprimer	CATCGCCCTTGGACTACGA		Integrated DNA Technology
Revprimer	GGAATCAAGGTAACGGACTTTAG		Integrated DNA Technology
Connector	TACTTAGACACGACGATTAGTTT		Integrated DNA Technology
Taqman probe	TGACGAACCGCTTGCCTGA	5'-FAM, 3'-MGB	Applied Biosystems

TABLE 2

PLA probes

PLA probe	DARPin	Target protein	Oligonucleotide
Probe 1	SNAP_G3	HER2	<i>Non-priming</i>
Probe 2	G3_SNAP	HER2	<i>Priming</i>
Probe 3	SNAP_9.01	HER2	<i>Priming</i>
Probe 4	SNAP_G3	HER2	<i>Biovic 1</i>
Probe 5	SNAP_9.01	HER2	<i>biovic 2</i>
PLA probe	5' modification	Specific for	Oligonucleotide
Probe 6	BC-maleimide	CLIP-tag	<i>Priming</i>
Probe 7	BG-hydrazine	SNAP-tag	<i>Non-priming</i>

37°C with a 30-fold molar excess of BG-hydrazine (5 mM in dimethylformamide (DMF); Covalys, Switzerland) in conjugation buffer (100 mM MES, 150 mM NaCl, pH 4.8). The BG-modified oligonucleotides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) using a 4.6 mm × 250 mm silica matrix column with a particle size of 5 μm (Lichrosphere RP18 column; Supelco, Inc.), a 60-min gradient of 2–60% B (A: 0.1 M triethylamine acetate (pH 7.0); B: acetonitrile) and a flow rate of 1 mL/min. The reactive cysteine in the SNAP-tag-DARPin fusion proteins (G3 and 9.01) were reduced in 20 mM DTT, and incubated overnight at 4°C in 1 mM DTT with a 3-fold molar excess of BG-modified oligonucleotides, resulting in PLA probes G3-SNAP-*priming*, *non-priming*-SNAP-G3, *priming*-SNAP-9.01, *biovic1*-SNAP-G3 and *biovic2*-SNAP-9.01 (Table 2). No purifications of the conjugates were undertaken, because remaining unconjugated oligonucleotides were removed by washes in the *in situ* and solid phase PLA reactions. The formation of conjugates was validated by separating the products in a SDS polyacrylamide gel (GeneGel Excel 12.5%; GE Healthcare), followed by protein silver staining (PlusOne™ silver staining kit; GE Healthcare) (Fig. 1A), band intensities were measured using Image J and the conjugation efficiency was calculated via the intensity of the conjugate band divided by the intensity of the non-conjugated SNAP-DARPin band. The anti-HER2 antibody pertuzumab and the anti-HER2 polyclonal antibody (AF1129, R&D Systems) were conjugated separately with aldehyde-modified *priming* and *non-priming* oligonucleotides and with aldehyde-modified *biovic 1* and *biovic 2* oligonucleotides (Table 1), respectively, as previously described [26]. In preparing probes for *in situ* analysis of FKBP and FRB interaction, 50 μL of 100 μM thiol-modified *priming* oligonucleotide was reduced in 20 mM DTT for 1 hour at 37°C. The reduced oligonucleotide was purified by MicroSpin G-50 column (GE healthcare) and incubated for 2 hours at room temperature (RT) with a 30-fold molar excess of BC-maleimide, followed by RP-HPLC purification as described above. The preparation of *non-priming*-BG was performed in the same way as for aldehyde-modified *non-priming* oligonucleotide (Table 2).

Cell cultures

The HER2 expressing breast cancer cell line (SK-BR-3) and ovarian cancer cell line (SK-OV-3) were grown in RPMI1640 medium and the human embryonic kidney cell line HEK 293T was grown in Dulbecco's modified Eagle's medium (DMEM). All media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and

1% penicillin-streptomycin (all reagents from Sigma Aldrich). The cells were grown at 37°C in a humidified 5% CO₂ environment.

Detection of HER2 in cells by *in situ* PLA

10,000 cells were seeded per well and cultivated overnight on Lab-Tek Chamber Slides (Nalge Nunc International). Cells fixed in EtOH were blocked in blocking buffer (1 mg/mL BSA, 20 μg/mL salmon sperm DNA, 2 mM cysteine, 0.05% Tween20 in TBS buffer) for 1 hour at 37°C, followed by incubation with 40 μL of DARPin PLA probes (*non-priming*-SNAP-G3 (2.4 nM) + G3-SNAP-*priming* (2.4 nM), *non-priming*-SNAP-G3 (4.8 nM) + *priming*-SNAP-9.01 (23 nM), Table 2) diluted in blocking buffer ON at 4°C. Circularization oligonucleotide 1 and 2 (125 nM each, Table 1) in ligation buffer (0.25 mg/mL BSA, T4 ligase buffer, 1 mM ATP, 250 mM NaCl, 0.05% Tween20, 0.05 U/μL T4 DNA ligase (Fermentas)) were incubated at 37°C for 30 min. After ligation rolling circle amplification (RCA) was performed (0.25 mg/mL BSA, phi29 polymerase buffer, 0.25 mM dNTP, 0.125 U/μL phi29 polymerase (Thermo Scientific)) for 90 min at 37°C followed by hybridization of 250 nM detection oligonucleotide (Table 1) in detection buffer (2 × SSC, 0.25 μg/μL BSA, 75 ng/μL polyA, 0.05% Tween20) to the single-stranded RCA product for 30 min at 37°C. All steps were separated by two washes in TBS + 0.05% Tween20 (TBST). The slides were counterstained with DAPI (100 μg/mL) in Vectashield mounting medium (Vector Laboratory). The same protocol was used for the pertuzumab PLA probe (10 nM of each probe).

Detection of HER2 in cell lysate by solid-phase PLA

1.2×10^5 SK-BR-3 cells or HEK 293T cells were lysed in 150 μL cold lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 0.5% Triton X-100, pH 7.4) supplemented with a Complete Protease Inhibitor Cocktail Tablet (Roche). Tubes (Robo-strip®, AJ Roboscreen) were coated with pertuzumab (1 ng/μL) in carbonate buffer (50 mM Na₂CO₃, 50 mM NaHCO₃, pH 9.6) for 1 hour at 37°C. After blocking for 1 hour at 37°C in PBS containing 1% BSA, 0.1% salmon sperm DNA and 0.05% Tween20, the tubes were incubated with 50 μL of cell lysate at 4°C ON. Subsequently, 50 μL of DARPin PLA probes (2 nM, *biovic1*-SNAP-G3 and *biovic2*-SNAP-9.01) or anti-HER2 antibody PLA probes (1 nM) was added to the tubes and incubated for 1.5 hours at 37°C. All steps were separated by three washes in PBS with 0.05% Tween20 (PBST). Thereafter, 50 μL ligation and PCR mix (PCR buffer (Invitrogen)), 100 nM connector oligonucleotide (Table 1), 2.5 mM MgCl₂, 0.4 units of T4 DNA ligase (Fermentas), 0.1 U uracil-DNA-glycosylase (Fermentas), 80 μM ATP, 0.2 mM dNTPs containing dUTP, 0.1 μM primers (*fwdprimer* and *revprimer*, Table 1), 220 nM Taqman probe (Table 1) were added. The real-time PCR was performed in a Mx-3000 instrument (Stratagene), with an initial incubation at 95°C for 2 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Solid-phase PLA was also performed with lysate buffer alone as a negative control. The results are presented as threshold cycle (Ct) values [27], reflecting the amount of PLA ligation products, and as ΔCt value where the Ct values for lysate buffer have been subtracted from the Ct value for the cell lysate.

Detection of FKBP and FRB interaction

FKBP-SNAP and FRB-CLIP fusion encoding plasmids were co-transfected together with a GFP-expressing vector and transiently

expressed in HEK 293T cells. Transfection using Lipofectamine[®] 2000 (Invitrogen) was carried out according to manufacturer's protocol using 2.5 μ g of plasmid and 7.5 μ L Lipofectamine and incubation of 50 μ L of plasmid-Lipofectamine complex for 24 hours. In this study, the FKBP-SNAP expressing plasmid (1.125 μ g) and the FRB-CLIP expressing plasmid (1.125 μ g) were co-transfected together with the GFP expressing vector (0.25 μ g) into HEK 293T cells. Thereafter, 15,000 cells/well were seeded on collagen-type 1-coated slides (BD Biocoat) and cultivated for 4 hours. Rapamycin (dissolved in DMSO, Sigma-Aldrich) was diluted in 200 μ L of antibiotics-free growth medium to concentrations 0.2 μ M, 1 μ M, 5 μ M, 20 μ M and 40 μ M and incubated with cells for 6 hours at 37°C in a humidified incubator with 5% CO₂, keeping the final concentration of DMSO at 0.1% (v/v). In parallel,

0.1% DMSO was included as a control for the effect of DMSO on the FKBP/FRB interaction. Cells were then fixed in 3% paraformaldehyde (PFA) (Sigma-Aldrich) for 15 min at RT, permeabilized for 15 min in 0.1% Triton X-100 (Sigma-Aldrich) in PBS, and briefly washed in PBS. 20 nM of *priming*-BC and 20 nM *non-priming*-BG in PBS was applied to the cells and incubation for 1 hour at 37°C was followed by 3 \times 5 min washing in TBST. The *in situ* PLA was performed as described above by steps of hybridization of circularization oligonucleotides, RCA and detection.

Image analysis

Images of all cell experiments were taken in a Zeiss Axioplan II epifluorescence microscope, using an AxioCam MRm CCD sensor and a 40x/1.3 Oil PlanNeofluar objective together with filters for

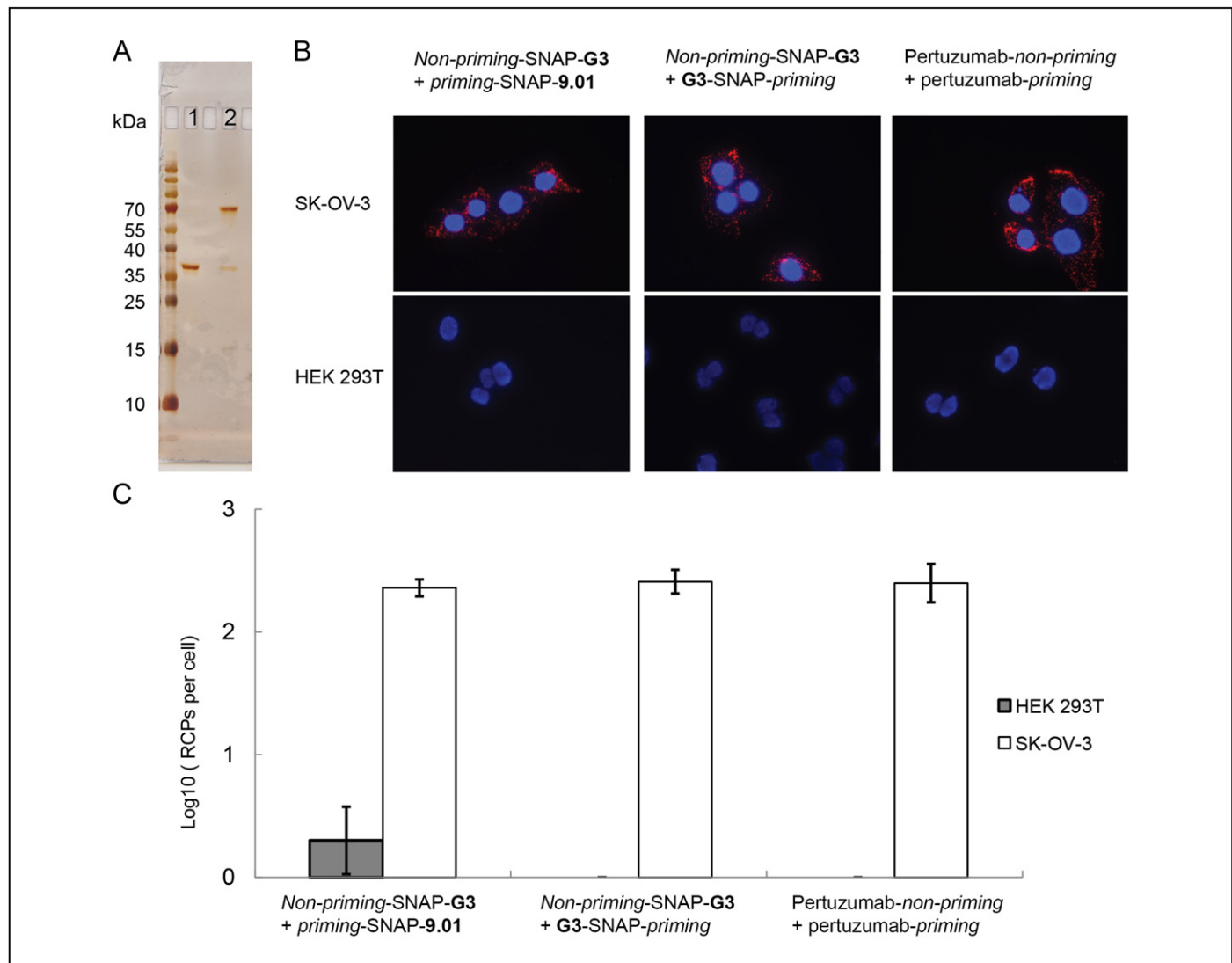


FIGURE 2

Detection of HER2 in cells using DNA-modified DARPins and antibodies. **(A)** SDS-PAGE gel demonstrating the conjugation of SNAP-9.01 (37.9 kDa) (lane 1) with the *biovic 1* oligonucleotide, for production of a PLA probe (lane 2). Molecular weights of marker proteins (Fermentas) are shown in kDa. Proteins were visualized by protein silver staining, and the conjugation efficiency was estimated using Image J. **(B)** *In situ* PLA showing HER2 proteins in SK-OV-3 and HEK 293T cells using two different DARPins, one binding to domain IV (*non-priming*-SNAP-G3) and another one binding to domain I (*priming*-SNAP-9.01) (left panel), both DARPins binding to domain IV (*non-priming*-SNAP-G3 and G3-SNAP-priming) (middle panel), and pertuzumab PLA probes (right panel). Red dots are RCA products representing HER2 proteins. **(C)** Numbers of RCA products representing HER2 proteins in SK-OV-3 cells (white bars) and HEK 293T cells (grey bars) detected using *in situ* PLA, as described in **(B)**. Data were generated in triplicates and the quantification was performed in Duolink ImageTool (Olink Bioscience). Y-axis of the plot is in log scale and error bars indicate standard deviations.

DAPI, FITC and Cy3. PLA signals from HER2 detection in cells were digitally quantified using Duolink ImageTool (Olink Bioscience, Sweden). For quantification of the number of PLA signals derived from FKBP/FRB interactions, only cells displaying signals both in the FITC channel (GFP transfected cells) and in the Cy3 channel (from the RCA products) were counted digitally using the Duolink ImageTool.

Results and discussion

Preparation of PLA probes using SNAP- and CLIP-tag technology and verification by *in situ* and solid-phase PLA

Simple and efficient methods to attach DNA strands to recombinant affinity proteins are needed to functionalize the reagents for use in methods such as immuno-PCR and PLA [28]. Here, we demonstrate a straightforward approach to convert recombinant affinity reagents into PLA probes by using SNAP-tag fusions of DARPIn affinity reagents for site-specific and stoichiometrically controlled conjugation of oligonucleotides. We wished to evaluate if the reactivity of the 20 kDa SNAP and CLIP tags, engineered to react covalently with benzyl-guanine or benzyl-cytosine derivatives, could be exploited to attach oligonucleotides to proteins. Two different approaches were used to obtain BG- (or BC-) derivatized oligonucleotides. Aldehyde-oligonucleotide was first coupled to hydrazine-BG and the product was purified via RP-HPLC. An analogous reaction was also carried out between thiol-modified oligonucleotide and the corresponding maleimide-BC derivative. The BG-coupled oligonucleotides were then incubated with DARPins fused to the SNAP domain tag, resulting in conjugates with a one-to-one ratio of oligonucleotides to affinity reagents. In this manner, 75–85% of total DARPIn-SNAP-tag fusion proteins were successfully coupled to an oligonucleotide each, as exemplified by the DARPIn PLA probe shown in Fig. 2A. Because the BG- and BC-modified oligonucleotides are stable for long periods they can be prepared in large batches, facilitating DNA

conjugation to many SNAP- or CLIP-labeled reagents. Two DARPins were used in these studies that both recognize the extracellular part of the human HER2 membrane protein, frequently overexpressed in, for example, breast cancer [29]. G3 has a K_D of about 100 pM to domain IV of HER2 [7,9], and 9.01 [8] binds domain I with a K_D of about 80 nM (C. Jost *et al.*, unpublished). Because it was not clear *a priori* whether the 20-kDa SNAP domain might interfere with binding by the DARPIn to HER2 on the cell, constructs were prepared with the SNAP domain either at the N- or C-termini of the DARPins. All products were obtained from *E. coli* at the same high yield as unfused DARPins.

The HER2-specific DARPIn-SNAP conjugates (PLA probes) were applied for HER2 expression analyses both by *in situ* PLA (schematic illustration of *in situ* PLA in Fig. 1A), and in solid-phase PLA of cell lysates with quantitative PCR readout (schematic illustration of solid-phase PLA in Fig. 1B). When two different DARPins are used to bind different epitopes of HER2, then both mono- and dimeric HER2 can be detected, while with DARPins binding to the same epitope (conjugated to different oligonucleotides), only homo-dimeric or clustered forms of HER2 would be detected. With HER2-overexpressing SK-OV-3 cells, the signals obtained using two different DARPIn-based PLA probes (referred to as *non-priming*-SNAP-G3 and *priming*-SNAP-9.01) or only one DARPIn (*non-priming*-SNAP-G3 and G3-SNAP-*priming*) are about the same. This suggests that most of the HER2 proteins are in a clustered state on this cell line (Fig. 2B,C). Clustering of highly overexpressed HER2 on tumor cells is consistent with previous observations [30]. Using pairs of PLA probes based on the therapeutic monoclonal antibody pertuzumab, similar results were obtained (Fig. 2B,C). By contrast, HEK 293T cells are known to express low but still measurable amounts of HER2 [31]. In these cells, HER2 is not expected to be oligomeric. Indeed, PLA signals were only seen when two different DARPins were used (Fig. 2B,C), but not with only one DARPIn or with pertuzumab.

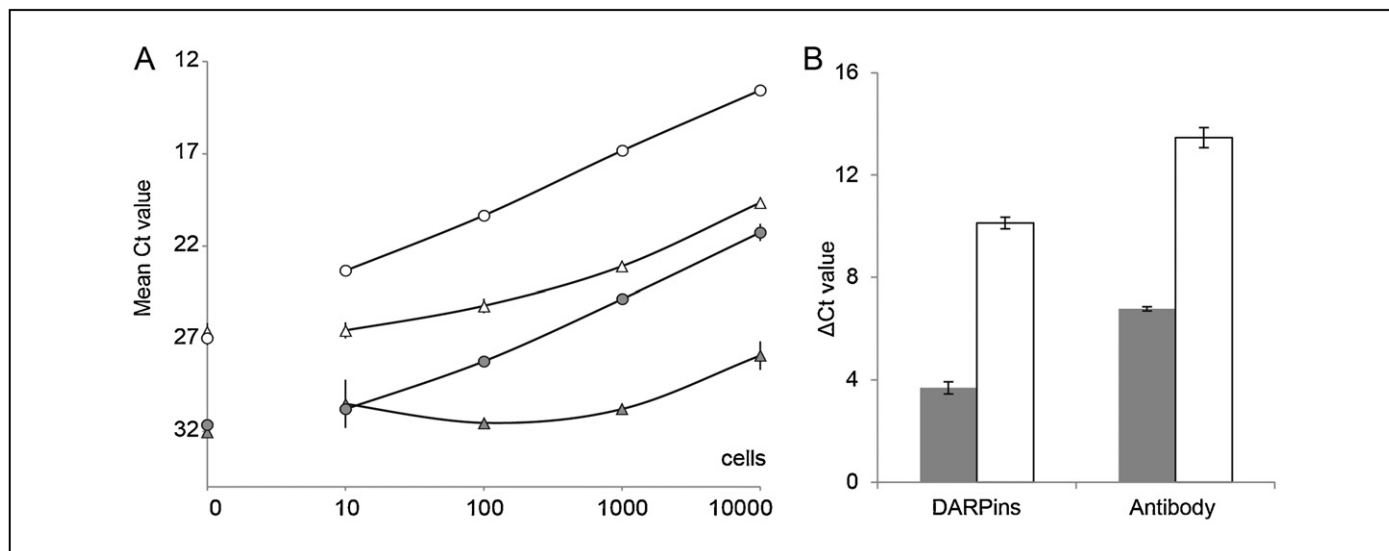


FIGURE 3

Comparison of HER2 detection in cell lysates using DARPins and antibody by solid-phase PLA. (a) Performance of solid-phase PLA in cell lysates using two different DARPins, one binding to domain IV (*biovic1*-SNAP-G3), the other binding to domain I (*biovic2*-SNAP-9.01) (in SK-BR-3 cell lysate, filled circles; in HEK 293T, filled triangles), or polyclonal antibody against HER2 (in SK-BR-3 cell lysate, open circles; in HEK 293T, open triangles). (b) Calculation of ΔCt from the data shown in (a) by subtracting the Ct value of lysis buffer from the Ct value of cell lysates (10^4 cells). Lysates from SK-BR-3 cells are shown in white bars and from HEK 293T cells in dark grey bars. Mean values with standard deviations for triplicate measurements are shown.

The DARPIn-SNAP PLA probes were also applied for protein measurements in cell lysates by solid-phase PLA with quantitative PCR readout. Here, HER2 levels in cell lysates from HER2 expressing SK-BR-3 cells and low HER2-expressing HEK 293T cells were compared. HER2 proteins were captured by pertuzumab immobilized on solid support and assayed using either two different DARPIn PLA probes, one binding to domain IV (*biovic1*-SNAP-G3) and the other binding to domain I (*biovic2*-SNAP-9.01), or an anti-HER2 polyclonal antibody divided in two aliquots, each conjugated to one of two different oligonucleotides. Both experiments revealed the expected difference in levels of HER2 proteins between the two lysates (Fig. 3). It should be pointed out that the two DARPins used both recognize conformational epitopes, and thus lysate preparations that even partially denature HER2 risk decreasing assay sensitivity. The

polyclonal anti-HER2 antibody (AF1129, RnD systems) that was used in this assay, together with pertuzumab as capture antibody on solid support, has been shown to detect HER2 in Western blots and must thus be able to detect denatured forms of HER2, or unfolded regions. The polyclonal antibody might simultaneously bind different epitopes of HER2 and there may be several oligonucleotides attached per antibody. By contrast, the DARPins are monoclonal and strictly contain only one oligonucleotide each, and will therefore produce less signals overall than the antibody-based assay, both for lysates from SK-BR-3 cells expressing high levels of HER2 and from HEK 293T cells (Fig. 3A). The differences in HER2 detection signals between SK-BR-3 and HEK 293T cells were similar, however, using the two classes of affinity reagents (Δ Cts of 6.4 and 6.7, for DARPins and antibodies respectively) (Fig. 3B).

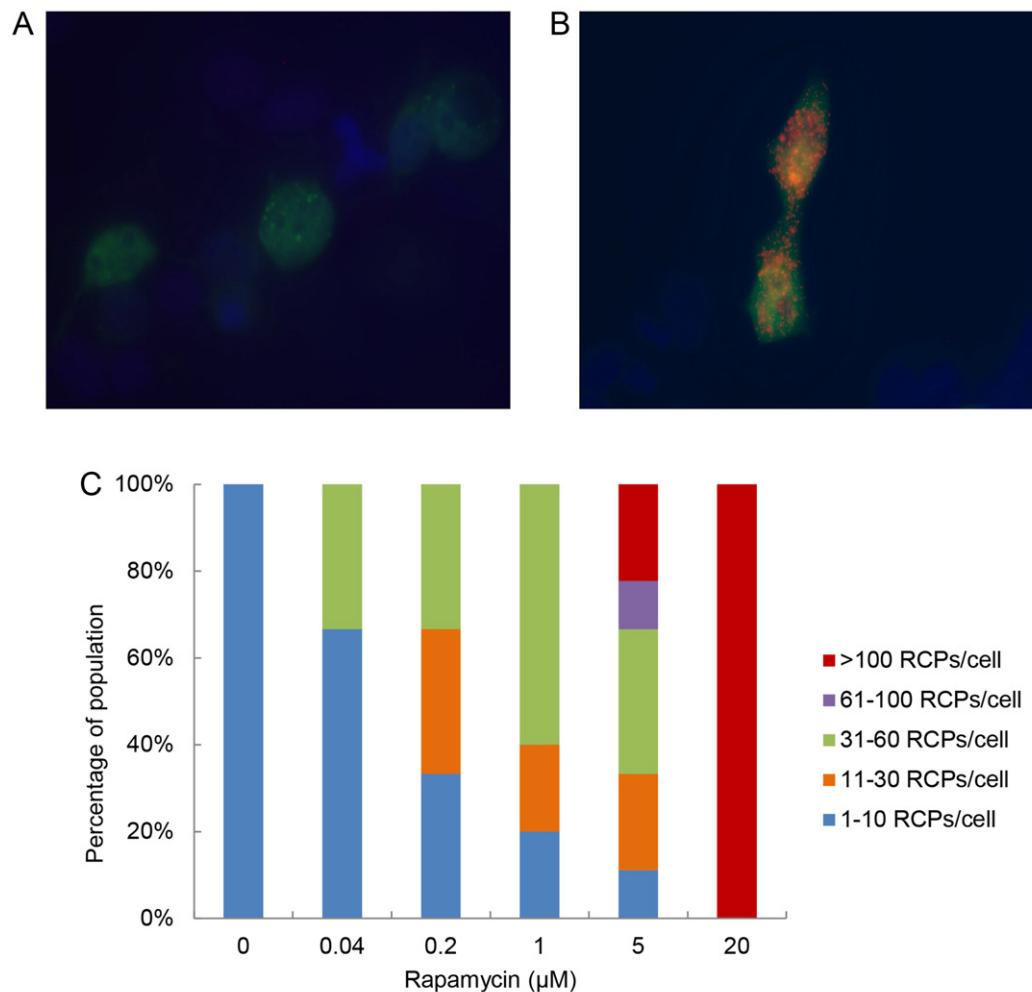


FIGURE 4

In situ detection of rapamycin-mediated interactions between FKBP and FRB fusion proteins via their fused SNAP and CLIP domains. HEK 293T cells were co-transfected with plasmids expressing FKBP-SNAP and FRB-CLIP fusion and with GFP-expressing plasmids. After 24 hours the cells were analyzed without (A) and with treatment with 20 μM of rapamycin (B) for 6 hours. *In situ* PLA with PLA probes (*priming*-BC and *non-priming*-BG) was applied for detection of interaction of FKBP and FRB fusion proteins in the absence and presence of rapamycin, and the RCA product detected with Alexa 555-labeled oligonucleotide (red) in GFP expressing cells (green). The cells were counterstained with DAPI (blue) to visualize the nuclei. GFP (green) served as an indicator of successful co-transfection and expression in cells. (C) Bar graph demonstrating the frequency of cells with the indicated numbers of RCA products (RCPs), as a function of the concentration of rapamycin in the culture media. On average 50 cells were investigated for each concentration of rapamycin in three experiments and analyzed using automated image analysis (Duolink ImageTool).

In situ conjugation of SNAP- and CLIP-fusion proteins

At times it is desirable to measure binding to and interactions among proteins for which no suitable affinity reagents exist. We investigated the possibility of transfecting cells with SNAP- and CLIP-fusion gene constructs to study protein interactions with no need for antibodies or any other specific protein-binding reagents. We tested this strategy using FKBP and FRP as model system, as interaction between these proteins can be induced by rapamycin. Rapamycin binds with high affinity ($K_D = 0.2$ nM) to the 12-kDa FK506 binding protein (FKBP) as well as to a 100-amino acid domain of the mammalian target of rapamycin (mTOR), known as the FKBP-rapamycin binding domain (FRB), thereby forming a FKBP-rapamycin-FRB ternary complex [32,33].

In cells transfected with SNAP- and CLIP-domains fused to FKBP and FRB proteins, respectively, the fusion proteins were conjugated *in situ* with BG- and BC-modified DNA oligonucleotides. Colocalization of the two oligonucleotides was investigated, by addition of two connector oligonucleotides, capable of being ligated into a DNA circle in reactions templated by the BG- and BC-oligonucleotides. We demonstrated successfully circularized oligonucleotides via RCA, followed by detection of the amplification products using fluorophore-labeled oligonucleotides, complementary to the repeated sequence of the RCA products. In the absence of rapamycin, the transfected HEK 293T cells, expressing FKBP-SNAP and FRB-CLIP, gave rise to only very few *in situ* PLA reaction products (1–10 RCPs per cell) (Fig. 4A), whereas upon incubation with 20 μ M rapamycin the numbers of signals per cell increased to greater than 100 per cell (Fig. 4B,C).

This approach provides a way to use *in situ* PLA where proteins of interest can be expressed as fusion proteins with the SNAP or CLIP domains after transfection. This strategy could also be combined with the use of specific affinity reagents in situation where such a reagent is only available against one partner in a protein interaction. The procedure, which works on cells that have been fixed and permeabilized, provides a complementary method to *in situ* interaction analysis of genetically modified living cells via Förster

resonance energy transfer (FRET) of pairs of fluorescent protein domains that are expressed as fusions to the proteins of interest [34,35] or by using complementing protein domains on fusion proteins [36]. Although the *in situ* PLA approach, unlike FRET and protein complementation techniques, is limited to fixed cells, the strong signal amplification can be of value because it enables detection of individual interaction events using standard microscopy.

Conclusion

Because the reaction of BG- or BC-modified oligonucleotides with SNAP or CLIP tags is a robust and specific procedure, this strategy will be helpful to functionalize a large range of recombinant affinity reagents for proteomics investigation through the addition of DNA strands. It thus provides a general means for production of highly stable, renewable DNA-conjugated affinity reagents with one oligonucleotide added in a site-specific fashion per affinity reagent molecule. This was demonstrated for HER2-specific DARPins PLA probes. We also demonstrate that BG- and BC-modified oligonucleotides can be used as general affinity reagents directly in cells transfected with fusion gene constructs.

Conflicts of interest

Ulf Landegren is a co-founder and stockholder of Olink Bioscience, having rights to the PLA technology. Andreas Plückthun is a co-founder and stockholder of Molecular Partners AG, having rights to the DARPins technology.

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