Heterobifunctional linker between antibodies and reporter genes for immunoassay development

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Abstract:

The amplification inherent in transcription and translation of DNA has already been exploited for the development of highly sensitive immunoassays by using a reporter gene as a label that, upon in vitro expression, generates multiple enzyme molecules in solution (expression immunoassay). The most challenging task in the development of an expression immunoassay is to link the antibody to a reporter gene that also contains control elements for transcription/translation. In this work, we prepare heterobifunctional linkers that consist of a modified avidin or streptavidin covalently attached to an oligonucleotide (dA)40. (Strept)avidin interacts with a biotinylated detection antibody whereas the oligonucleotide hybridizes with a complementary poly(dT) tail added enzymically to the 3′ end of the reporter gene. The linker is evaluated in a model two-site (sandwich-type) immunoassay performed in microtiter wells. A 4.3 kb plasmid containing the firefly luciferase cDNA is used as a reporter.

Keywords: immunoassay | in vitro expression | bioluminescence | chemistry | biochemistry

Article:

1. Introduction

A reporter gene is a DNA fragment coding for a protein that can be readily detected at low concentrations. Most widely used reporter genes encode enzymes (e.g. chloramphenicol acetyl transferase, alkaline phosphatase, luciferase, etc.), fluorescent proteins (GFP) and photoproteins (aequorin). Analytical applications of reporter genes include [1], (i) the spatial and temporal

monitoring of gene expression in vivo[2] and [3], (ii) the investigation of the strength and developmental regulation of promoters/enhancers fused to the reporter gene [2] and [3], (iii) the construction of light-emitting biosensors based on whole cells transfected with a reporter gene that is fused to an inducible promoter [1] and [4], and (iv) the development of in vitro immunoassays and hybridization assays for determination of antigens and nucleic acids [5], [6],[7] and [8].

Expression immunoassay uses a reporter gene as a label [5], [6] and [7]. Following completion of the immunoreaction, the reporter gene undergoes an in vitro (cell-free), coupled transcription and translation to generate multiple active enzyme molecules per immunocomplex. Therefore, gene expression provides another significant amplification step in addition to substrate turnover, thereby improving greatly the detectability of the assay. All reagents required for gene expression are added as a mixture in a single step. It has been shown [5], [6] and [7] that, although the transcription and translation process consists of a series of reactions that require the concerted action of several factors, the final outcome is a reproducible, linear relationship between the amount of input DNA and the activity of the synthesized enzyme.

Currently, the most challenging task in the development of an expression immunoassay is to link the antibody to the reporter gene (or the entire vector), which is a several kilobase long double-stranded DNA fragment that also contains control elements for transcription and translation. A functional macromolecular complex between antibody and reporter gene should retain both the binding affinity for the antigen and the expressibility of the DNA fragment. In previous reports [5] and [7], a streptavidin–reporter gene complex was prepared by using a series of tedious and time-consuming steps. A plasmid was first linearized followed by a fill-in reaction using Klenow DNA polymerase and biotin-dNTP to produce fragments that were biotinylated at both 3′ termini. There are four binding sites on each (strept)avidin molecule [9]. In order to avoid cyclization and polymerization of biotinylated DNA fragments during complexation, one of the DNA termini was first removed by digestion. The digested fragments were separated by gel electrophoresis, and the fragment containing the reporter gene was purified directly from the gel. The complexation was carried out with an excess of streptavidin and the complexes were purified by size-exclusion HPLC. The overall yield was 10–20% due to the multiple purification steps involved. The difficulty in preparing the complex constitutes the main obstacle for the wider use of gene expression as a highly sensitive amplification system in immunoassays.

The objective of the present work was to eliminate all the above difficulties by preparing a heterobifunctional linker between antibody and reporter gene. It consists of (strept)avidin covalently attached to an oligonucleotide $(dA)_{40}$. The linker is universal because (strept)avidin interacts with any biotinylated antibody or any biotinylated binder in general, such as peptides or receptors. The oligonucleotide $(dA)_{40}$ hybridizes with a complementary poly(dT) tail added enzymically to any reporter gene. Both biotinylation of binders and tailing of DNA are reactions performed readily without interfering with the respective biological activity. The linker is

applied to a model sandwich-type immunoassay for prostate specific antigen (PSA) using a 4.3 kb plasmid containing the firefly luciferase cDNA as a reporter.

2. Experimental

2.1. Instrumentation

Immunoasssays were performed in microtiter wells using the Amerlite shaker/incubator (Amersham, Oakville, Ont., Canada). The microtiter plate washer, model EAW II, was from SLT-Lab Instruments, (Salzburg, Austria). Luminescence measurements were carried out using a liquid scintillation counter (model LS-6500, Beckman Instruments Inc., Fullerton, CA) in the single-photon monitoring mode.

2.2. Materials

Bgl I was obtained from New England Biolabs (Beverly, MA). Terminal deoxynucleotidyl transferase, streptavidin (SA), bovine serum albumin (BSA), and blocking reagent (cat. #1096176) were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). *N*-Succinimidyl-*S*-acetylthioacetate (SATA), sulfo-*N*-hydroxysuccinimide ester of biotin (sulfo-NHS-LC-biotin), sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) and maleimide-activated neutravidin were from Pierce (Rockford, IL). T7 RNA polymerase, deoxyribonucleotides (dNTPs, 100 mmol/l solutions), NAP columns were from Amersham Pharmacia Biotech (Piscataway, NJ). Centricon-30 concentrators were from Amicon Inc. (Beverly, MA). Solutions of various concentrations of PSA were prepared by diluting PSA (Scripps Laboratories, CA) in 50 mmol/l Tris, pH 7.8, 60 g/l BSA, and stored at 4 ° C. Monoclonal capture (cat. #8301) and detection (cat. #8311) anti-PSA antibodies were obtained from Diagnostic Systems Laboratories (Webster, TX) [10]. Linear DNA markers (lambda-DNA digested with Eco RI and Hind III and containing fragments from 1.2 to 21.2 kbp), EDTA, ethidium bromide, coenzyme A, tricine, and hydroxylamine hydrochloride were from Sigma (St. Louis, MO). U-bottom, transparent, polystyrene microtiter wells (Nunc, Maxisorp) were obtained from Life Technologies (Burlington, Ont., Canada). The TNT T7 rabbit reticulocyte lysate, used for the in vitro transcription‑translation reactions, and beetle luciferin are available from Promega Corp. (Madison, WI).

The oligodeoxynucleotides $(dA)_{40}$ and a 17mer (5′ -GTAGGCCTCAGCTGGAA-3′), both modified with a primary amino group at the 5′ end, were synthesized by Oligos Etc Inc. (Wilsonville, OR).

The wash solution consisted of 50 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, and 1 ml/l Tween-20. The assay buffer contained 50 mmol/l Tris, pH 7.8, 60 g/l BSA, 0.5 mmol/l KCl, 0.5 g/l NaN_3, and 0.5 g/l Triton X-100. The conjugation buffer contained 0.1 mol/l sodium phosphate, pH 7.0, and 5 mmol/l EDTA. The blocking solution consisted of 10 g/l blocking reagent, 100 mmol/l maleic acid, and 150 mmol/l NaCl, pH 7.5. The hybridization buffer contained 60 mmol/l sodium citrate, 0.6 mol/l NaCl and 10 g/l blocking reagent. The firefly luciferase substrate

solution contained 20 mmol/l tricine, pH 7.8, 1.1 mmol/l magnesium carbonate pentahydrate, 2.7 mmol/l $MgSO_4$, 0.1 mmol/l EDTA, 33 mmol/l dithiothreitol, 270 μmol/l coenzyme A, 530 μmol/l ATP, and 470 μmol/l luciferin [11].

2.3. Preparation of poly(dT)-tailed DNA encoding firefly luciferase (poly(dT)-Luc DNA)

A DNA plasmid (4.3 kbp Luc DNA) containing the T7 RNA polymerase promoter upstream from the firefly luciferase gene, was linearized as follows: 100 μg of the plasmid were mixed with 200 units of Bgl I in 325 μl of 50 mmol/l Tris‐HCl, pH 7.9, 100 mmol/l NaCl, 10 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol. The reaction mixture was incubated at 37°C for 2 h. The linearized DNA fragment was purified by ethanol precipitation. The 3′ ends of the purified DNA were subsequently tailed with dTTP by using terminal deoxynucleotidyl transferase. The reaction mixture (20 μl) consisted of 10 μg of Luc DNA, 700 pmol dTTP, 25 units of terminal transferase, 25 mmol/l Tris‐HCl, pH 6.6, 200 mmol/l potassium cacodylate, 5 mmol/l $CoCl_2$, and 250 mg/l BSA. The mixture was incubated at 37°C for 60 min, and the reaction was terminated by adding 2 μl of 2 mmol/l EDTA. The tailed DNA fragment was used without purification.

2.4. Biotinylation of anti-PSA antibody

The monoclonal anti-PSA antibody (0.2 mg) was dialyzed overnight against 3.5 l of 0.1 mol/l sodium bicarbonate at 4°C. The antibody was then diluted with 0.5 mol/l carbonate buffer, pH 9.1, to a final concentration of 0.5 g/l. For biotinylation, 1 mg of sulfo-NHS-LC-biotin was dissolved in 50 μl dimethyl sulfoxide and 12.5 μl (0.25 mg) aliquot was added to the antibody solution. The mixture was incubated for 2 h at room temperature. The biotinylated antibody was stored at 4°C and used without purification.

2.5. Preparation of the heterobifunctional linkers

The 5′ amino group of $(dA)_{40}$ was first derivatized with SATA in a 40 μl reaction mixture containing 2.5 nmol of $(dA)_{40}$, 250 nmol of SATA, and 0.1 mol/l carbonate buffer, pH 9.1. SATA was added to the mixture as a 20 mmol/l freshly prepared solution in 0.1 mol/l carbonate and 50% dimethylformamide. The mixture was incubated at room temperature for 60 min. The derivatized $(dA)_{40}$ was purified from excess SATA by size-exclusion chromatography using a NAP column with an elution buffer containing 10 mmol/l ammonium carbonate, pH 6.8. The $(dA)_{40}$ solution was lyophilized to dryness and then reconstituted in conjugation buffer. The SATA-derivatized $(dA)_{40}$ (2.5 nmol) was mixed with 0.25 nmol of maleimide-activated avidin in the conjugation buffer (total volume 50 μl) and the reaction was initiated by deacetylation with hydroxylamine (final concentration 0.1 mol/l) to deprotect the sulfhydryl group. The reaction was allowed to proceed for 60 min at room temperature. Afterwards, the volume was increased to 2 ml with 0.1 mol/l sodium phosphate (pH 7.0) and the free oligo was removed by ultrafiltration using Centricon-30. The purification step was repeated twice. The linker was stored at −20°C.

A streptavidin-$(dA)_{40}$ linker was prepared as follows: streptavidin (0.96 nmol) was first derivatized with 38 nmol SMCC in 0.1 mol/l phosphate buffer, pH 7.0 (24 μl reaction mixture). After a 60 min incubation at room temperature, the free SMCC was removed by ultrafiltration using Centricon-30. The derivatized streptavidin was conjugated to the SATA-derivatized $(dA)_{40}$ as described above for avidin.

2.6. Application of the heterobifunctional linker to a sandwich-type immunoassay

Microtiter wells were coated overnight at room temperature with 25 μl of 5 mg/l capture anti-PSA antibody, diluted in 50 mmol/l Tris, pH 7.8, and 0.5 g/l NaN_3. Before use, the wells were washed six times with wash solution. A 5 μl aliquot of PSA standard (or diluted serum sample), and 20 μl of 0.5 mg/l biotinylated anti-PSA antibody, diluted in assay buffer, were added into each well. The immunoreaction was allowed to proceed for 60 min with continuous shaking. At the end of the incubation, any unbound biotinylated anti-PSA was removed by washing the wells as above. Afterwards, 25 μl of 8 nmol/l linker (diluted in blocking solution), was added into each well and incubated for 15 min followed by washing. Subsequently, 25 μl of 1.6 nmol/l poly(dT)-Luc DNA, diluted in hybridization buffer, were added into each well and incubated for 15 min. The wells were then washed eight times with wash solution and two times with 50 mmol/l potassium acetate. The bound firefly luciferase-coding DNA was expressed into active luciferase molecules by an in vitro coupled (one-step) transcription-translation reaction. The 25 μl of transcription-translation mixture was added into each well and incubated at 30 $^{\circ}$C for 90 min. The activity of synthesized luciferase was measured by mixing 2 μl of the transcription-translation reaction product with 50 μl of luciferase substrate solution (at room temperature) and monitoring the emitted luminescence for 1 min using the liquid scintillation counter.

2.7. Analysis of clinical specimens

Serum samples were diluted 10-fold in blocking solution prior to the analysis. The PSA concentration of the serum samples was determined by using a calibration curve constructed from PSA standards, whose concentrations were confirmed by the Abbott IMx PSA assay (Abbott Laboratories, IL).

3. Results and discussion

The series of chemical reactions that are involved in the preparation of the heterobifunctional linker is summarized in Fig. 1. In the first step, the *N*-hydroxy succinimide ester functional group of SATA reacts with the primary amino group at the 5′ end of $(dA)_{40}$ to produce a protected sulfhydryl derivative. The acetyl protecting group is then removed by a treatment with hydroxylamine and the released □SH group reacts with maleimide groups on (strept)avidin which were introduced by treatment with SMCC. Fig. 1 also presents the configuration of a microtiter well-based sandwich-type immunoassay using the (strept)avidin-$(dA)_{40}$conjugate as a

heterobifunctional linker between a biotinylated detection antibody and an enzyme-coding reporter gene.

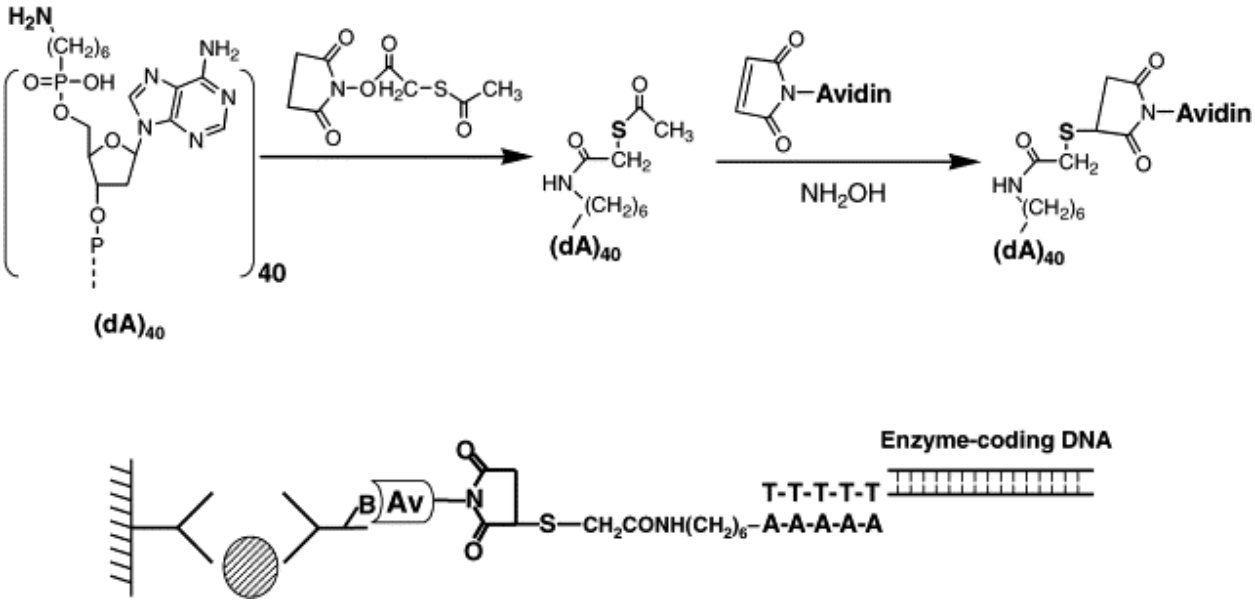

Fig. 1. Upper panel: a schematic diagram of the series of reactions involved in the preparation of the heterobifunctional linker. 5′ Amino-modified oligonucleotide $(dA)_{40}$ is first derivatized with SATA to give an acetylated sulfhydryl group that, in turn, is deprotected with hydroxylamine, and conjugated to maleimide-activated avidin or streptavidin. Lower panel: sandwich-type immunoassay configuration using the heterobifunctional linker between the detection antibody and a reporter gene. After completion of the immunoreaction, the reporter gene is subjected to one-step in vitro transcription/translation and the activity of synthesized enzyme is monitored. B: biotin; Av: avidin.

For (strept)avidin-$(dA)_{40}$ conjugate to be suitable as a linker between an antibody and a reporter gene, the following requirements must be fulfilled; (a) (strept)avidin retains its affinity for biotin after conjugation, (b) the conjugation does not interfere with the hybridization of $(dA)_{40}$ to the poly(dT)-tailed reporter gene, (c) the bound reporter gene is accessible for in vitro transcription and translation and (d) the non-specific binding of the linker and the reporter gene to the solid phase used in the assay is low.

A modified avidin (neutravidin) was chosen in this study because it has a significantly lower isoelectric point (p_I=6.3). As a consequence, its non-specific binding to the solid phase is relatively low.

The SATA to $(dA)_{40}$ molar ratio required for complete derivatization was studied in the range of 20‑400. The SATA-derivatized $(dA)_{40}$ was conjugated to avidin and the linker was used in the immunoassay as described in Section 2. The highest signal was obtained with molar ratios

greater than 100. The signal dropped by 60% when the ratio decreased to 20 because of the incomplete derivatization of the oligonucleotide.

The SATA-derivatized $(dA)_{40}$ to maleimide-activated avidin molar ratio was studied in the range of 2–50 (Fig. 2). Initially the signal increases when the SATA-$(dA)_{40}$ to avidin ratio is increased because the fraction of avidin molecules that are conjugated to $(dA)_{40}$ increases. The maximum signal is obtained at a ratio of 10. At higher ratios, the signal remains practically constant because the maximum number of Luc DNA molecules that can be bound to the immunocomplex on the well is achieved. At molar ratios greater than 10, the signal/background ratio (S/B) drops sharply. This was attributed to an increase of the non-specific binding of the linker to the solid phase.

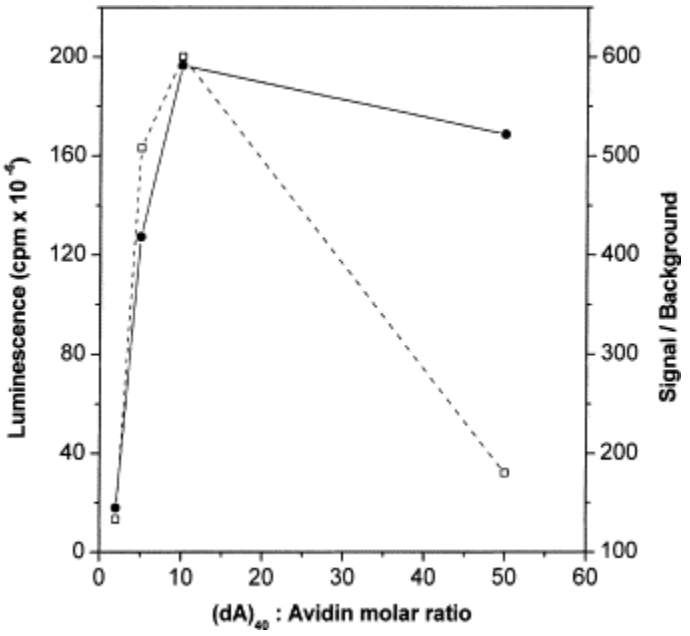

Fig. 2. Optimization of the molar ratio of $(dA)_{40}$ to avidin required for the preparation of the linker. Linkers were prepared by reacting a constant amount of maleimide-activated avidin with various concentrations of the oligonucleotide $(dA)_{40}$ and then used in the sandwich-type immunoassay of 25 pg of PSA as described in Section 2. The solid and dashed lines represent the luminescence and the S/B ratio, respectively.

For the preparation of streptavidin-$(dA)_{40}$ linkers, maleimide groups were first introduced to streptavidin by reacting with SMCC. The reaction was studied at molar ratios of SMCC to streptavidin ranging from 5 to 100. It was observed that the highest signal and S/B ratio were obtained at a molar ratio of 40. At higher SMCC concentrations the signal decreased because of the interference with the biotin–streptavidin interaction. Optimization experiments of the molar ratio of SMCC-derivatized streptavidin to SATA-$(dA)_{40}$ showed that the maximum signal was obtained at a ratio of 10 (similar to avidin above).

The effect of the linker concentration used in the immunoassay was studied in the range of 0.4–80 nmol/l. The results are presented in Fig. 3A. The signal increases with the linker concentration and

reaches to a plateau at 8 nmol/l. At higher concentrations, the signal remains constant, but the S/B ratio decreases due to the increase of the non-specific binding of the linker.

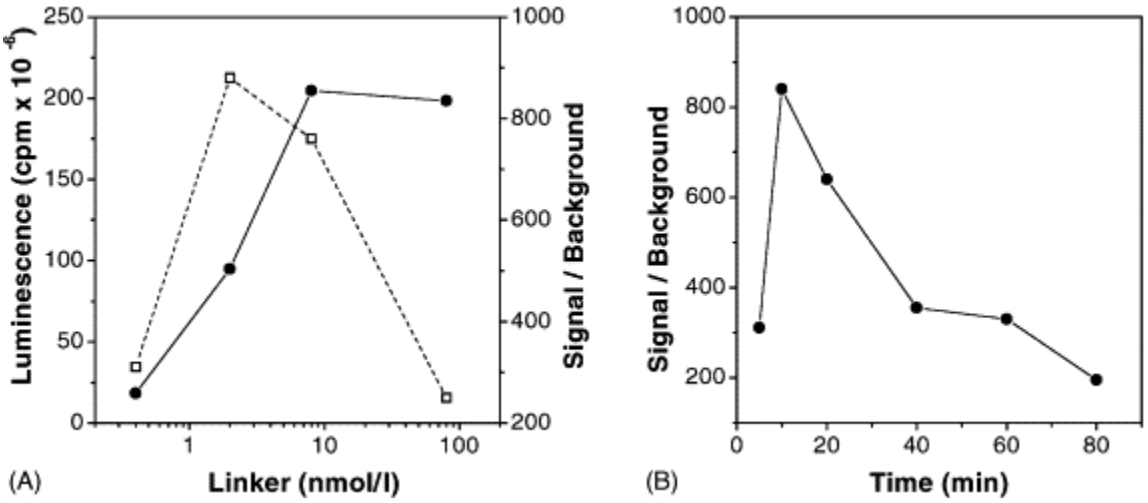

Fig. 3. (A) Effect of linker concentration used in the immunoassay. The signal (solid line) and the S/B ratio (dashed line) were plotted as a function of the linker concentration. (B) Time dependence of the binding of the linker to the biotinylated anti-PSA antibody. The immunoassays were carried out as described in Section 2 with 25 pg of PSA.

The time required for the binding of the linker to the immunocomplex was studied in the range of 5–80 min. The results are presented in Fig. 3B. It was observed that 40% of the linker binds to the immunocomplex in the first 5 min incubation. A 15 min incubation period generates the highest S/B ratio. With longer incubation periods, the S/B ratio drops due to the increased non-specific binding of the linker.

The effect of the poly(dT)-Luc DNA concentration was studied in the range of 0.3–10 nmol/l (Fig. 4A). In this study, the linker concentration was kept constant at 8 nmol/l. A continuous increase of the S/B ratio is observed with increasing poly(dT)-Luc DNA concentration and a maximum is reached at 1.2 nmol/l. At higher concentrations, the S/B ratio decreases because of the increased non-specific binding of Luc DNA.

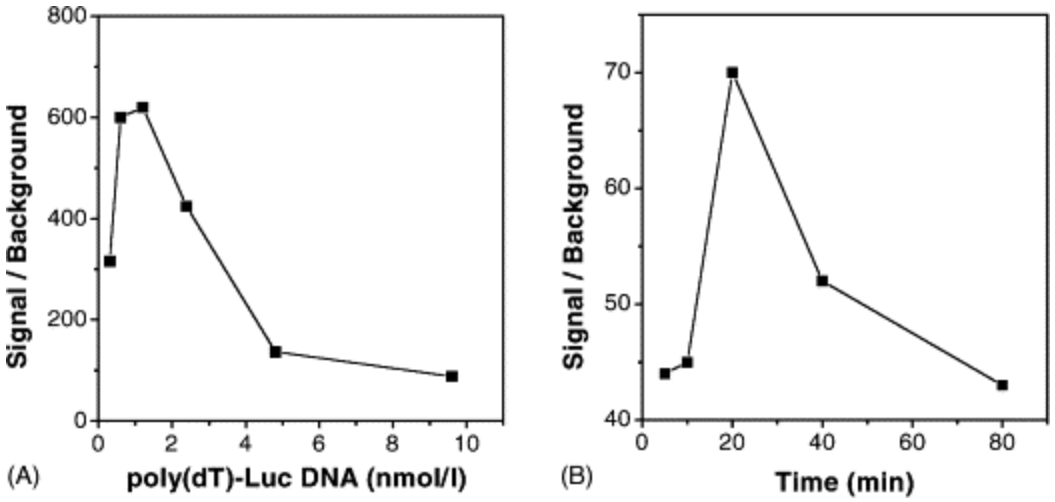

Fig. 4. (A) Optimization of the concentration of the poly(dT)-Luc DNA in the immunoassay. (B) Time-dependence of the hybridization of the poly(dT)-tailed reporter gene to the linker. The immunoassay was performed as described in Section 2 with 25 pg of PSA.

We further studied the time required for hybridization of the poly(dT)-Luc DNA to the linker (Fig. 4B). Within 5 min, 50% of the Luc DNA is bound to the linker and the highest S/B ratio is obtained after 15 min incubation. Longer incubation times result in increased non-specific binding of the poly(dT)-Luc DNA and lower S/B ratios.

The calibration curve for a "two-site" (sandwich-type) immunoassay for PSA was constructed by analyzing PSA standard solutions. In Fig. 5A, the luminescence was plotted as a function of the PSA concentration in the assay mixture. As low as 20 ng/l of PSA were detectable with a S/B ratio of 2.6 and the linear range extended up to 20,000 ng/l. The linker was tested further by analyzing (after appropriate dilution) 24 serum samples with PSA concentrations ranging from 5 to 50 μg/l by the proposed method and another widely used method (Abbott IMx) (Fig. 5B). The linear regression equation was $(\text{proposed method}) = 1.16 + 1.12$(IMx) with a correlation coefficient of 0.98.

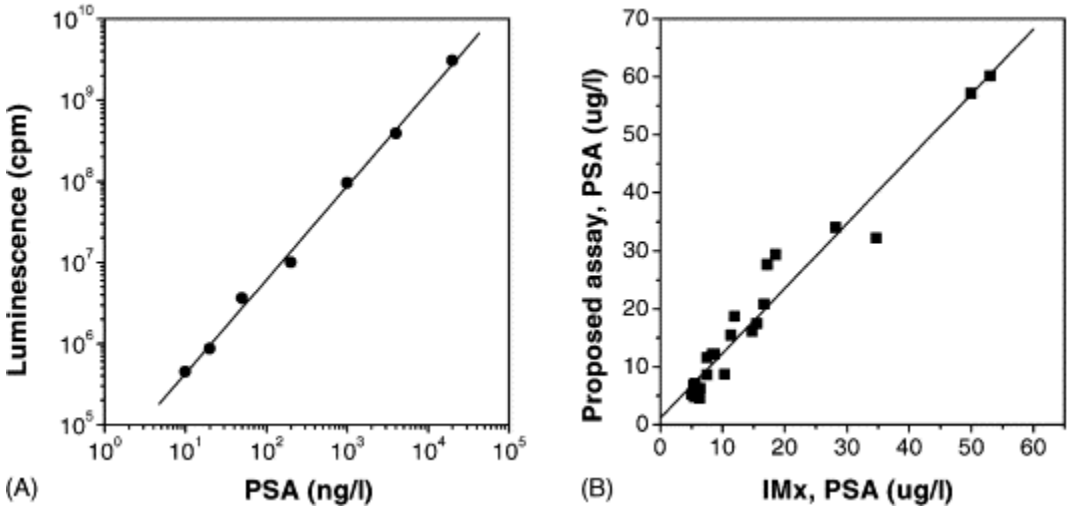

Fig. 5. (A) Calibration graph for the proposed immunoassay using the $(dA)_{40}$-avidin as a linker between the detection antibody and the reporter gene. The luminescence (corrected for the background) was plotted as a function of the concentration of PSA present in the well. (B) Correlation of PSA concentrations obtained by the proposed immunoassay and the IMx method (*n*=24).

To assess the reproducibility of the immunoassay, we analyzed serum samples containing 5.4, 15.5 and 49.9 μ g/l PSA. The CVs were 11.8, 9.5 and 5.0%, respectively (*n*=9).

The performance of the avidin-$(dA)_{40}$ and streptavidin-$(dA)_{40}$ linkers was practically the same. We have also prepared an avidin-$(dA)_{25}$ linker by following the same protocol as described for the $(dA)_{40}$ in Section 2. The luminescence decreased by 25% with the shorter oligonucleotide.

In addition, we have investigated whether the performance of the linker in the immunoassay would benefit by increasing the distance between the oligo(dA) sequence and avidin. A random 17mer oligo was derivatized with SATA at the 5′ end and conjugated to avidin as described for $(dA)_{40}$ in Section 2. The linker was then formed by tailing the conjugate (30 pmol) with dATP (3 nmol) using terminal transferase (25 units). Therefore, this linker contained a single stranded DNA spacer arm between avidin and the poly(dA) tail. Another linker was also prepared with a double-stranded DNA spacer arm between avidin and poly(dA) to ensure a more rigid structure for the spacer. A 17 bp double-stranded DNA fragment was first prepared by annealing two complementary 17mer oligos, one of them containing a $\square NH_2$ group at the 5′ end. The DNA was derivatized with SATA, conjugated to maleimide-activated avidin and tailed with dATP. The results obtained with both linkers were practically the same as with the original $(dA)_{40}$ linker.

The preparation of the reporter gene used in the assay was carried out by simply linearizing the appropriate vector with a restriction enzyme (single cutter), preferably one that could create 3′ protruding ends, and then tailing with dTTP using terminal transferase. Thus, both ends of the gene are tailed simultaneously. The poly(dT)-reporter gene can be used directly in the assay without purification.

Stability studies showed that the linker does not lose its activity after storage for at least 1 year at -20° C.

The most sensitive assays for proteins are based on the interaction of the analyte with a specific binder (antibody, receptor or peptide) that is linked to a signal-generating molecule. In recent years, there is an increased research activity on the selection of binders with high affinity and specificity for a large number of proteins. Various approaches include recombinant antibodies selected by phage-display [12] or ribosome-display [13], RNA or DNA aptamers [14], and small organic compounds selected through combinatorial library methods [15] and [16]. In most cases, the extent or the position of biotinylation of these binders can be optimized to avoid interference with the interaction between the binder and the protein. Thus, the proposed (strept)avidin-$(dA)_{40}$ linker may be used as a universal reagent to attach a highly detectable reporter gene to practically any binder molecule targeted to a protein analyte. The microtiter well format facilitates automation and high-throughput analysis.

Enzymes are the most widely used labels in immunoassays. Consequently, efforts toward improving the immunoassay detectability have been focused (for decades) on the synthesis of novel substrates. Substantial improvements have arisen from the replacement of chromogenic substrates with fluorogenic and chemiluminogenic ones. Expression immunoassay, instead of providing an alternative means for monitoring enzyme activity, entails an increase in the number of enzyme molecules per immunocomplex, thus introducing additional amplification. Furthermore, by using an enzyme-coding DNA fragment as a label the problem of enzyme inactivation during conjugation to antibodies is eliminated and the enzyme remains free in solution. This is particularly important for firefly luciferase, which is inactivated by the chemical reactions involved in direct labeling of antibodies or antigens. Moreover, expression immunoassay has shown considerably higher detectability when compared directly with fluorescence and chemiluminescence immunoassays and hybridization assays [5], [6] and [17].

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