Development and evaluation of a versatile receptor-ligand binding assay using cell membrane preparations embedded in an agarose gel matrix and evaluation with the human adenosine A_1 receptor

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Abbreviations:

B_{max} = total density (concentration) of receptors in a sample

CGS 21680 = 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid

CHO-K1 cells = Chinese hamster ovary cells

DPCPX = 8-cyclopentyl-1,3-dipropylxanthine

G418 = Geneticin

GPCRs = GTP-binding-protein coupled receptors

GTP = Guanosine-5'-triphosphate

hA_1AR = human A_1 adenosine receptor

$hA_{2A}AR$ = the human A_{2A} adenosine receptor

IC_{50} = inhibitory concentration - the concentration (of an inhibitor) a ligand required to reduce the rate of (an enzymatic reaction) a binding of a radioligand by 50%

K_D =equilibrium dissociation constant; K_i, inhibition constant; k_{off}, kinetic constant for dissociation

k_{on}= kinetic constant for association

LMP-agarose = low melting point agarose

NECA = 1-(6-amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuranuronamide

one-way ANOVA = one-way analysis of variance; PBS, Phosphate-buffered saline

PET = Positron Emission Tomography

SDS = Sodium dodecyl sulfate

SPECT = Single Photon Emission Computed Tomography; TRIS, tris(hydroxymethyl)aminomethane

ZM 241385 = 4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl)phenol

Abstract

GTP-binding protein-coupled receptors (GPCRs) are the target of up to 40% of prescribed medications worldwide. To evaluate the suitability of novel receptor ligands, frequently elaborate, time consuming, and expensive receptor-ligand interaction studies have to be carried out. The present work describes the development and proof of principle of a rapid, sensitive, and reliable receptor-ligand binding assay. CHO cells were stably transfected with a construct encoding the human adenosine A_1 receptor (hA_1AR). For ligand binding assays, membranes from these cells were prepared and embedded in low melting point agarose. These “immobilized” samples were incubated with tritiated 8-cyclopentyl-1,3-dipropylxanthine ($[^3H]$DPCPX), a well-established receptor antagonist. The K_D and B_{max} values as well as kinetic parameters (k_{on}, k_{off}) of receptor-ligand interaction were determined. Unspecific binding of various radiotracers to either the carrier material or the agarose gel matrix was negligible. The dissociation constant (K_D) for $[^3H]$DPCPX at the hA_1AR was determined by saturation-, competition binding-, and kinetic experiments. These studies resulted in K_D values of ~3 nM which is in good accordance with previously published data obtained from conventional receptor-ligand binding assays. The procedure described here simplifies classical binding studies to a kit-like assay. The receptors retained their binding properties even when preparations were dried completely. Transport and delivery of the material is conceivable without loss of biological activity. Therefore, other laboratories can perform binding studies without special equipment or the necessity to run a cell culture lab and/or to dissect tissue on their own.

1 Introduction

With more than 2000 members, GTP-binding-protein coupled receptors (GPCRs) are encoded by the largest gene family present in mammalian genomes. These membrane proteins are the target for 30 – 40% of prescription medicines.[1] Thus, there is an inherent demand to precisely determine the binding interaction between a specific GPCR and its potential ligands.[2] In the present work, a purinergic receptor, the human A_1 adenosine receptor (hA_1AR), was used as an example. In mammals, four genes encode adenosine receptors: A_1AR, $A_{2A}AR$, $A_{2B}AR$, and A_3AR.[3] All isoforms share the cognate structural features of GPCRs with seven membrane-spanning domains.[4]

The development of new ligands for molecular imaging (*Positron Emission Tomography* (PET) or *Single Photon Emission Computed Tomography* (SPECT)), especially for the adenosine A_1 receptor, which has a large clinical potential (sleep-wake regulation, cognitive performance, neurogenerative diseases (e.g. Alzheimer's disease), epilepsy, tumor growth, ischemia, stroke, heart disease),[5–10] is one of our main research topics[11–13].

A critical step in uncovering the in vivo function of a GPCR is to gain precise knowledge about its tissue and/or cellular localization. In recent years the resolution of non-invasive in vivo imaging techniques has tremendously increased and allows to determine the distribution pattern of a target protein with high precision. Therefore, it is an essential goal to develop novel or to improve known radioligands suitable either as therapeutics or imaging agents for PET or SPECT.[14] However, before clinical application, an extensive evaluation of a ligands' properties has to be carried out. In order to determine the interaction of a ligand with its destined receptor, usually ligand binding assays are performed. For this purpose, radioactively labeled ligands are incubated with samples of native tissue where target protein expression is expected, or with genetically modified cell lines in which the target protein is overexpressed. The main challenge in these experiments is the separation of the "free" from the "bound" ligand.

Among centrifugation and filtration procedures, immobilization techniques, especially for "High Throughput" assays, are playing an increasingly important role.[15–17] A major disadvantage of immobilization techniques, however, is the need for chemical manipulation either of the target proteins or the ligands. Thus, a system that retains the properties of the target protein or the ligand without preceding chemical modifications would be advantageous since it would come closer to native conditions. Today, major efforts are made to avoid the use of radioactivity and thus making the methods faster, more efficient, and less harmful. In this context fluorescence-based techniques are of particular interest.[18]

The aim of the present work was to develop a method that allows binding studies to be carried out reliably, fast, and efficiently. With regard to an application of newly developed ligands in humans, we reasoned to use human receptors to avoid inter-species differences of receptor binding affinities. To achieve this goal, CHO cells were stably transfected with a construct encoding the hA_1AR. For ligand binding assays, membranes from these cells were prepared and embedded in low melting point agarose (LMP-agarose). These “immobilized” samples were incubated with tritiated 8-cyclopentyl-1,3-dipropylxanthine ($[^3H]$DPCPX), a well-established A_1AR antagonist and the K_D and B_{max} values as well as kinetic parameters (k_{on}, k_{off}) of receptor-ligand interaction were determined.[19,20]

Due to the diversity of target proteins and ligands, a general and standardized method for the implementation of binding studies does not exist.[21,22] The assay was successfully carried out on membrane fractions containing hA_1ARs, with homogenates from animal tissue, and with membrane suspensions of CHO cells which constitutively expressed the human A_{2A} adenosine receptor ($hA_{2A}AR$).[13]

When developing radioactively labelled ligands for molecular imaging with PET and SPECT, the technique of classical radioligand binding still represents the gold standard for the evaluation of new compounds.

Therefore, we envision that the assay presented here is valuable to a wide range of applications requiring only a few optimization steps when other ligands and/or target molecules are used.

2 Methods

2.1 Chemicals and radiolabeled ligands

Low melting point agarose (LMP-agarose) with a melting temperature of ≤ 65°C and a congealing temperature of 26-30 °C as well as solvents and reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany). The radiolabeled ligands [^{3}H]DPCPX (4438 GBq / µmol), [^{3}H]ZM 241385 (1850 GBq / µmol), [^{3}H]NECA (962 Gbq / mmol) , and [^{3}H]CGS 21680 (1498 GBq / µmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). GEL-FIX™ strips were supplied by SERVA Electrophoresis GmbH (Heidelberg, Germany) and used as a supporting material for agarose gel spots. Scintillation cocktail (Ultima Gold) was obtained from Perkin Elmer (Solingen, Germany). Unless otherwise stated all manipulations were performed at ambient temperature.

2.1 Cell culture

Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection, Manassas, VA, USA) were propagated in 10 cm Petri dishes supplied with Ham's F12 Nutrient Mixture, containing 10% (v/v) fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM) in a humidified incubator at 37 °C, 5% CO_2/95% air. Cells were split two or three times weekly at a ratio between 1:5 and 1:20 when the culture reached approximately 90% confluency.

2.2 Stable transfection of CHO cells

A pcDNA3.1(+) plasmid containing the cDNA encoding the full-length human adenosine A_1 receptor (hA_1AR) was obtained from cDNA Resource Center (www.cdna.org, Bloomsberg, PA, USA). Recombinant plasmids used for cell transfections were amplified in E. coli cells (JM109 (K12), Promega, Madison, Wisconsin, United States). Purification of plasmid DNA from E. coli cultures was performed with a NucleoBond® Plasmid Purification Kit (Macherey and Nagel, Düren, Germany) following the suppliers protocol. Purified plasmid DNA was solubilized in TE (10 mM Tris-HCl, 1 mM EDTA; pH 7.4) buffer.

For transfection, we used a modified version of the calcium phosphate precipitation method.[23,24] Briefly, 4 x 10^5 CHO-K1 cells were seeded in 6 cm Petri dishes and transfected with 8 µg of hA_1AR-encoding plasmid DNA for 20 h at 37°C. Selection of stably transfected cells was initiated with 1 mg/mL geniticin (G418) until cell colonies had formed. From these colonies, single clonal lines were isolated by limiting dilution. Propagation of receptor expressing cells was performed in medium containing 450 µg/mL G418. Expression of hA_1ARs was verified by ligand binding and Western blotting. Western blotting of membrane fractions obtained from transfected or non-transfected cells showed specific immunological staining against the recombinant A_1 receptor (Anti-Adenosine A_1 Receptor antibody obtained from Abcam, Berlin, Germany) and was only observed in the lane containing material from the transfected cell line.

2.3 Membrane preparation

2.3.1 Membrane preparation of CHO cells

Ligand binding assays were performed on membrane preparations obtained from hA_1AR-expressing cells as well as on non-transfected CHO-K1 cells, for control. Briefly, the medium was removed and cells were washed with PBS buffer (pH 7.4). Cells were mechanically scraped off the dish in 1 mL PBS and collected by centrifugation (200 x g, 5 min, 4°C). The PBS was discarded. Cell pellets were snap frozen in liquid N_2 and stored at -80°C until further use.

Membrane preparations for ligand binding experiments followed a modified established protocol: Frozen cell samples were thawed and homogenized on ice (Ultra-Turrax, 1 x 30 sec at full speed)[20] The homogenate was centrifuged at 600 x g for 10 min at 4°C. To collect the membrane fraction, the supernatant was centrifuged at 50,000 x g for 60 min at 4°C. The resulting membrane pellet was re-suspended in 50 mM Tris/HCl buffer (pH 7.4), frozen in liquid N_2 at a protein concentration of 6 mg/mL and stored at -80 °C. Protein content was determined with a naphthol blue black photometric assay after solubilization in 15% NH_4OH containing 2% SDS (w/v) using human serum albumin as a standard.[25]

2.3.2 Membrane preparation of brain tissue

The preparation was carried out according to a described procedure.[26]

Frontal cortices from brains of male Wistar rats were homogenized for 1 min in 10 volumes of ice-cold 320 mM sucrose, containing a protease inhibitor (SIGMAFAST™ Protease Inhibitor from Merck) by a Potter at 20,000 rpm under external ice cooling. The homogenate was centrifuged at 1,000 g for 10 min at 4 °C (Beckmann Optima L, SW41Ti rotor). The pellet was discarded, and the supernatant was centrifuged at 50,000 g for 15 min at 4°C. The resulting pellet was washed once with buffer (50 mM Tris/HCl, pH 7.4) and resuspended in 5 volumes of buffer (50 mM TRIS/HCl, pH 7.4). Aliquots (120 µL) were stored at –80°C until further use. Protein amount was determined as described above.

2.4 Interaction of ligands with the substrate material

To test for a potential interaction of different tritiated ligands ([^{3}H]DPCPX, [^{3}H]ZM 241385, [^{3}H]NECA, and [^{3}H]CGS 21680) with the GEL-FIX™strips, radioligands were added to strips in a concentration of 0.5 nM (6,000 – 26,000 DPM) and incubated for 30 min at room temperature in 1.5 mL Tris/HCl (0.05 M, pH 7.4) buffer. After this incubation step, strips were washed for 30 sec with distilled water and transferred to a Packard vial containing 5 mL of scintillation cocktail. Radioactivity was measured in a β-scintillation counter for 5 - 10 min per sample.

2.5 Preparation of agarose-loaded GEL-FIX™ strips

The GEL-FIX™ material was cut into small strips (0.7 x 6.3 cm) and fixed to a sheet of paper by adhesive tape. Stock solutions of LMP-agarose (2.5% (w/v)) were prepared in distilled water. For control experiments LMP-agarose solutions were diluted with incubation-buffer (1 Vol +1 Vol). For binding studies LMP-agarose and the same volume of the membrane suspension was mixed resulting in a final concentration of 1.25 % (w/v) agarose that was applied to the strips.

Thus, the concentration of agarose applied to the strips was always 1.25 % (w/v).

For ligand binding experiments, cell membranes in Tris/HCl (0.05 M, pH 7.4) buffer at a twofold higher protein concentration than the final experimental concentration were mixed with an equal volume of the LMP-agarose solution at 37°C and 5 µL aliquots were pipetted onto the GEL-FIX™ strips. In experiments without membrane suspensions, 5 µL of LMP-agarose solution was applied to the strip surface. Typically, three LMP-agarose spots were applied per GEL-FIX™ strip. To maintain humidity of the spots, strips were placed in a humidity chamber and kept at 4°C. Alternatively, spots were dried in a refrigerator at 4°C.

Incubation procedures and conditions

All incubations were performed in Tris/HCl buffer (0.05 M, pH 7.4) and at room temperature (22°C), unless otherwise stated.

In order to examine unspecific retention of radioligands in LMP-agarose spots, rinsing conditions of the samples were tested. GEL-FIX™ strips with LMP-agarose spots were incubated in 2 mL Eppendorf vials in 1.5 mL Tris/HCl buffer containing 1 nM [^{3}H]DPCPX for 30 min. Strips were removed and rinsed with distilled water for 1, 2, 5, 10, 15, 20, 30, 60, 90, and 120 seconds. Individual LMP-agarose spots were cut out from the strips and transferred to Packard scintillation vials containing 5 ml of scintillation cocktail. Radioactivity was quantified in a β-scintillation counter. For the determination of background values and the activity adhering to the material, stripes without LMP-agarose spots (blank values) have been also incubated with [^{3}H]DPCPX and processed in the same way as membrane-containing samples.

To examine for the loss of protein from LMP-agarose spots loaded with membrane fractions from CHO cells, strips were incubated in distilled water. Each LMP-agarose spot contained 7.5 µg total protein. Strips were removed after 0, 30, 60, 90, 120, 150, and 180 min of rinsing and air-dried at room temperature. Protein staining was performed in a solution of naphthol blue black for 2 min at room temperature. Samples were washed in methanol and air-dried again. LMP-agarose spots containing a dilution series of the membrane suspension, and which were not incubated in water, served as a calibration standard. Staining intensity was registered and digitized with a transmitted light scanner. Image files were evaluated densitometrically using ImageJ software (https://imagej.net/).

2.6 Examination of ligand-receptor interactions

The amount of total cell membranes required in individual LMP-agarose spots to examine ligand-receptor binding was determined experimentally. GEL-FIX™ strips with LMP-agarose spots containing protein amounts between 0.42 µg and 20 µg were used. The strips were incubated in 1.5 mL Tris/HCl buffer containing 1 nM [^{3}H]DPCPX for 30 min at room temperature. After rinsing the strips for 60 sec with distilled water, LMP-agarose spots were immediately cut-out from the strips and transferred to Packard scintillation vials containing 5 mL of scintillation cocktail. Radioactivity was quantified in a β-scintillation counter.

In order to determine the association of radioligands to hA$_1$ARs, GEL-FIX™ strips with dried LMP-agarose spots containing 7.5 µg total protein (i.e., cell membranes) were incubated in Tris/HCl buffer containing 0.51 nM [^{3}H]DPCPX. Individual strips were removed from the incubation medium at given time points and washed for 60 sec with distilled water. Gel-spots were cut-out from the strips and radioactivity was measured in a β-scintillation counter.

To determine the dissociation course, samples were first pre-incubated for 60 min in incubation buffer containing the radioligand. Then, strips were removed and transferred to Tris/HCl buffer without radioligand. Ligand dissociation was examined between 0 and 60 min after transferring strips to the radioligand-free solution. The final treatment of the samples was as described for the association experiments. Data were analyzed by a computer-assisted curve fitting program (GraphPad Prism (version 4.0); GraphPad Software, Inc., La Jolla, CA, USA).

2.7 Saturation- and competition-binding experiments

Samples were prepared as described above. Strips with dried LMP-agarose spots containing 7.5 µg – 15 µg total protein/gel spot were incubated in Tris/HCl buffer containing [^{3}H]DPCPX and a competitor of given concentration (if necessary) in 2 mL Eppendorf vials at a final volume of 1,5 mL. The incubation was stopped after 60 min by removing the film strips from the solution and rinsing the samples for 60 sec with distilled water. Strips were air-dried, the LMP-agarose spots were cut out from the strips and transferred to the scintillation cocktail. Radioactivity was quantified in a β-scintillation counter.

2.8 Control experiments

For these experiments, membrane fractions of CHO cells expressing the human A_1 adenosine receptor and membrane fractions of non-transfected CHO (wild-type) cells were used. The strips were prepared as described above. The protein content in each spot was 7.5 µg. The concentration of the radioligand [^{3}H]DPCPX was 1.36 nM and the incubation time was 1 hour. To determine the level of unspecific binding, unlabelled DPCPX in a final concentration of 1 µM was added to the membrane spots. The further procedure was as described above.

2.9 Long-term stability of dried samples

To obtain information about the shelf-life of the samples, 12 strips were prepared as described above. Immediately after drying, three strips were used to examine total binding of [^{3}H]DPCPX and another three strips were used to determine unspecific binding using the same concentration of [^{3}H]DPCPX in the presence of 1 µM non-labeled DPCPX. The experiment was repeated with the remaining six strips stored in a refrigerator at 4°C for 13 days.

2.10 Assay procedure

The outline of the assay is schematically shown in Fig. 1. After the tissue or cells have been collected and processed, the membrane homogenate is mixed with LMP-agarose at 37°C. Three 5 µL aliquots are pipetted onto the carrier material. After drying, these samples can be stored at 4°C or used immediately. After incubation with, e.g., radioactively labeled ligands, the strips are rinsed with water and the individual spots are cut-out. The spots are transferred into scintillation cocktail and radioactivity is measured in a β-scintillation counter. The obtained values were corrected as described under 2.5.

A photo of an original sample with three dried 5 µL LMP-agarose spots is shown in Fig 2.“

2.11 Calculation fundamentals for binding experiments

Kinetic experiments

The simplest model of a ligand-receptor interaction is described by a second order reaction:[27,28]

(1) $L + R \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftarrows}} B$

Where (L) is the concentration of free ligand, (R) is the concentration of free receptor sites, and (B) is the concentration of ligand-receptor complexes; k_{on} and k_{off} are the kinetic constants for association and dissociation (rate constants).

The time course of the reaction can be described by equation 2:

(2) $\frac{dB}{dt} = k_{on} \cdot L_t \cdot R_t - k_{off} \cdot B_t$

Where L_t is the free ligand concentration, R_t the free receptor concentration and B_t the concentration of the ligand-receptor complex as a function of time.

The kinetic association constant, k_{on}, can be determined from the time course of ligand association to the receptor by integrating the rate equation.

Usually, however, an approximation is used that is valid when less than 10 % of the ligand is bound to calculate k_{on}.[28] This condition is also met in the present assay examining [^{3}H]DPCPX binding to hA$_1$AR:

(3) $B_t = B_\infty \cdot (1 - e^{-k_{ob} \cdot t})$

This equation contains an auxiliary parameter (k_{ob}) that can be determined directly from the experiment. B_∞ represents the equilateral concentration of the receptor-ligand complex ("bound"). With the additional knowledge of k_{off} and the ligand concentration (L) the association constant k_{on} can be calculated according to equation 4.

(4) $k_{on} = \frac{k_{ob} - k_{off}}{L}$

If, when determining the dissociation constant k_{off}, the forward reaction can be neglected, e.g., if there is no free ligand present at the beginning of the experiment, the equation can be simplified describing a "pseudo" first order reaction kinetic (5).

(5) $$B_t = B_0 \cdot \mathrm{e}^{-k_{off} \cdot t}$$

Saturation experiments

In chemical equilibrium, the relationship as described by equation (6) is valid for the "binding isotherm". With (R) the concentration of free receptor sites, (L) the concentration of free ligand, (B) the concentration of ligand - receptor complex and B_{max} the total concentration of receptors (B_{max} = B + R).

(6) $$B = \frac{B_{max} \cdot L}{K_D + L}$$

For competition experiments the IC_{50} value is determined in advance. At this ligand concentration, the half-maximal displacement of the radioligand is achieved.

Non-linear regression of the concentration-response curve according to equation (7) was used to determine the IC_{50} value, where (B) is the concentration of bound ligand as a function of the concentration of the inhibitor/competitor (I), B_0 is the maximal value of bound ligand (total binding without competitor), *Bottom* and *Top* are the plateaus at high and low inhibitor/competitor concentrations, respectively.

(7) $$B = Bottom + \frac{Top - Bottom}{1 + 10^{\log(I) - \log(IC_{50})}}$$

To determine the K_I value of DPCPX, the "Cheng-Prusoff" equation (8) was used, although it is an approximation.[29,30]

(8) $$IC_{50} = K_I \cdot (1 + \frac{L}{K_D})$$

2.12 Data analysis

All values are given as mean ± SD. For statistical analyses, GraphPad Prism (version 4.0) (GraphPad Software, Inc., La Jolla, CA, USA) was used. Significant differences between the means of individual test groups were assessed by one-way analysis of variance (ANOVA). The significance level was set to 0.05.

3 Results and discussion

Agarose is a sugar-based biopolymer that is usually used for gel electrophoretic separations of nucleic acids and proteins. In this paper, agarose was used as a matrix to immobilize membrane suspensions obtained from eukaryotic cells or tissue to a carrier material, namely transparent GEL-FIX™ strips. These samples were used to determine kinetic as well as saturation binding parameters of human A_1 adenosine receptor (hA_1AR) – ligand interactions. Immobilization of proteins to agarose has already been described [31,32]. However, proteins were typically covalently bound to chemically modified agarose matrices. The goal of the present project was to embed functional biological samples in an agarose matrix without prior chemical modification or conjugation step. Low melting point (LMP) agarose turned out to be a suitable substrate because it can be handled at 37°C, a temperature compatible with eukaryotic proteins. Embedding hA_1AR-containing membrane suspensions in LMP-agarose spots allowed to determine the K_D for $[^3H]$DPCPX. With ~2.99 nM this value was very similar to that obtained from conventional binding studies (3.86 nM).[20] However, the experiments could be performed in shorter time and with reduced radioactive waste production.

3.1 Interaction of radioligands with the GEL-FIX™ carrier material and LMP-agarose

In a first set of experiments, the direct interaction of radioligands with the GEL-FIX™ strips was investigated. Individual strips were incubated for a given time in buffered solutions containing tritiated radioligands. Well known, documented, and chemically varying ligands which have been used previously in binding studies on adenosine A_1 and A_{2A} receptors were used in this study: ZM 241385 - an adenosine A_{2A} receptor specific antagonist with high affinity for the receptor, NECA a potent adenosine receptor agonist with almost equal binding affinity for A_1 and A_{2A} receptors, and CGS 21680 an adenosine A_{2A} receptor specific agonist.[33]

After one washing step with distilled water the residual radioactivity remaining on the strip was measured in a β-scintillation counter. Table 1 summarizes the results of the experiments; binding of all radioligands to the GEL-FIX™ material was almost negligible. The remaining radioactivity on the strips was noticeably lower than the unspecific binding measured in subsequent binding studies (see below).

In a next set of experiments, LMP-agarose spots without embedded cell membranes were applied to GEL-FIX™ strips. They were incubated in a buffer solution containing 1 nM [^{3}H]DPCPX. After 60 min, which corresponds to the duration of a typical binding assay, the strips were rinsed with distilled water for different time intervals and the remaining radioactivity was measured in a β-scintillation counter. Both, wet and dry LMP-agarose spots were examined to determine the required rinsing times to wash out unspecifically bound radioligand. The results, depicted in Fig. 3, reveal that the washout of radioactivity is less efficient and slower for moist (Fig. 3A) than for dry gel spots (Fig. 3B).

This result can be explained by differences in the diffusion processes of moist and dried LMP-agarose spots, because the dried spots have a considerably smaller inherent volume. After a rinsing time of 60 sec, the level of remaining radioactivity of both, the moist and the dried LMP-agarose spots had dropped to a value of the respective unspecific binding. Therefore, in all following experiments, except when determining the dissociation kinetics, a rinsing time of 60 sec was applied. Notably, the washout experiments also demonstrated a stable contact between the LMP-agarose spot and the GEL-FIX™ strips, both in the moist and dried state, because the detachment of a spot would have resulted in a complete loss of detectable radioactivity.

3.2 Optimization of the protein amount in cell membranes for binding studies

Fig. 4 shows the results of binding experiments performed with moist and dried LMP-agarose spots containing different amounts of cell membranes (= protein) obtained from a cell line constitutively expressing the hA$_1$AR.

Protein content was varied from 0.42 to 20 µg per spot. For labeling hA$_1$ARs, [^{3}H]DPCPX was used as the radioligand. As expected, the total binding of [^{3}H]DPCPX increased almost linearly with increasing protein content. In order to differentiate between specific and unspecific binding, experiments were performed in the presence of 1 µM unlabelled DPCPX.

It turned out that the level of unspecific binding between moist and dried LMP-agarose spots differed considerably (Fig. 4), but in both cases, the values were almost constant for all protein amounts examined (Fig. 4A, 4B). Therefore, specific binding, was calculated directly from the slopes of the linear fits and determined for graph A with 46.6 +/- 3.5 (95% confidence interval 37.6-55.7) DPM / µg protein and for graph B with 50 +/- 2.1 (95% confidence interval 44.6-55.5) DPM / µg protein. These results suggest that dried spots were favorable because the signal-to-noise ratio was considerably better. This is advantageous especially when protein amounts become a limiting factor.

Drying the cell membranes in the LMP-agarose spot obviously had no effect on [^{3}H]DPCPX binding to hA$_1$ARs, however, total [^{3}H]DPCPX binding in moist LMP-agarose spots resulted in higher values of detected radioactivity (see Fig. 4). Since strips with dried LMP-agarose spots are rather convenient, easy to handle and, moreover, show better signal-to-noise ratios, subsequent experiments have been carried out exclusively with dried LMP-agarose spots.

3.3 *Studies on protein loss*

Due to the permeability of agarose, protein loss during incubation in aqueous solutions cannot be excluded. Since protein loss during a binding assay would significantly impair the value of the results, e.g., an inaccurately estimated receptor density in the examined membrane fraction, the protein content in dried LMP-agarose spots was determined for various incubation times between 0 and 180 min. The protein amount in dried LMP-agarose spots was adjusted to 7.5 µg and samples were immersed into a water bath with constant agitation. After given time points strips were removed, dried, and the spots stained with naphthol blue black. For calibration, a dilution series from the same membrane preparation was used, embedded in LMP-agarose, dried, and stained with naphthol blue black. Protein quantification was carried out densitometrically. As depicted in Fig. 5, the protein amount in individual LMP-agarose spots is rather constant up to an incubation time of 150 min. Longer incubation times (180 min) resulted in a loss of approximately 20% of total protein. For examining kinetic parameters of hA_1AR and ligand interactions, however, an incubation time of 150 min is sufficiently long.

3.4 Kinetics of hA_1AR ligand interaction.

Determination of the dissociation constant, K_D, was carried out in two approaches addressing the association and dissociation kinetics of [^{3}H]DPCPX to hA_1ARs. Figs. 6A/B show the temporal course of the association and dissociation of the receptor present in membrane fractions embedded in LMP-agarose spots. The incubation times chosen were not influenced by protein loss from LMP-agarose spots (see Fig. 5). The temporal progression of ligand association and dissociation also provides information about a reasonable incubation time for binding studies conducted at chemical equilibrium. In addition, the dissociation kinetics provides information about the rinsing time that does not result in radioligand wash-out effects.

From the association experiment, the k_{ob} value for [^{3}H]DPCPX binding to hA_1AR was calculated by non-linear regression of the binding curve. This resulted in a value of 0.054 +/- 0.007 s^{-1}.

The calculated k_{off} value for [^{3}H]DPCPX dissociation from hA_1AR was 0.043 +/- 0.00225 s^{-1}. Using both kinetic values that were determined at a [^{3}H]DPCPX concentration of 0.51 nM, the association constant k_{on} according to equation 4 gives a value of 0.0215 s^{-1} nM^{-1} and the dissociation constant (K_D) for [^{3}H]DPCPX was derived from equation (9).

(9) $K_D = k_{off}/k_{on}$

The value is very similar to K_D values that have been previously determined using a transfected cell line (3.86 nM) or measured by a mass spectrometrically based ligand assay for DPCPX (3.34 nM)[20,34]

3.5 Assays in chemical equilibrium

3.5.1 Saturation binding experiments

Using the previously determined assay parameters, a series of saturation binding experiments were conducted. Results of a typical experiment are shown in Fig. 7.

Unspecific binding was measured in the presence of 1 µM non-labeled DPCPX. Specific binding was calculated as the difference between total and unspecific binding. A K_D value of 2.99 +/- 0.27 nM was determined by non-linear regression according to equation 6 and nicely fits to the value obtained from the kinetic experiments, i.e., 1.9 nM (see previous chapter).

3.5.2 Competition binding assays

Competition binding experiments were conducted to determine the affinities of non-radioactively labeled compounds. In this case, radioligand and competitor were isotopologues and thus chemically identical. In Fig. 8, a typical result for [^{3}H]DPCPX and its non-labeled orthologue is shown. The graph has a sigmoidal shape, as expected for such an experiment. The concentration of the competitor at the turning point of the graph represents the IC_{50} value. At this ligand concentration, the half-maximal displacement of the radioligand is achieved.

For the experiment depicted in Fig. 8 an IC_{50} value of 2.89 +/- 0.03 nM was calculated. To determine the K_I value of DPCPX, the "Cheng-Prusoff" equation 8 was used.[29]

At a radioligand concentration (L) of 0.41 nM and a K_D for [^{3}H]DPCPX of 2.89 nM , the K_I value for DPCPX was 2.54 nM.

3.6 Stability of membrane preparations in dried LMP-agarose spots

Initial shelf life tests have shown that strips containing dried LMP-agarose spots with membrane preparations from hA_1AR-expressing cells could be stored for several days. Strips that had been stored at 4°C for 13 days revealed similar binding properties (total binding 2244 +/- 441 DPM, unspecific binding 84 +/- 11 DPM) as freshly prepared samples (total binding 1838 +/- 193 DPM, unspecific binding 90 +/- 12 DPM).

Each value was obtained from 6 independent measurements corrected with the respective blank values. Since neither the total binding groups nor the non-specific binding groups differ significantly ($p>0.05$), it can be concluded that the samples ca be stored for at least 13 days without losing their binding characteristics.

However, strips produced under the same conditions but containing membrane homogenates from rat brain had a significantly shorter shelf life. In these preparations, a reduced total binding of the radioligand was already observed after 7 days (data not shown). Therefore, the storage period without impairing the functional properties of a sample obviously depends on the biological source and, thus, must be checked individually beforehand, especially if longer storage periods are required. Although we have not addressed this issue here, it might be reasonable to add specific protease inhibitors to the samples avoiding protein degradation during storage.

3.7 Control experiments

In order to verify the specificity of the binding experiments, control experiments were performed with processed cell membranes derived from non-transfected CHO wild-type cells. The results are shown in Fig.9. The level of total binding of [^{18}F]DPCPX to homogenates from cells constitutively expressing hA$_1$AR differs significantly from the control sample (Fig. 9, bar A and C; $p < 0.001$). Notably, the amount of bound [^{18}F]DPCPX to membrane preparations was almost not discernible from values measured on samples from hA$_1$AR-expressing and wild-type cells in the presence of non-labeled competitor (Fig. 9, bar B-D; $p > 0.05$).

3.8 Saturation binding experiments with rat cortical membrane preparations

The assay conditions described above - performed exclusively with membrane preparations of CHO cells expressing the human adenosine A$_1$ receptor - were also used for binding studies with animal tissue samples. The result of a representative saturation binding experiment with [^{3}H]DPCPX as radioligand and membrane preparations of rat cortices is shown in Figure 10. From three independent experiments a K_D value of 2.07 nM with a standard deviation of 0.33 nM was determined. This value is significantly lower ($p<0.05$) than that determined with CHO cells. This result is in accordance with a recently published study, in which a higher affinity of DPCPX to adenosine A$_1$ receptors from rat cortices compared to human A$_1$ receptors has also been described.[35]

4 Conclusions

Cell or tissue membrane preparations embedded in low melting point agarose and immobilized on transparent GEL-FIX^{TM} strips have been established as a simple, fast, and efficient approach to carry out receptor-ligand binding studies.

Neither the carrier material nor the low melting point agarose gel did adsorb radiotracers to a considerable extent. Notably, no protein loss from the gel matrix was detected during incubation times for up to 150 minutes in distilled water.

Dissociation constants for [^{3}H]DPCPX on adenosine A_1 receptors were determined from kinetic as well as equilibrium-binding (saturation and competition) experiments. Using membrane preparations either from hA_1AR-expressing cells (K_D ~ 2.9 nM) or rat cortical membranes (K_D = 2,07 +/- 0.33 nM) resulted in K_D values that were in good agreement with previously published data.

The versatility of the current approach is further supported by the fact that samples can be dried and stored at 4°C for long time periods without impairing the properties of the biological material.

Disclosure

The authors declare that there is no conflict of interest.

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Tables

Table1. Residual radioactivity

Radioligand	Remaining activity [%]	Standard deviation
$[H^3]$DPCPX	0.033	0.011
$[H^3]$ZM241385	0.113	0.030
$[H^3]$NECA	0.124	0.039
$[H^3]$CGS21680	0.132	0.048

Figure Legends

Figure 1. Schematic representation of the practical implementation of the assay.The essential steps for the execution of the assay are shown schematically. After processing the target tissue, cell membranes are mixed with agarose at 37°C and defined aliquots are pipetted onto strips. After drying at 4° the strips are ready for binding studies. Following incubation with ligands, the strips are rinsed with water. Individual gel spots are cut out and the radioactivity is measured immediately in a β-scintillation counter.

Figure 2. Photograph of a prepared film strip.The high contrast picture shows a strip with three dried 5 µL agarose gel spots next to a scale.

Figure 3. Optimization of the rinsing time. Influence of the rinsing time on the remaining activity of [^{3}H]DPCPX using “wet” (A) and “dry” (B) agarose spots. Spots were prepared from agarose without membrane suspensions. All tests were performed in triplicate and the standard deviations are represented by the error bars.

Figure 4. Binding experiments performed with moist and dried LMP-agarose. Increasing amounts of membrane protein using “wet” (A) and “dry” (B) spots were used in this experiment. Protein amounts were varied between 0.4 µg and 20 µg. The strips were incubated for 70 min in Tris/HCl buffer containing 0.35 nM [^{3}H]DPCPX at room temperature (total binding ●). Unspecific binding (✖) was measured in the presence of 1 µM non-labeled DPCPX. After rinsing the strips for 60 sec with distilled water, the radioactivity in LMP-agarose spots was immediately quantified in a β-scintillation counter. All tests were performed in triplicate and the standard deviations are represented by the error bars.

Figure 5. Protein loss depending on the rinsing time. Protein content in dried agarose spots containing hA_1AR-expressing cell membranes was determined as a function of the washing time with double-distilled water. The initial protein concentration was 7.5 µg/spot. The protein content was determined densitometrically after staining with naphthol blue black. The small graphical insert shows the calibration curve. All tests were performed in triplicate and the standard deviations are represented by the error bars.

Figure 6. Binding kinetics. Graph (A) shows a time course of the [^{3}H]DPCPX association at a concentration of 0.51 nM. The K_{ob} value determined by nonlinear regression for the displayed data is 0.053 s^{-1}. Graph (B) shows a time course of the [^{3}H]DPCPX dissociation. The K_{off} value determined by nonlinear regression for the displayed data is 0.042 s^{-1}.The values from association and dissociation result in a K_D value of 1.9 nM. Experiments were performed with dried agarose spots containing cell membranes obtained from hA_1AR-expressing cells (total amount 7.5 µg protein). Measurements were performed in triplicate and the error bars represent the standard deviations.

Figure 7. Saturation experiment. Dried agarose spots containing 15 µg of membrane protein derived from hA_1AR-expressing cells were incubated with [^{3}H]DPCPX as radioligand. Unspecific binding was measured in the presence of 1 µM non-labeled DPCPX. Specific binding (▼) was calculated as the difference between total (●) and unspecific binding (✖). The mean value for K_D determined in three independent assays was 2.9 nM (standard deviation ± 0.27 nM). Each assay was performed in triplicate and the error bars represent the standard deviations.

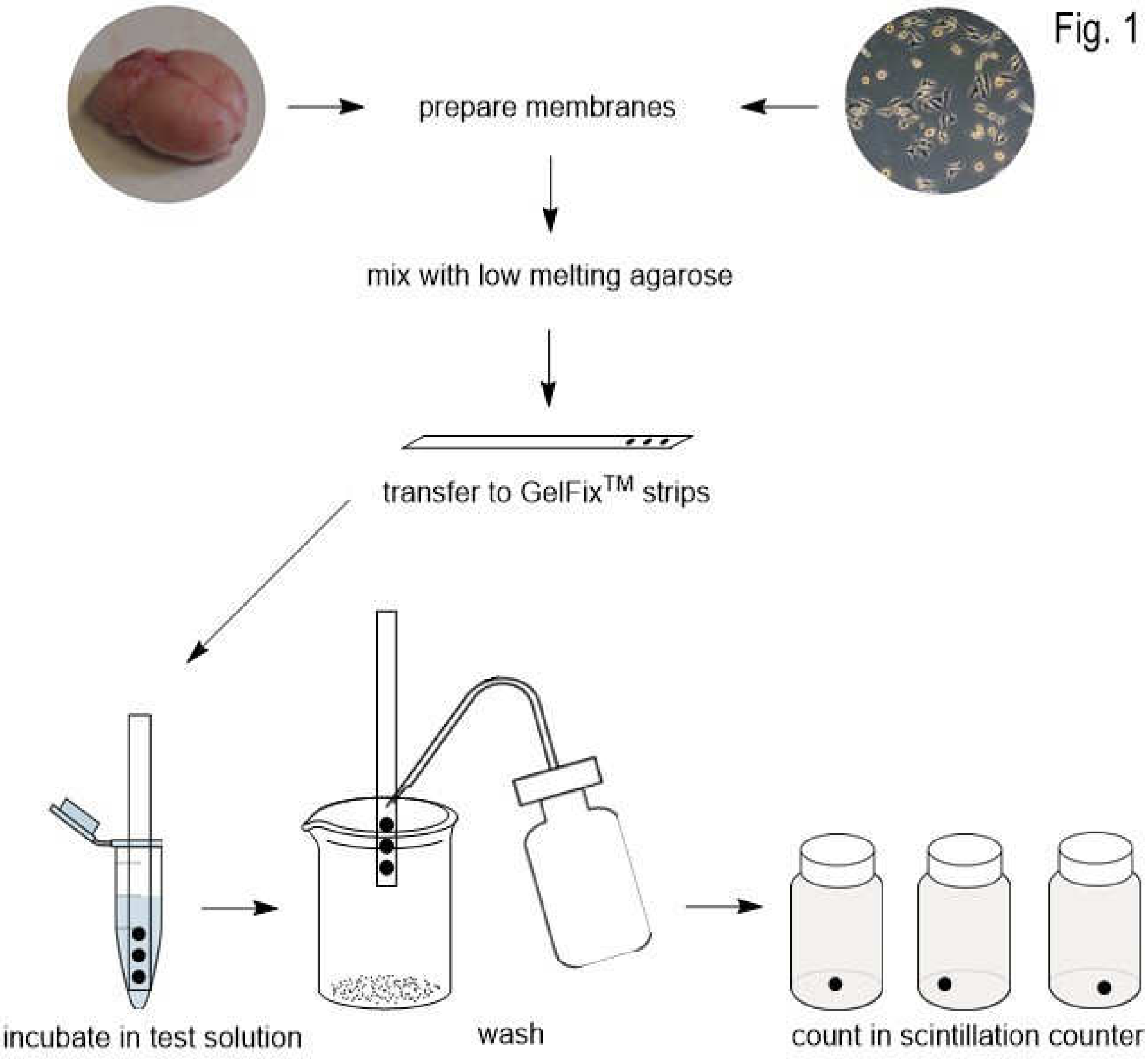
Figure 8. Competition experiment. In this experiment [^{3}H]DPCPX was used as radioligand at a concentration of 0.41 nM and unlabelled DPCPX as competitor on dried agarose spots containing 7.5 µg of membrane protein derived from hA_1AR-expressing cells. Non-linear regression of the concentration-response curve according to equation 8 was used to

determine the IC_{50} value of 2.9 nM with a standard error of 0.27 nM using the K_D value depicted in Fig. 7. Measurements were performed in triplicate and the error bars represent the standard deviations.

Figure 9. Control experiment. The gel spots of preparations A and B contained membrane suspensions obtained from cells constitutively expressing hA_1ARs. Samples in C and D were prepared from non-transfected wild-type cells. The radiotracer [^{18}F]DPCPX in a concentration of 1.36 nM was used for all preparations. Samples B and D additionally contained unlabelled DPCPX in a concentration of 1 μM (unspecific binding).
Total binding to the hA_1AR-containing sample (A) was 2658 +/- 244 DPM and 90.0 +/- 6 DPM in the presence of competitor (= unspecific binding). Total binding and non-specific binding in samples obtained from wild-type cells were almost identical to (B), i.e. non-specific binding to hA_1AR-containing samples.

Figure 10. Saturation experiment using rat cortical membrane preparations. Dried agarose spots containing 15 μg of membrane protein derived from rat cortices were incubated with [^3H]DPCPX as radioligand. Unspecific binding was measured in the presence of 1 μM non-labeled DPCPX. Specific binding (▼) was calculated as the difference between total (●) and unspecific (✖) binding. The mean value for K_D determined in three independent assays was 2.07 nM (standard deviation ± 0.33 nM). Each assay was performed in triplicate and the error bars represent the standard deviations.

Fig. 1


prepare membranes
mix with low melting agarose
transfer to GelFix^{TM} strips
incubate in test solution
wash
count in scintillation counter

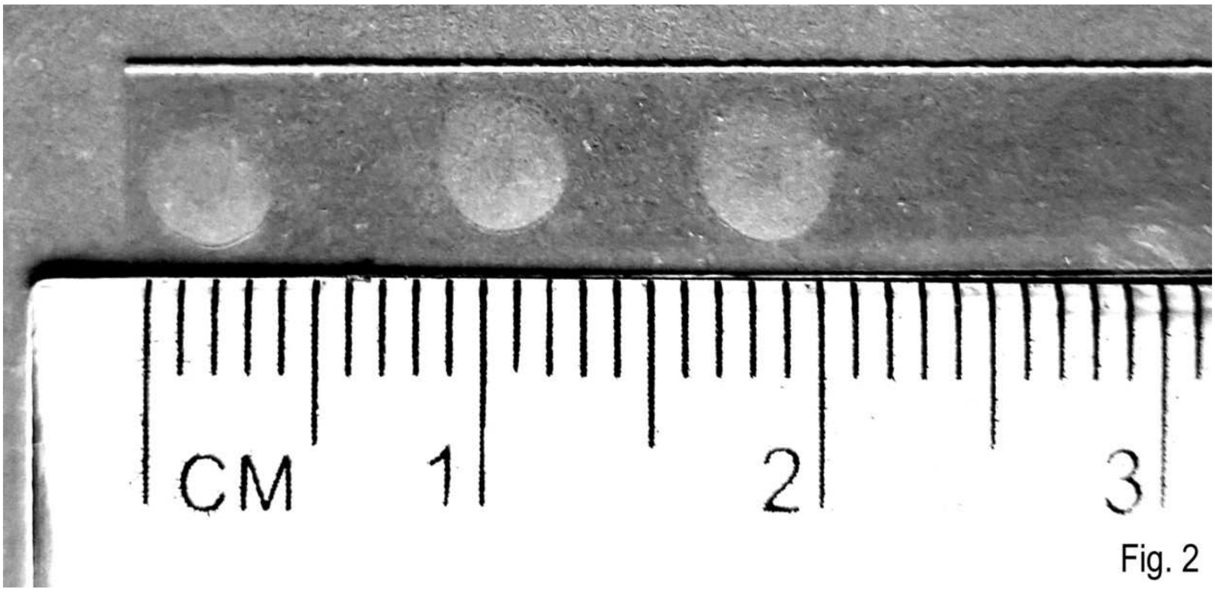


Fig. 2

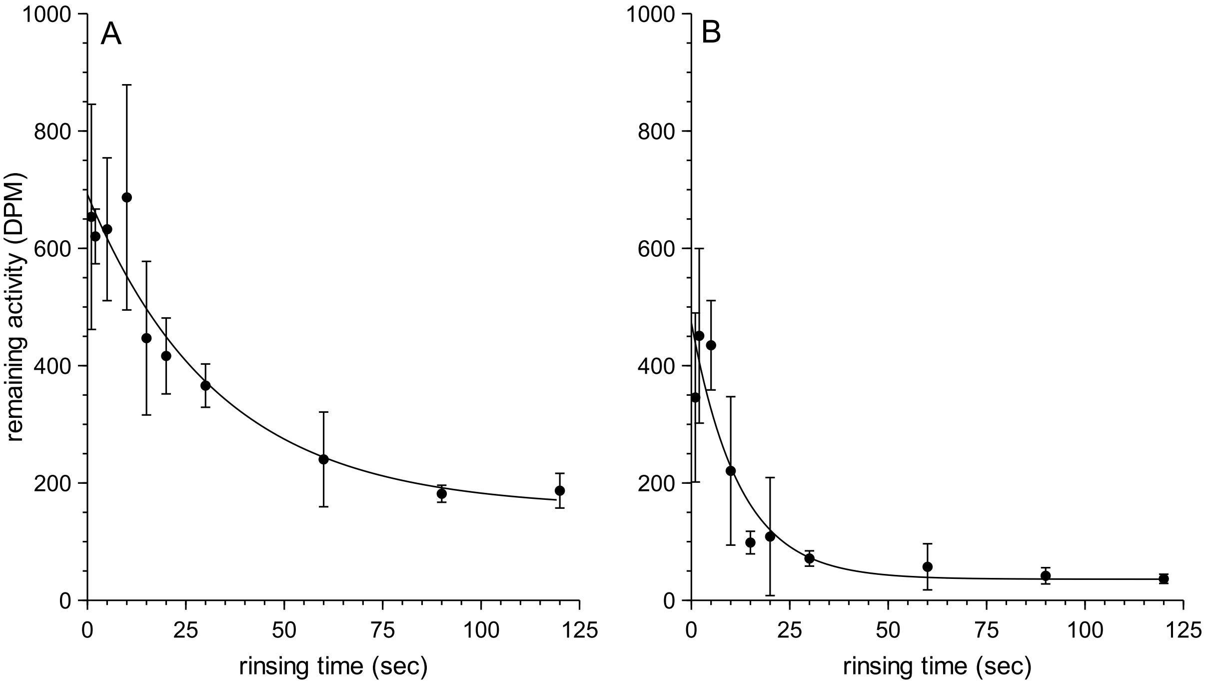

A
B
remaining activity (DPM)
0
200
400
600
800
1000
0
25
50
75
100
125
rinsing time (sec)

Fig. 3

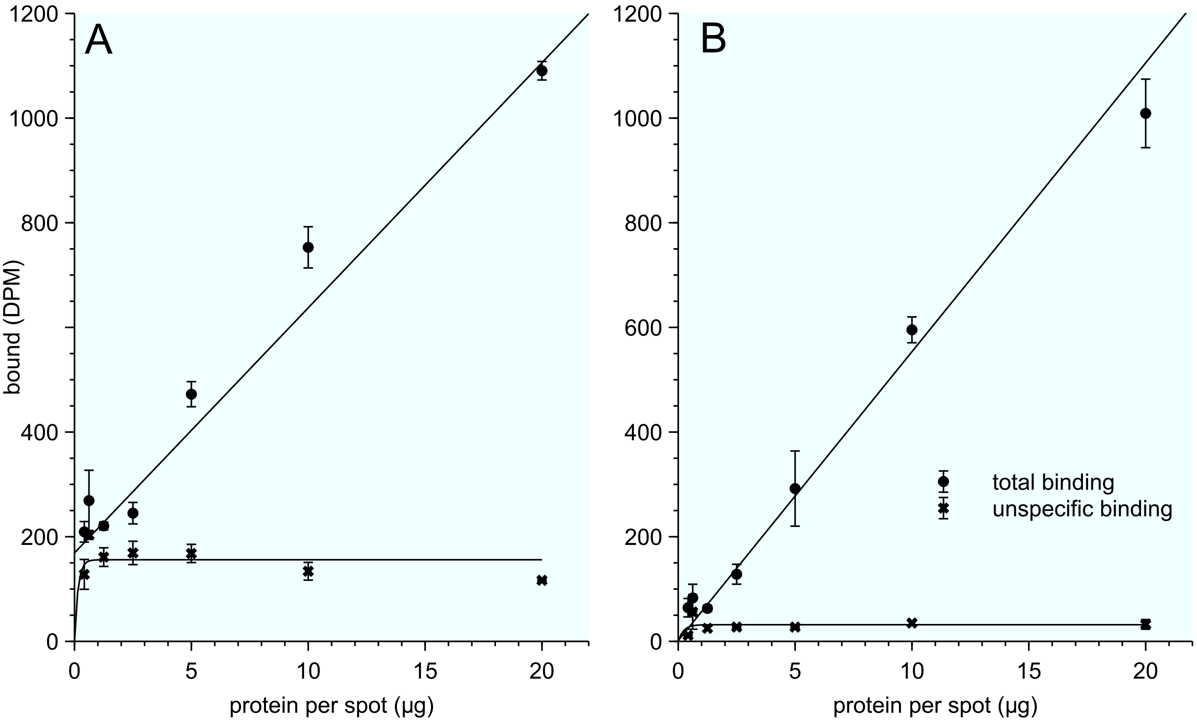
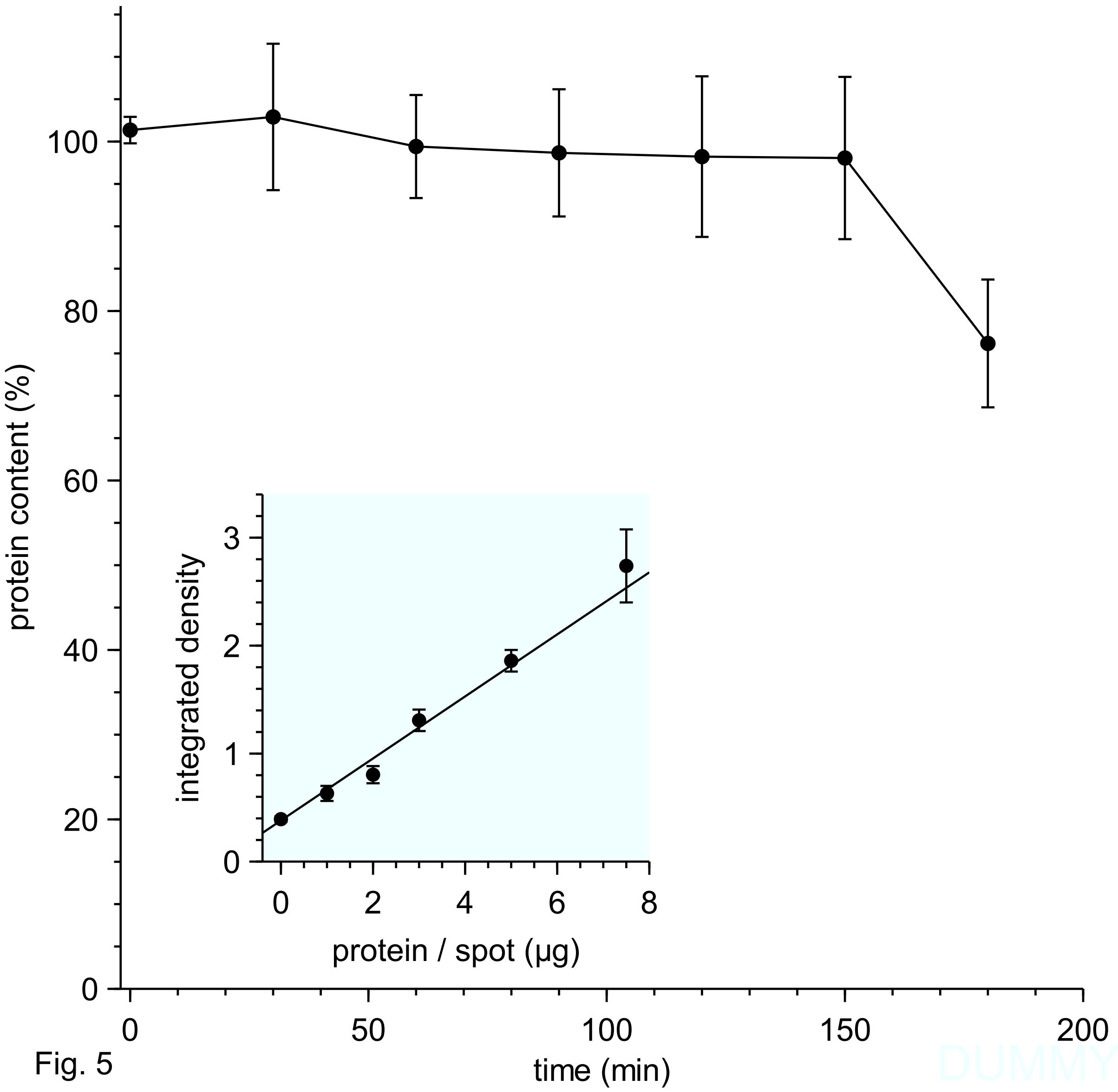

A
B
bound (DPM)
0
200
400
600
800
1000
1200
protein per spot (µg)
0
5
10
15
20
total binding
unspecific binding

Fig. 4



protein content (%)
0
20
40
60
80
100
0
50
100
150
200
time (min)
integrated density
0
1
2
3
0
2
4
6
8
protein / spot (µg)

Fig. 5

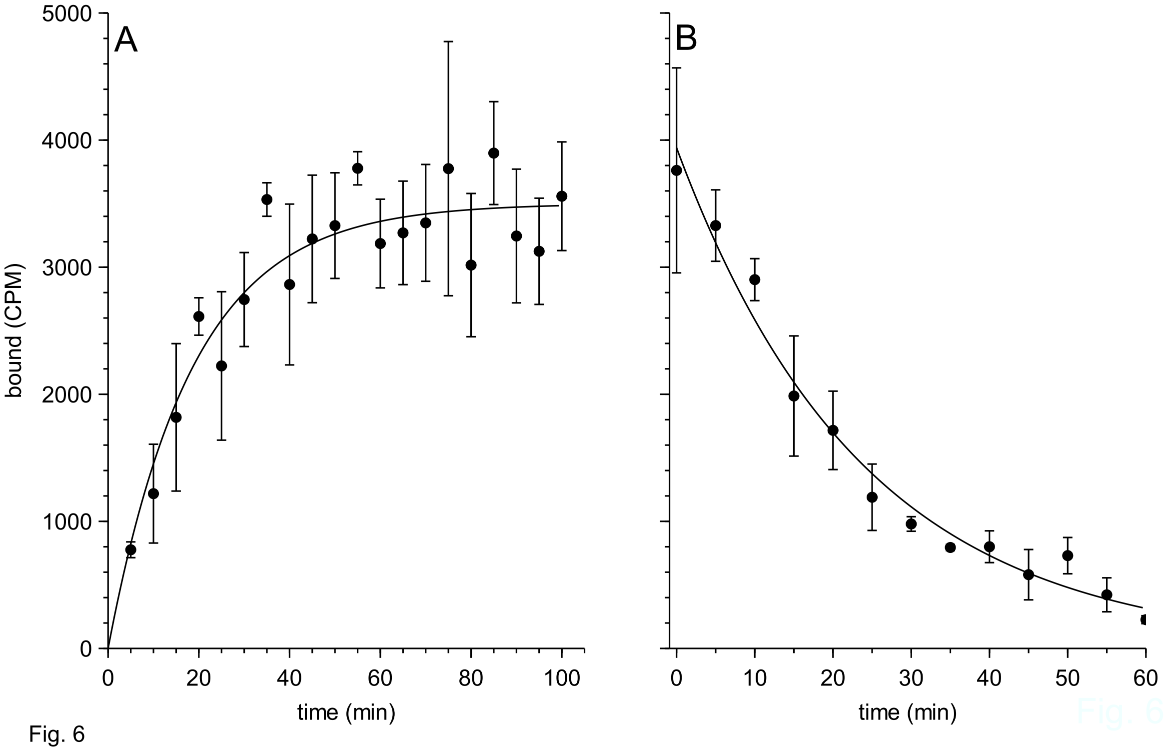

A
B
5000
4000
3000
2000
1000
0
bound (CPM)
0
20
40
60
80
100
time (min)
0
10
20
30
40
50
60
time (min)

Fig. 6

Fig. 6

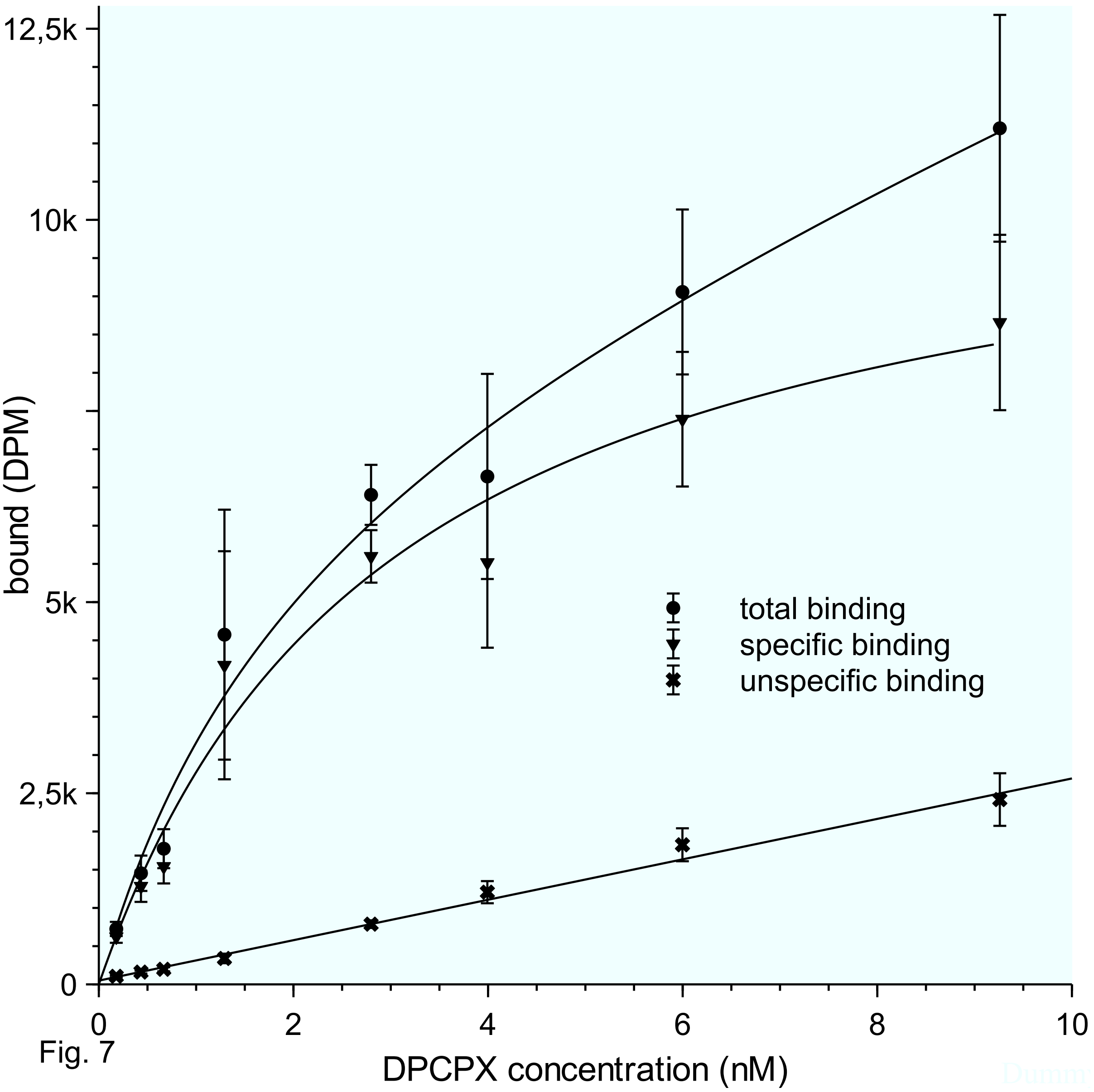

12,5k
10k
5k
2,5k
0
bound (DPM)
0
2
4
6
8
10
DPCPX concentration (nM)
total binding
specific binding
unspecific binding
Dumm

Fig. 7

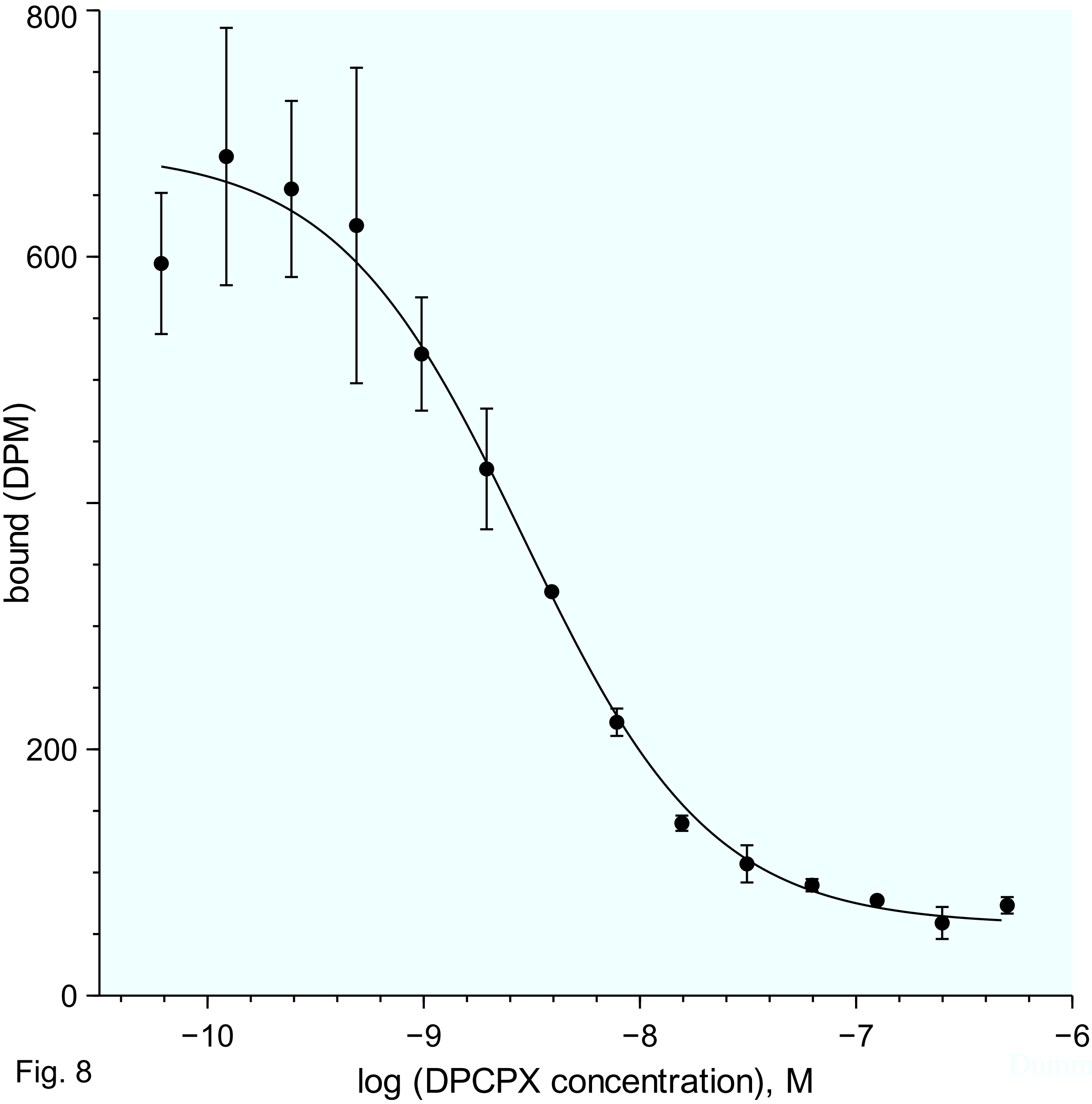


Fig. 8

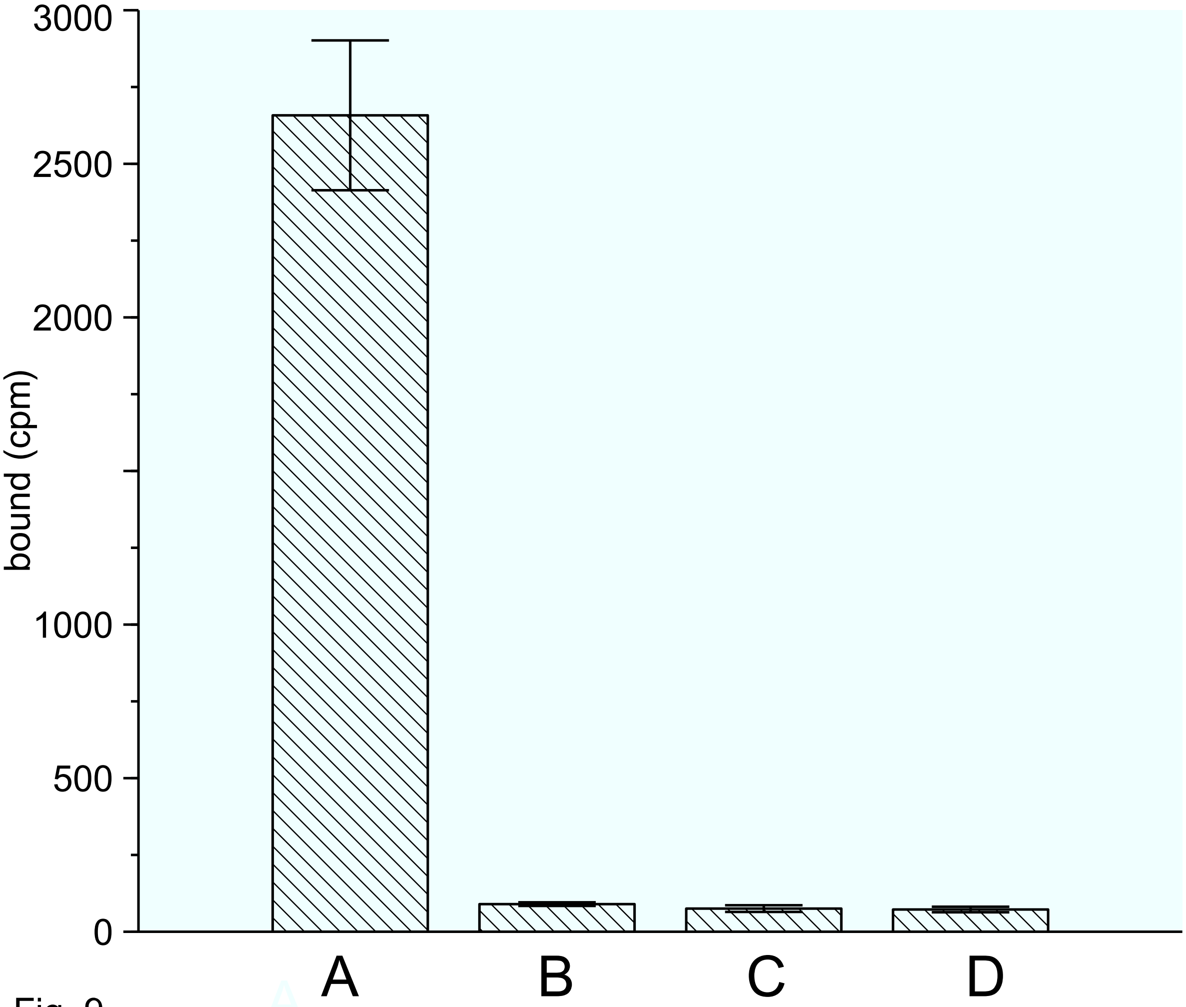


Fig. 9

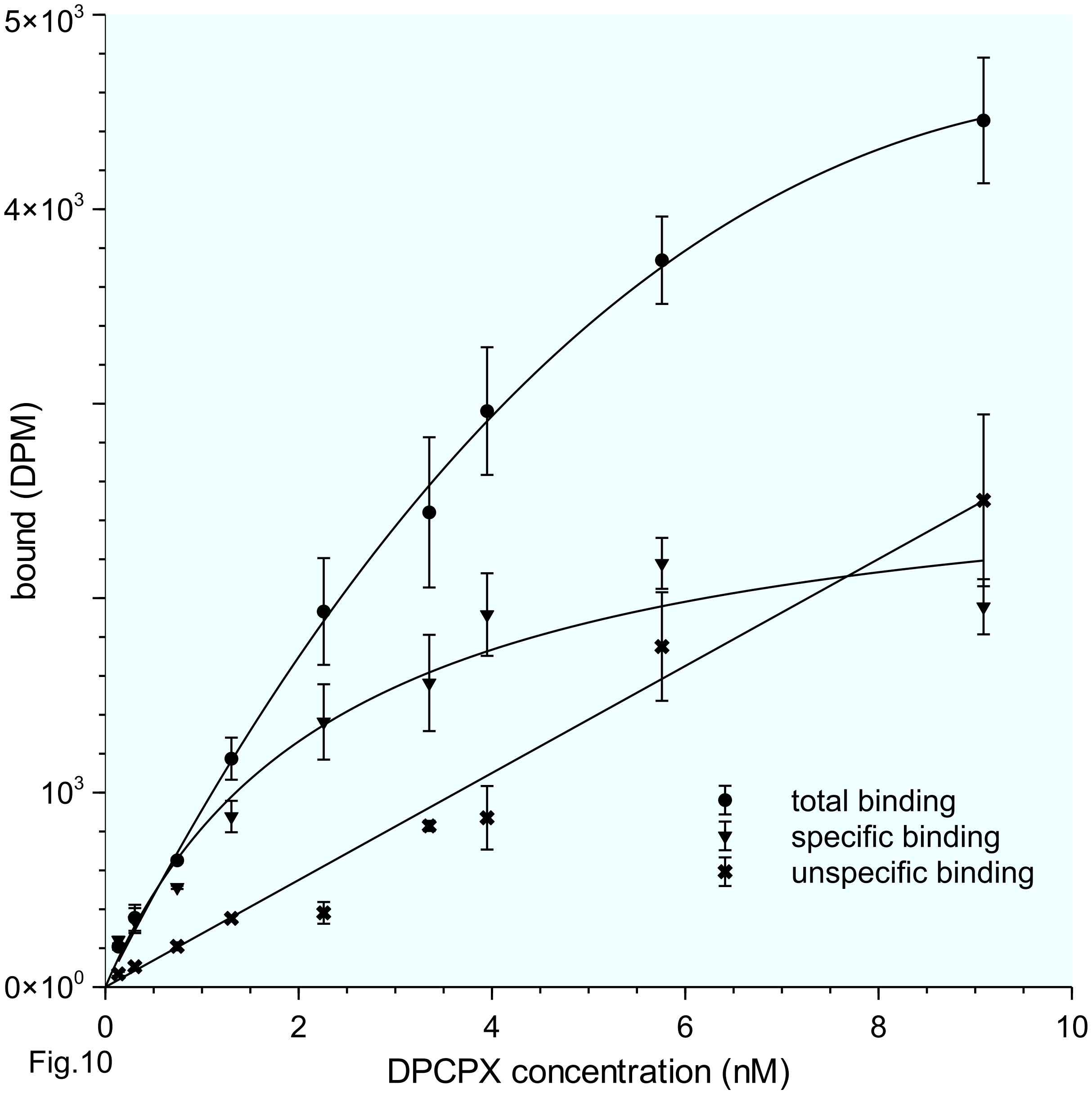

5×10³
4×10³
10³
0×10⁰
bound (DPM)
0
2
4
6
8
10
DPCPX concentration (nM)
total binding
specific binding
unspecific binding

Fig.10