Journal of Chromatography, 594 (1992) 97–103
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 831

Ligand–receptor interactions in affinity cell partitioning

Studies with transferrin covalently linked to monomethoxypoly(ethylene glycol) and rat reticulocytes

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(First received July 17th, 1991; revised manuscript received October 29th, 1991)

ABSTRACT

The partitioning of rat reticulocytes in poly(ethylene glycol) (PEG)–dextran two-phase systems increases into the PEG-rich top phase when the cells are incubated with transferrin covalently modified with monomethoxy-PEG (MPEG–transferrin) prior to partitioning. Two observations support the suggestion that such an increase in top-phase partitioning is due to the specific interaction of the MPEG–transferrin conjugate with the transferrin receptor on the surface of the reticulocyte: first, the MPEG–transferrin conjugate competes with [^{125}I]transferrin for the transferrin receptor on reticulocytes ($K_a = 6.28 \cdot 10^6$ l mol^{-1}); and second, the MPEG-modified transferrin is unable to change the partitioning of rat erythrocytes, cells lacking the transferrin receptor. This example illustrates the feasibility of manipulating the partitioning of a selected cell population when ligand–receptor interactions are exploited. The increase in the partitioning of the reticulocytes takes place within a narrow range of MPEG–transferrin bound per cell, *viz.*, 10.2–11.3 fg per cell. The latter range corresponds to *ca.* 80 000–89 000 molecules of MPEG–transferrin bound per cell.

INTRODUCTION

The partitioning of cells in poly(ethylene glycol) (PEG)–dextran aqueous two-phase systems can be specifically directed towards one of the phases by coating the cells with a ligand which partitions into that phase [1–3]. Several examples have demonstrated the feasibility of this affinity partitioning methodology for the extraction of a selected cell population into the PEG-rich top phase of a biphasic system in which the bulk cells partition into the interface [4–9]. Complete separation of artificial mixtures of red blood cells from two species has been achieved by multiple extractions with the counter-current distribution procedure [4–6]. This affinity cell partitioning technique has been demonstrated to distinguish cells on the basis of the expression of the target protein on their surface [7]. Strategies for isolating cells present at low abundance (1%) have been identified [9]. In all these examples the ligands were antibodies raised against one or several proteins on the surface of the targets cells. The antibodies were covalently linked to monomethoxy-PEG (MPEG) to produce MPEG–antibody conjugates with high partitioning into the PEG-rich phase. More recently, soluble metal–chelate–PEG conjugates have been used to increase the partitioning of erythrocytes from different species [10].

Ligand–receptor interactions, however, have not been exploited to manipulate the partitioning behaviour of a selected cell population. A positive selection of cells in the top phase of the biphasic system by using a natural ligand might have advan-

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tages over the immunoaffinity approach where antibodies, either polyclonal or monoclonal, have to be produced for each single antigen.

Two experimental approaches have been used to apply immunoaffinity cell partitioning. In the first the cells are partitioned in a biphasic system containing the MPEG–antibody conjugate, *i.e.*, the cell–antibody interaction and the partitioning occur simultaneously [5,7]. In the second approach the cells are first incubated with the MPEG–antibody conjugate, to allow cell–antibody interaction, and then the cells are recovered and introduced into the biphasic system for partitioning [4,6,8,9]. In most of the examples the MPEG–antibody conjugate has to be isolated from the non-physiological reaction mixture (excess of activated MPEG and phosphate–borate buffer) prior to use [4–7]. A simplified version of the second approach uses MPEG activated with tresyl chloride (TMPEG), which can be linked to the protein in phosphate–buffered saline (PBS) at pH 7.5, the excess of TMPEG being quenched by reaction with bovine serum albumin, leading to a reaction mixture suitable for direct incubation with the cells [8,9].

We have examined the feasibility of ligand–receptor interactions to manipulate the partitioning of a cell population using transferrin as the ligand and rat reticulocytes as the target cells. MPEG is first covalently linked to transferrin using TMPEG to produce a MPEG–transferrin conjugate with increased partitioning into the PEG-rich top phase. The feasibility of the MPEG–transferrin conjugate as an affinity ligand was studied by examining the partitioning of rat reticulocytes. To test the specificity of the MPEG–transferrin conjugate for the transferrin receptor, two independent approaches were undertaken. Erythrocytes, a related cell type lacking the relevant receptor [11,12], were subjected to the same process. Second, the affinity of the MPEG–transferrin conjugate for the transferrin receptor on rat reticulocytes was addressed. Finally, the value for the association constant (K_a) of MPEG–transferrin for the transferrin receptor was used to establish mathematically the number of MPEG–transferrin molecules required to be bound per cell to produce an increase in the partitioning.

EXPERIMENTAL

Chemicals

PEG (relative molecular mass, M_r = 6000) was obtained from Serva (Heidelberg, Germany), dextran T-500 (lot NK 05164) from Pharmacia (Uppsala, Sweden), MPEG (M_r 5000) (record 398) from Union Carbide (New York, USA), 2,2,2-trifluoroethanesulphonyl (tresyl) chloride from Fluka (Buchs, Switzerland), iodine-125 (sodium salt) from Amersham (Amersham, UK) and human transferrin (iron saturated) and bovine serum albumin (globulin free) from Sigma (St. Louis, MO, USA). All other chemicals were from Merck (Darmstadt, Germany).

Preparation of rat reticulocytes and erythrocytes

Male Wistar rats of weight 175 g received an intraperitoneal injection of phenylhydrazine (30 mg kg^{-1}) during five consecutive days to produce lysis of the erythrocytes. Two days after the final injection the blood with a content of more than 95% reticulocytes [13] was taken over heparin, plasma separated by centrifugation and the pellet of reticulocytes washed twice with 0.15 *M* sodium chloride and finally resuspended in Hank's balanced salt solution (HBSS). Erythrocytes were obtained similarly from the blood of untreated rats.

Affinity cell partitioning

The procedure for affinity cell partitioning includes two major stages: first, modification of the transferrin with MPEG and neutralization of the excess of activated MPEG and second, incubation of the cells with the MPEG–transferrin and partitioning. A similar experimental protocol has been used previously for immunoaffinity cell partitioning [8,9].

The activated (tresylated) MPEG (TMPEG) is obtained by reaction of MPEG with tresyl chloride in anhydrous dichloromethane with pyridine as acid scavenger as reported previously [14]. To produce the MPEG–transferrin conjugate, TMPEG is made up in PBS [0.05 *M* sodium phosphate buffer (pH 7.5) in 0.125 *M* sodium chloride] to a concentration of 216 mg ml^{-1} and mixed with 3 mg ml^{-1} transferrin in PBS, in a 1:1 volume ratio. An excess of 20 mol of TMPEG per mole of lysine residues in the transferrin is used to obtain a high degree of substitution of the protein with TMPEG. The solution is gently

stirred for 2 h at room temperature. An aliquot of this preparation is then removed to measure the partition coefficient of the MPEG–transferrin conjugate. To neutralize the excess of TMPEG, 90 mg ml^{-1} BSA in PBS is added at a 1:1 volume ratio and the solution is gently strirred for a further 2 h at room temperature. The BSA is added in a molar excess of four lysine residues per active group in TMPEG. This strategy has been used previously for immunoaffinity cell partitioning [8,9], as the unreacted TMPEG reacts with proteins on the surface of the cells leading to non-specific increases in their partitioning [15]. To calculate the molar ratio of TMPEG to lysine residues in transferrin and BSA, molecular weights of 76 500 and 60 000 are used, respectively. Transferrin has been described as a protein having 50 lysine residues per molecule whereas BSA has 60 lysine residues per molecule [16]. The concentration of MPEG–transferrin is always expressed as weight of transferrin per unit volume.

To proceed with the incubation of the cells with the MPEG–ligand, the solution containing the MPEG–transferrin is first diluted with PBS to the required concentration in a volume of 400 μl and then 100 μl of HBSS containing the appropriate number of cells (see figure captions) are added. The incubation is carried out at 4°C in a rotary mixer for 2 h, after which the cells are recovered by centrifugation. The supernatant is discarded and the pelleted cells resuspended in 1 ml of top phase. This top phase is sampled to determine the number of cells added to the biphasic system. Then 0.8 ml of top phase containing cells is mixed with 0.8 ml of fresh bottom phase by gentle inversions. The system is allowed to settle for 20 min at 4°C into the top and bottom phases and then the top phase is sampled to determine the number of cells recovered. Partitioning is expressed as the percentage of cells which distribute to the top phase. Determination of cell numbers is done by impedance cell counting with a Coulter Counter Model ZBI.

Preparation of the biphasic system for cell partitioning

The two-phase system consisting of 4.75% PEG, 4.75% dextran, 0.01 *M* sodium phosphate and 0.15 *M* sodium chloride (non-charged system) is prepared at a total of 250 g from the following stock solutions: 40% (w/w) PEG, *ca.* 20% (w/w) dextran (the exact concentration of the stock solution is determined by polarimetry), 0.44 *M* sodium phosphate buffer (pH 6.8) and 0.6 *M* sodium chloride. The total weight is achieved by addition of distilled water. The system is mixed and then allowed to settle into the top PEG-rich and the bottom dextran-rich phases at 4°C for 4–6 h. The top and bottom phases are then separated and stored at 4°C until required. For cell partitioning, the biphasic system is reconstituted by mixing top and bottom phases in a 1:1 volume ratio.

Association constant of transferrin and MPEG-modified transferrin for the transferrin receptor

A competition assay with ^{125}I-labelled transferrin is used. [^{125}I]Transferrin with a specific activity of $1.56 \cdot 10^7$ cpm μg^{-1} is obtained as described previously [13]. Reticulocytes ($1 \cdot 10^8$) were resuspended in 0.5 ml of HBSS containing 1% BSA, 0.06 μg ml^{-1} [^{125}I]transferrin and increasing concentrations of either transferrin or MPEG–transferrin conjugate (0.1–200 $\mu g\ ml^{-1}$). Before sealing the tubes, the air is purged with oxygen–carbon dioxide (95:5, v/v) (gas cylinder supplied by SEO, Spain) and the incubation is carried out at 4°C with constant shaking for 90 min. Reticulocytes are then washed three times in ice-cold PBS. The amount of bound [^{125}I]transferrin is determined in the last pellet of cells (500 *g*, 10 min, 4°C) by using a gamma counter. Bound [^{125}I]transferrin is expressed as a percentage of the amount bound in the absence of transferrin or MPEG–transferrin.

The association constant of the MPEG–transferrin conjugate for the transferrin receptor is calculated from the association constant of transferrin ($2 \cdot 10^8$ l mol^{-1} [13]) and the concentrations of transferrin and MPEG–transferrin conjugate required to displace 50% of the [^{125}I]transferrin form the receptor. This approach has been described by Koteman [17].

The saturation curve of the transferrin receptor with MPEG–transferrin conjugate is simulated by solving the equation for the binding equilibrium and considering a value of 100 000 binding sites per reticulocyte [13].

Partition coefficient of transferrin and MPEG–transferrin

Two-phase systems consisting of 4.75% PEG-6000, 4.75% dextran T-500, 0.15 *M* sodium chloride and 0.01 *M* sodium phosphate buffer (pH 6.8) (non-charged system) are prepared on a weight for weight basis (1 g total) from stock solutions of 40% (w/w) PEG, 20% (w/w) dextran, 0.44 *M* sodium phosphate buffer (pH 6.8), 0.6 *M* sodium chloride, distilled water and 0.1 g of solutions of either the native transferrin or the MPEG-modified transferrin in PBS. After inversion 30–40 times, the mixture is left to settle at room temperature until complete separation of the phases is observed (15–20 min). Aliquots from the top and bottom phases are then analysed for protein concentration by the Coomassie Brilliant Blue assay [18]. The partition coefficient is defined as the ratio between the protein concentrations in the top and the bottom phases.

RESULTS

In order to use transferrin as an affinity ligand to change the partitioning of a cell population towards the PEG-rich top phase of a PEG–dextran biphasic system, the protein requires at least two features (a) high partitioning into the PEG-top phase and (b) affinity for the receptor on the surface of the cells with maintenance of the ligand–receptor interaction in the biphasic system.

Transferrin in its native form favours the dextran-rich bottom phase of a non-charged two-phase system of 4.75% PEG, 4.75% dextran, as shown by its low partition coefficient of 0.6 ± 0.05 [mean ± standard error of the mean (S.E.M.) of three observations]. However, as a result of a 2-h incubation of the protein with TMPEG, the partition coefficient of the tansferrin increases to 6.1 ± 0.5 (mean ± S.E.M. of three observations). Hence, the incubation with TMPEG produces a transferrin that favours the PEG-rich top phase of the system, therefore fulfilling one of the requirements for affinity partitioning purposes.

To evaluate the feasibility of the MPEG–transferrin conjugate for affinity partitioning, rat reticulocytes were used as a model system (Fig. 1). A constant concentration of MPEG–transferrin (75 μg ml^{-1}) was first used and the number of cells per incubation was varied from $7.5 \cdot 10^7$ to $2.5 \cdot 10^7$ cells

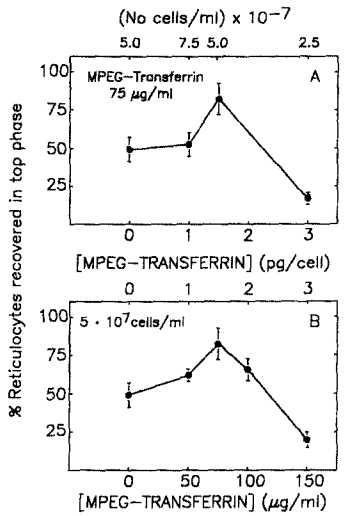

Fig. 1. Partitioning of rat reticulocytes after incubation with the MPEG–transferrin preparation: influence of the concentrations of cells and MPEG–transferrin conjugate. (a) Constant concentration of MPEG–transferrin (75 μg ml^{-1}) and decreasing number of cells and (b) constant number of cells ($5 \cdot 10^7$ cells ml^{-1}) and increasing concentration of MPEG–transferrin. Data are means ± S.E.M. of three experiments.

ml^{-1} (Fig. 1A). A maximum in the partition coefficient, far above that of resting reticulocytes, is observed for the incubation with $5 \cdot 10^7$ cells ml^{-1}. When the number of cells was reduced to $2.5 \cdot 10^7$ cells ml^{-1} the partitioning of the reticulocytes was below that of the resting cells (Fig. 1A). The influence of increasing concentrations of MPEG–transferrin was then studied using the optimum concentration of cells (Fig. 1B). The partitioning of reticulocytes increased with increasing concentration of MPEG–transferrin to a maximum for a ratio of 1.5 pg per cell. Higher concentrations of the MPEG–transferrin preparation led to reductions in the partition coefficient of the reticulocytes, which decreased below that of the resting reticulocytes for a ratio of MPEG–transferrin of 3 pg per cell (Fig. 1B).

To demonstrate that the increased partitioning of reticulocytes was due to a specific interaction of the MPEG–transferrin conjugate with the transferrin receptor, two approaches were used. First the partitioning of rat erythrocytes (cells lacking the transferrin receptor) was studied under conditions leading to increased reticulocyte partitioning. Second, compe-

tition studies between MPEG–transferrin and [^{125}I]-transferrin for the receptor were carried out.

The partitioning of resting erythrocytes (48.4 ± 3.2, mean ± S.E.M., n = 6) did not change on incubation with MPEG–transferrin at concentrations of 0.75 pg per cell (48.8 ± 1.2, mean ± S.E.M., n = 3) or 2 pg per cell (42.5 ± 3.7, mean ± S.E.M., n = 3). These observations exclude coating of the cell surface with any of the components in the MPEG–transferrin preparation.

Fig. 2 shows the displacement of [^{125}I]transferrin from the transferrin receptor on reticulocytes by increasing concentrations of either native transferrin or MPEG–transferrin. Both ligands compete similarly for the transferrin receptor (as shown by the parallel slopes), although MPEG–transferrin shows a lower affinity.

The interaction of the MPEG–transferrin preparation with the transferrin receptor and the lack of effect on erythrocyte partitioning strongly support the suggestion that the increased partitioning of reticulocytes is a result of specific coating of the cells with MPEG via binding of the MPEG–transferrin conjugates to the transferrin receptor.

The association constant for the MPEG–transferrin conjugates is $6 \cdot 10^6$ l mol^{-1}, two orders of magnitude below that of unmodified transferrin ($2 \cdot 10^8$ l mol^{-1} [13]). Knowing the association constant for the MPEG–transferrin conjugates and the number of binding sites on the reticulocyte, the curve for the saturation of the receptor with MPEG–transferrin is easily simulated (Fig. 3). This allows us to interpolate the values for MPEG–transferrin bound per cell under those incubation conditions leading to increased partitioning of reticulocytes (Fig. 3). The

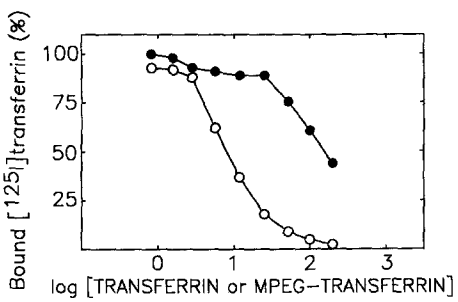

Fig. 2. Displacement of [^{125}I]transferrin (0.06 μg ml^{-1}) from transferrin receptor on rat reticulocytes ($2 \cdot 10^8$ cells ml^{-1}) by (○) transferrin and (●) MPEG-modified transferrin.

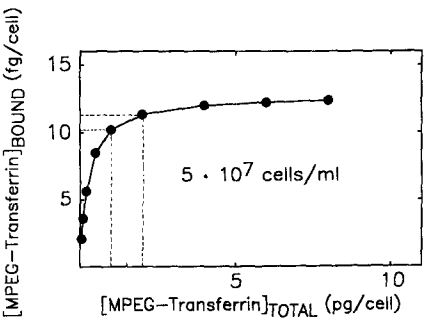

Fig. 3. Simulation of the saturation of the transferrin receptor with MPEG–transferrin in an incubation with $5 \cdot 10^7$ reticulocytes ml^{-1}.

number of MPEG–transferrin molecules bound per cell with the corresponding amounts of MPEG–transferrin bound per cell are summarized in Table I.

DISCUSSION

Transferrin covalently linked to MPEG has been successfully applied as an affinity ligand for affinity cell partitioning. The covalent linkage of MPEG to transferrin is demonstrated by the increase in the partition coefficient in PEG–dextran aqueous two-phase systems and presumably takes place via the ε-amino groups of lysine residues in the protein. Such attachment has been demonstrated previously for BSA [14].

The MPEG–transferrin conjugate is a suitable ligand for affinity cell partitioning, as shown by its ability to increase the partitioning of rat reticulocytes. Such an increase is due to a specific interaction of the MPEG–transferrin conjugate with the trans-

TABLE I

MPEG–TRANSFERRIN BOUND TO TRANSFERRIN RECEPTOR AFTER INCUBATION WITH RAT RETICULOCYTES ($5 \cdot 10^7$ CELLS ml^{-1}): INFLUENCE OF THE TOTAL CONCENTRATION OF MPEG–TRANSFERRIN

MPEG–transferrin in incubation (pg per cell)	MPEG–transferrin bound (fg per cell)	MPEG–transferrin bound (molecules per cell)
1.0	10.2	80 217
1.5	10.9	85 884
2.0	11.3	89 034

ferrin receptor because first, the increased partitioning only takes place with the cells expressing the receptor, and second, the MPEG–transferrin conjugate retains affinity for the receptor.

The reduced affinity of the MPEG–transferrin conjugate for the transferrin receptor was not unexpected, although its molecular basis is unclear. It is well established that the covalently linkage of MPEG to enzymes leads to a severe reduction in their activity, presumably by preventing the formation of the substrate–enzyme complex owing to steric hindrance [19–21]. It is also known that the apparent binding activity of a monoclonal anti-rabbit Fc fragment antibody is considerably decreased when PEG-1900 is covalently attached to the molecule [22], but this is the first study in which the affinity of the protein for its receptor after covalent linkage of MPEG is reported.

Although these results demonstrate the principle of affinity cell partitioning, the experimental approach adopted here does not provide the spectacular increase in partitioning observed when MPEG–antibodies were used instead of MPEG–transferrin [9]. In addition, with MPEG–antibodies the increased partitioning did not decrease at high concentrations of the ligand [9]. The BSA added in a large excess to quench the unreacted TMPEG might be responsible for the reduction in partitioning of rat reticulocytes at concentrations of MPEG–transferrin of 2 pg per cell or higher. It is known that BSA partitions into the bottom phase of the biphasic system used in this study [14]. The cells coated with the BSA will then partition towards the bottom phase, thereby opposing the affinity effect of the MPEG–transferrin. A decrease in the partitioning of liposomes with MPEG covalently linked to their surface has been observed when the liposomes were exposed to plasma, and this effect has been attributed to absorption of albumin to the lipid bilayer [23]. The number of transferrin receptors per reticulocyte is much lower than the number of antigenic determinants per erythrocyte used for the immunoaffinity approach [9]. This difference might explain why the negative effect of BSA was not seen in the latter.

The immediate alternative to quenching with BSA is the isolation of the MPEG–ligand from the excess of activated TMPEG. However, this is not a trivial issue, as the MPEG in the mixture interferes with the resolution of chromatographic techniques such as gel filtration [24,25]. In addition, despite the low molecular weight of the MPEG, its exclusion radius is substantially greater than that of proteins of even greater molecular weight [26] and this complicates the use of other conventional molecular sieve methods such as dialysis or ultrafiltration. Quenching with other small nucleophiles rather than BSA will affect the osmolarity of the preparation and then again isolation of the PEG–ligand conjugate will be required. These problems have not yet been resolved. New chromatographic media incorporating PEG in the matrix [27] might help to isolate the PEG–transferrin conjugate from the reaction mixture (and this might increase the effectiveness of the ligand for affinity cell partitioning).

To summarize, we have shown the feasibility of taking advantage of ligand–receptor interactions for affinity cell partitioning. Despite the drastic reduction in the affinity of the MPEG–transferrin for the transferrin receptor, the MPEG–transferrin conjugate can efficiently increase the partitioning of the reticulocyte. This model system has demonstrated the feasibility of using affinity cell partitioning to change the partitioning of a cell when about 80 000 molecules of the PEG–ligand are attached to the surface. To optimize affinity cell partitioning further, suitable methodologies to produce individual MPEG–ligand conjugates free of spent TMPEG or quenching agent will be required.

ACKNOWLEDGEMENTS

This work was supported by a grant awarded by the Ministerio de Educacion y Ciencia to C.D. and by grants from the Comision Interministerial de Ciencia y Tecnologia (Spain). The authors thank Dr. Angel Herraez for critical reading of the manuscript.

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