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Study of dissociation of human chorionic gonadotropin monoclonal antibody complexes using nitrocellulose as an insoluble support

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A method for the determination of real-time kinetic parameters of antigen–antibody reaction using ^{125}IhCG and immobilized monoclonal antibody (MAb) has already been reported. Improvizations were needed to make it amenable for automation. These included the development of nitrocellulose (NC) as a solid support for the adsorption of MAb, utilization of a lead shield to specifically quantify the radioactivity released in the presence of the radioactive complex and the development of a computer program for analysis of the data. A method was developed for the determination of the kinetic constants with the combined use of the NC adsorbed MAb, ^{125}IhCG and the lead shield. Kinetic parameters derived from this method compared well with the earlier method, and promise the feasibility of developing an inexpensive indigenous equipment for the measurement of real-time kinetic analysis of ligand–ligate interaction.

STUDY of real-time kinetics of ligand–ligate interaction has assumed importance in scientific investigations because of its great potential and the availability of the automated equipment for these studies[1,2]. Our studies on the dissociation of the human chorionic gonadotropin-monoclonal antibody (hCG-MAb) system adopting a classical approach have indicated that the dissociation of the hCG-MAb fits into a two-step reaction[3–5], contrary to the generally accepted view of a single-step reaction. This method measures the rate of release of ^{125}IhCG from an immobilized MAb–^{125}IhCG complex and calculates the rate constants of the dissociation. This method could be developed as an inexpensive alternate for the BIAcore, if automated. The method needed improvements in several lines to make it viable for automation. One of them was the support for immobilization, which in the earlier approach was virgin plastic through immunochemical bridge (ICB)[3,6]. While the ICB was satisfactory for most of the high affinity antigen–antibody systems, it had limited reach and was adaptable for high affinity ligand–ligate systems. An alternate support for adsorption of ligand was nitrocellulose (NC). NC is known to adsorb proteins and nucleic acids well with high capacity and finds routine use in Western and Southern blots. Hence NC as a matrix for adsorption of MAb was investigated. The immobilized MAb–^{125}IhCG complex (on NC) was studied for dissociation, and the results are presented in this communication. A second disadvantage for automation was that the experimental protocol needed repeated pipetings for measurement of the radioactivity released.

An alternate method for measurement of the release of radioactivity was investigated which could make the method amenable for automation, making the radiolabelled approach a practical inexpensive alternate for the BIAcore.

Human chorionic gonadotropin (hCG) was prepared from early pregnancy urine using immunoaffinity chromatography. Its characteristics have already been reported[7]. Iodination grade hCG was kindly provided by NIH, USA. Iodination was carried out by the standard procedure using Iodogen[8].

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NC discs (6 mm diameter) were punched out and used for coating the monoclonal antibodies. The discs (60 in number) were taken in a 6 ml scintillation vial and coated with 2 ml MAb VM4 ascites at 1/1 k dilution in RIA buffer containing 0.3% bovine serum. If the discs were coated in buffer, the consistency of binding was found to be poorer. Addition of 0.3% bovine serum in the coating buffer did not only improve the consistency but also the quantity of the ^{125}IhCG bound to immobilized MAb. The discs were left overnight on a rotator and washed next day with buffer. Then 2 ml of 1% BSA solution was added for blocking and left overnight on the rotator. These discs were stored for 3–4 months in RIA buffer until use.

The MAb immobilized disc was taken in a plastic tube (3 ml) and 300 μl of ^{125}IhCG (50,000–100,000 cpm) in RIA buffer was added and incubated overnight. The disc was washed three times with 1 ml RIA buffer to remove all the unbound ^{125}IhCG. This NC–MAb–^{125}IhCG complex (NC_{com}) was subsequently used in all dissociation experiments. The NC_{com} obtained under these conditions had 20–30% saturation of the MAb bound to the NC.

NC_{com} (6 discs) was taken in a scintillation vial (6 ml capacity) and counted in a gamma counter. Dissociation of the complex was started by adding 3 ml of 1 μg/ml hCG solution. This quantity of the hCG corresponded to a 1000-fold excess of the label and 200–300-fold excess of the total MAb that was adsorbed on the NC. At different time intervals 0.5 ml of the solution from the scintillation vial was pipetted into a plastic tube and counted. This measures the radioactivity dissociated from the complex at that point of time. After counting, contents of the tube was transferred *in toto* back into the scintillation vial. The release of radioactivity with time was subsequently used for the determination of kinetic constants of dissociation.

Figure 1 shows a diagrammatic representation of the lead shield (LS). Lead quantitatively blocks gamma radiation; 1 mm lead can block the radioactivity by more than 99%. Since the radioactivity of NC_{com} is localized on the NC disc a 'radioactive' disc (NCMAb–^{125}IhCG) placed at the bottom of the LS measures very low radioactivity in a gamma counter. However when this immobilized ^{125}IhCG is released into the medium (1.2 ml), the radioactivity is spread over the whole solution. Thus, part of this radioactivity is in the volume of the solution not masked by the LS and hence easily measured by the counter. Thus the rate of release of radioactivity during dissociation can be obtained. However the recovery of radioactivity is only partial, since the radioactivity in that volume of the solution covered by the LS is not measured.

The NC_{com} disc was taken in a 3 ml plastic tube and radioactivity was measured in a gamma counter. This was taken in a LS, placed in the well of the gamma counter and the radioactivity was again measured

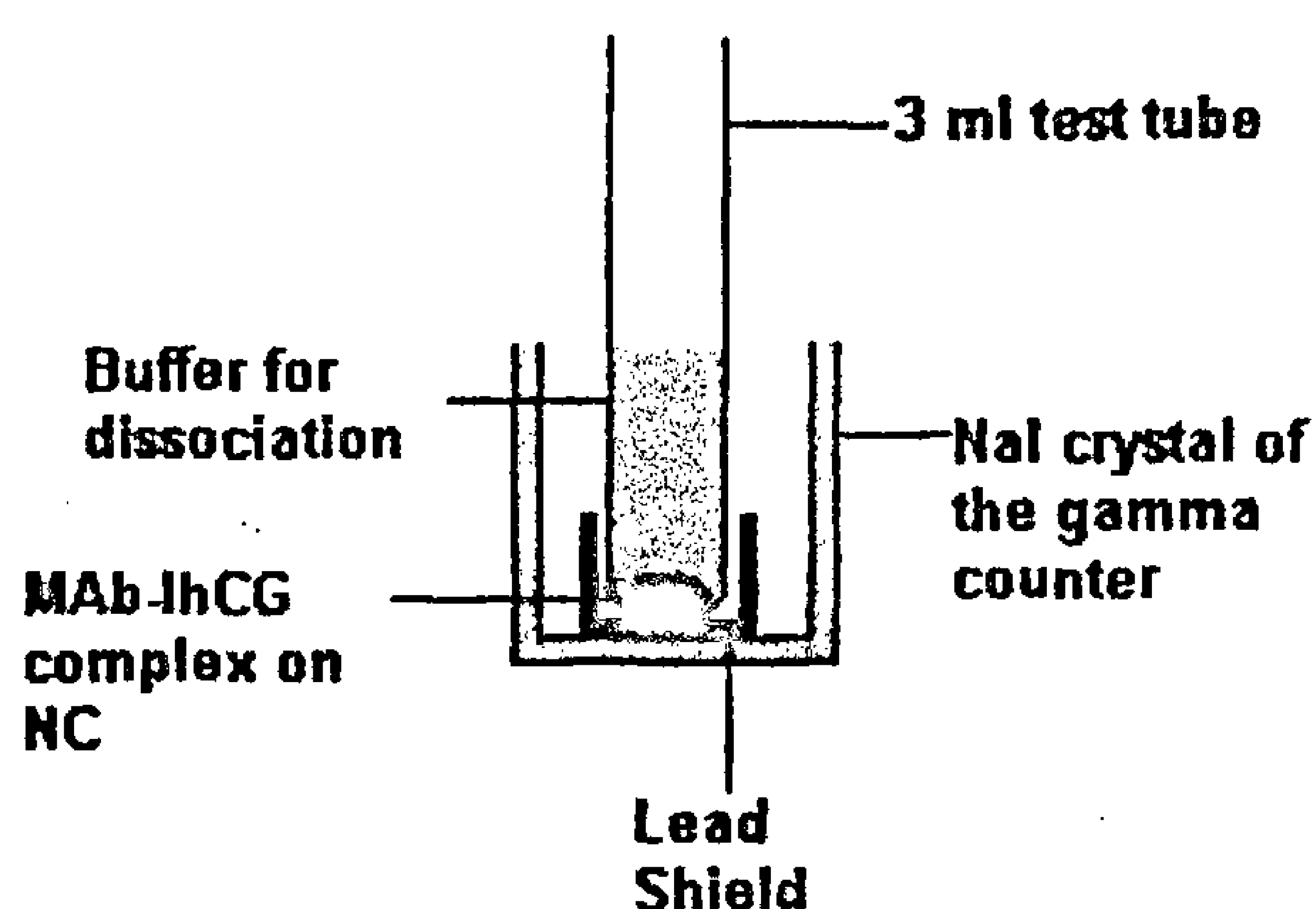
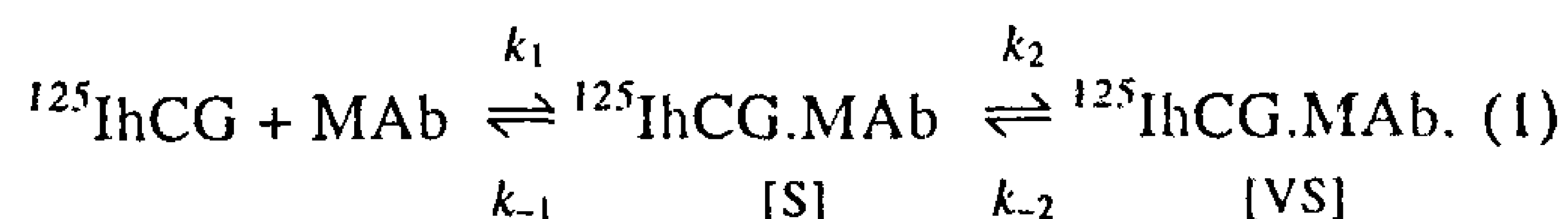


Figure 1. Schematic representation of the functioning of the lead shield. Dimensions of the various parts are as follows: 3 ml tube – ID 7 mm, length 70 mm, Lead shield-ID 10 mm, OD 13 mm, length 17.5 mm, thickness 1.5 mm; NaI crystal (well of the gamma counter) – ID 16 mm: length 50 mm; Nitrocellulose disc (Nc_{com}) – dia. 6 mm: For further details see the text.

(< 0.5% of total radioactivity). This represented the background, which was always well below 1000 cpm. Dissociation was started by adding 1.2 ml of 10 μg/ml hCG solution in RIA buffer to the tube already placed in the LS. The solution was gently vortexed each time before measurement to ensure that the released radioactivity is uniformly distributed, and the release was monitored with time. Care was taken to see that the NC disc settles at the bottom of the tube before measuring the counts. Once the dissociation was started, the LS was not removed from the well of the counter until the completion of the experiment.

The reaction scheme between an antigen and antibody is considered to be a two-step reaction, and the mechanism proposed earlier[5] shown in eq. (1), has been used in the analysis of the dissociation data.



$$^{125}\text{IhCG} + \text{MAb} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{[\text{S}]}{^{125}\text{IhCG.MAb}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{[\text{VS}]}{^{125}\text{IhCG.MAb.}} \quad (1)$$

where the k's are respective association and dissociation constants, S and VS are the 2 forms of the complex.

Equation (2) was used in fitting the dissociation[5]

$$x = a_1[1-\exp^{(-k_{-1}t)}] + a_2[1-\exp^{(-k_{-1}t)}]*[1-\exp^{(-k_{-2}t)}], \quad (2)$$

where x is the radioactivity released at time t, k_{-1} is the rate constant of dissociation of S to reactants, k_{-2} is the rate constant of VS–S transformation; a_1 is the initial concentration of S at $t = 0$; a_2 is the initial concentration of VS.

A computer program for curve fitting was used. At the beginning of the dissociation the NC_{com} will have the

two complexes in unknown proportions and the dissociation of the S complex releases the ^{125}IhCG into solution directly. However release of the ^{125}IhCG from the VS complex goes through the S complex and hence the initial part of the graph presents predominantly the dissociation from the S complex. For curve fitting purposes, the maximum amount of ^{125}IhCG which can dissociate was considered to be the amount dissociated over a 24 h period $(a_1 + a_2)$. A computer program was written which considered several values of k_{-1}, k_{-2}, a_1 and a_2 (such that summation of $(a_1 + a_2)$ is the overnight dissociation measured) and obtained a theoretical graph. The error (%) at each point of time between the theoretical and experimental plots was obtained. Best fit was considered to be the one in which the summation of the square of the errors was minimum. In order to obtain the accurate values of $(a_1 + a_2)$, for different values of the dissociable counts $(a_1 + a_2)$ ranging between 100 and 120% of the overnight value, the minimum of the summation of the squares was measured by the program and those values of k_{-1}, k_{-2}, a_1 and a_2 which gave the minimum error were taken as true values. (For details see Table 1).

Table 1. Determination of $(a_1 + a_2)_{max}$ from the dissociation data

$a_1 + a_2$ (cpm)	a_1 (cpm)	a_2 (cpm)	k_{-1} (/min)	k_{-2} (/min)	SD
18500	6345	12155	0.00895	0.000780	2.55
19000	6365	12635	0.00885	0.000754	2.45
19500	6370	13130	0.00875	0.000743	2.43
19850	6429	13420	0.00870	0.000732	2.55
20000	6610	13390	0.00870	0.000627	2.60
20500	6620	13880	0.00860	0.000611	2.46

Choice of maximum and minimum values to be considered was made as follows.
The minimum value of k_{-1} was calculated assuming that the total dissociation observed represented [S]. k_{-1} was calculated by the equation $(dx/dt)_0 = k_{-1}*[S]$, where $(dx/dt)_0$ represents the initial slope of the dissociation graph.
The maximum value of the k_{-1} was chosen on the basis that only 20% of the dissociated ^{125}IhCG is represented by [S]. k_{-1max} was calculated using the equation $(dx/dt)_0 = k_{-1max}*[S]$, where S = 20% of $(a_1 + a_2)$. The choice of k_{-2} was taken as 5–35% of the k_{-1} value in all calculations.

Table 2. Effectiveness of lead shield in blocking the gamma radiation

Mode of reading	Radioactivity on NC (cpm)	Radioactivity dispersed in 1.2 ml buffer (cpm)
Normal	98950	97880
Lead shield	780	28900
% Recovery	0.05	30.5

The effectiveness of the LS to prevent the passage of the gamma radiation was complete (Table 2). The radioactivity recovered with the LS by the counter from NC_{com} was 0.05%, while the same amount of radioactivity dispersed in 1.2 ml of solution resulted in 30% recovery of radioactivity by the counter.

Dissociation profile of the NC_{com} presented by the points in the aliquot method (Figure 2) and LS method (Figure 3) fit well with the best fit theoretical plot for the two-step mechanism. Rate constants obtained in the two methods were compared with those obtained using the immunochemical bridge method (Table 3).

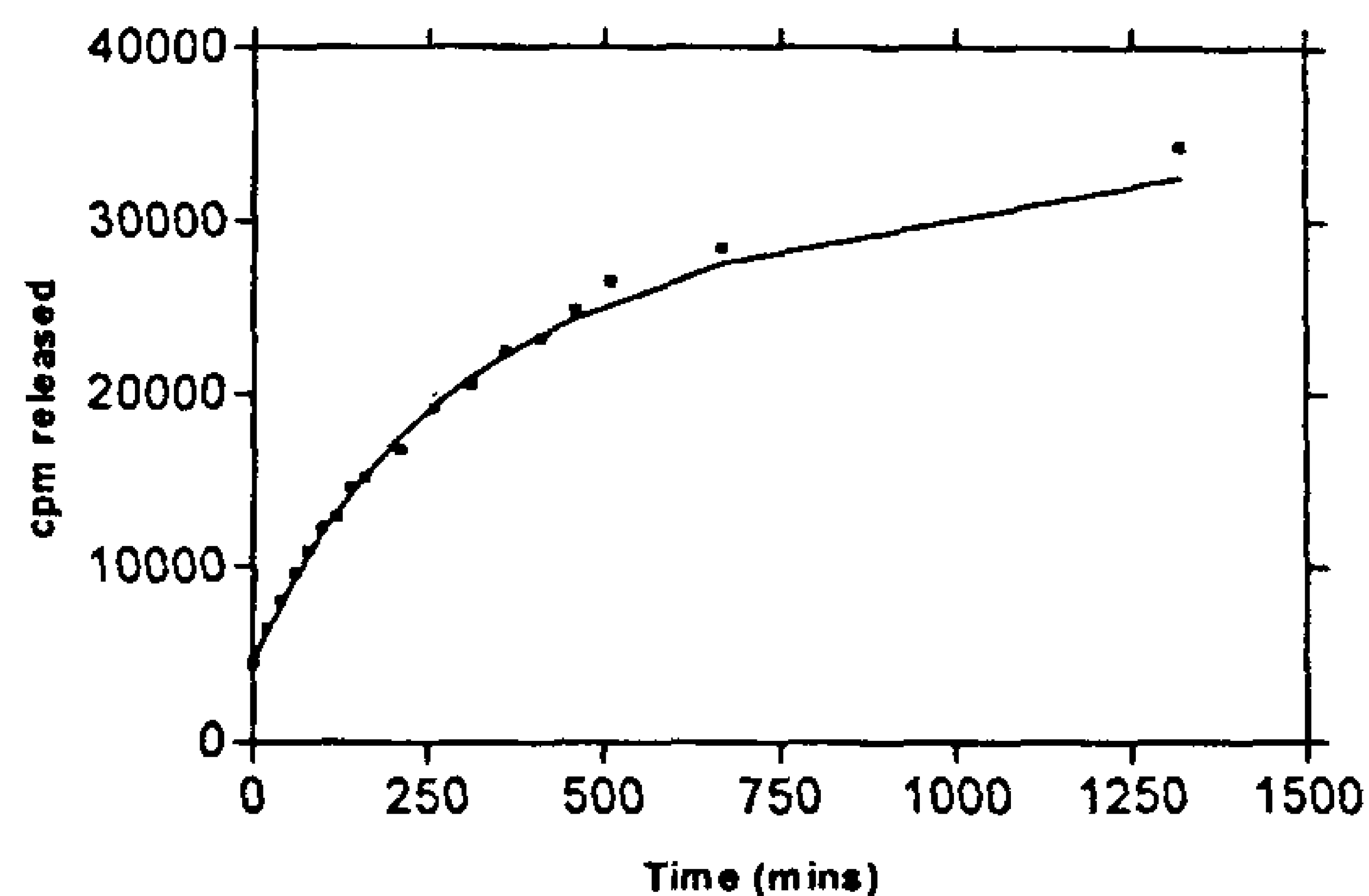


Figure 2. Dissociation profile of the NC_{com} using the aliquot method. The NC_{com} was obtained by incubating 11 lakhs cpm of ^{125}IhCG with the antibody coated NC discs (6 no) in a total volume of 1 ml. Total radioactivity bound was 2.77 lakhs. Dissociation was done in a total volume of 3 ml and 0.5 ml aliquots were taken for the measurement of radioactivity at various time points. The curve presents the best-fit for the experimental data (points). Values obtained for the kinetic parameters are shown in Table 3.

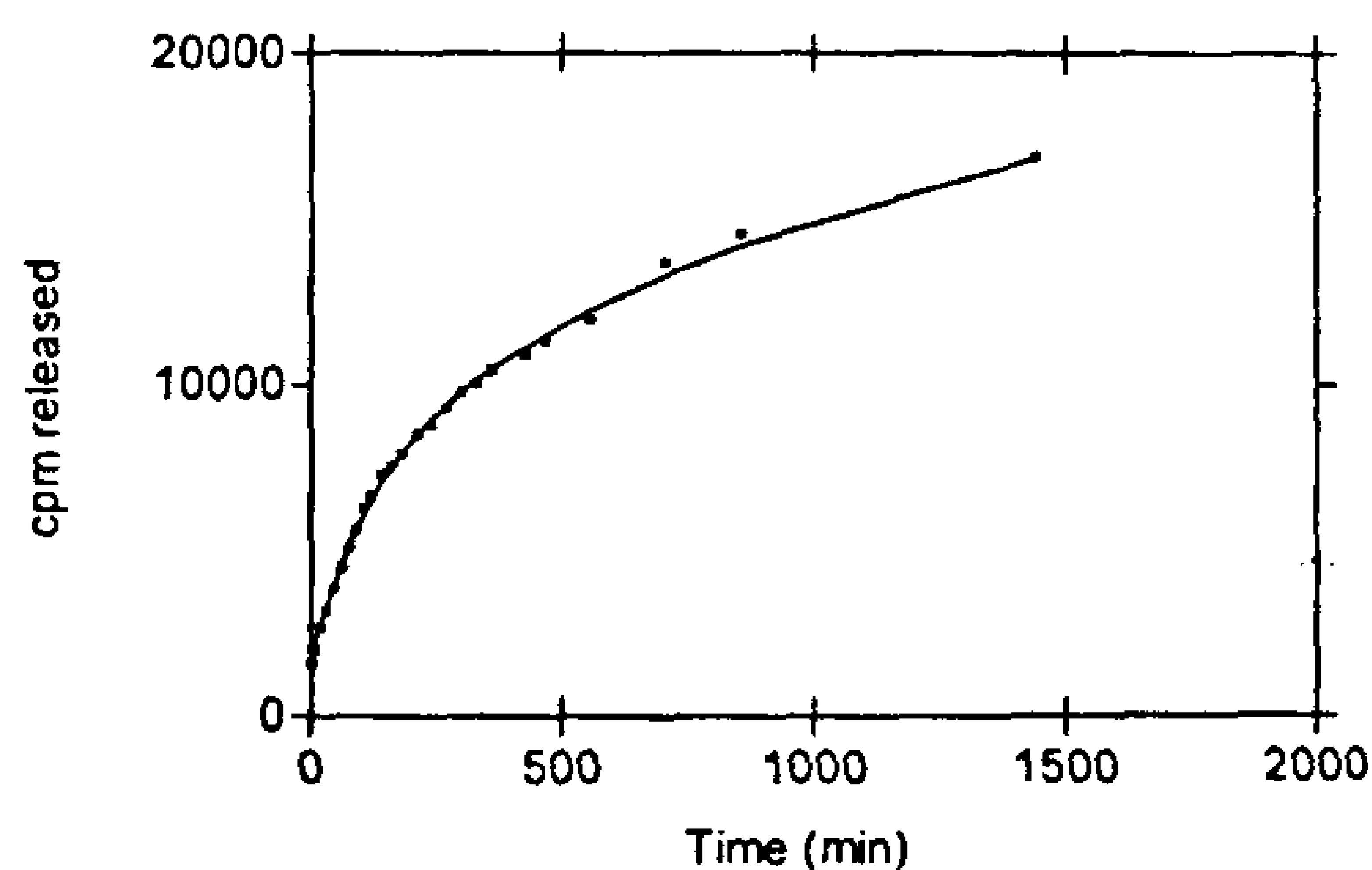


Figure 3. Dissociation profile of the NC_{com} using lead shield. Binding was done using a MAb-NC disc and 2.8 lakhs cpm of ^{125}IhCG in a total volume of 0.3 ml overnight in RIA buffer. The bound NC_{com} (75000 cpm) was placed in a 3 ml tube and dissociation was started by adding 1.2 ml of hCG solution of 1 µg/ml concentration. The lead shield placed in the well of the counter at the start of the experiment was not disturbed until the end. The curve presents the best fit for the experimental data (points) and the values of kinetic constants obtained are as shown in Table 4.

Table 3. Evaluation of kinetic parameters of dissociation of ^{125}IhCG-MAb complexes obtained from different methods

Method used	k_{-1}/min	k_{-2}/min
ICB method*	0.0080	0.0010
Aliquot method**	0.0090	0.0015
LS method***	0.0089	0.0009

*Taken from ref. 5; **Value derived from Figure 2; ***Value derived from Figure 3.

Table 4. Dissociation data using lead shield method

Input (cpm)	TCB (cpm)	$a_1 + a_2$ (cpm)	a_1 (cpm)	a_2 (cpm)	k_{-1} (/min)	k_{-2} (/min)	SD	a_1/a_2
273058	69976	13500	5960	7540	0.009	0.001	2.44	0.79
273782	70118	13500	5230	8270	0.011	0.0015	2.23	0.63
273261	70701	14500	6415	8085	0.009	0.0011	2.18	0.79
280500	70934	14000	5725	8275	0.011	0.0012	2.77	0.69
272391	75944	13000	5280	7720	0.011	0.0011	2.74	0.68

Input = 272930 ± 617; k_{-1} = 0.0102 ± 0.001; k_{-2} = 0.001186 ± 0.000189; Bound, 70197 ± 483.

The rate constants are comparable in all the three systems. Of the four parameters obtained in this method, k_{-1} and a_1 are measured with greater certainty. The values of k_{-2} and a_2 depend on the extent of the errors in the values of $(a_1 + a_2)$ chosen for calculations. In fact this is one of the major drawbacks of this method, where accurate values of $(a_1 + a_2)$ cannot be determined, and overnight value is an approximation. In the analysis of the results presented in the paper the value taken for $(a_1 + a_2)$ is not available for experimental verification. It is the value of the radioactivity that would be released if the reaction were to be carried for a very long time. Ideally the increase in radioactivity released should plateau off with time, and after some time there should not be any increase in the radioactivity measured. This took about 2 days to stabilize. While this is possible in theory, it is not a practical approach to determine $(a_1 + a_2)$. Hence to obtain the best-fit, value of $(a_1 + a_2)$ was varied stepwise (2%) up to ±20% of the dissociation observed overnight, and the value of $(a_1 + a_2)$ which gave minimum SD was considered as the best value for obtaining the parameters. One such optimization is shown in Table 1.

Consistency of the parameters obtained in the LS method is satisfactory (Table 4). The rate constant k_{-1} was accurate and consistent over several determinations. It was observed here that though the counts bound are high the radioactivity that is dissociated over 24–48 h is only 50% even after 48 h of dissociation. This apparent nonreversible portion appears to increase with the storage time of ^{125}IhCG. However the kinetic parameters obtained by the method were independent of the storage time of ^{125}IhCG. It can be indicated here that in BIAcore, a majority of the antigen–antibody interactions are essentially nonreversible[9–11] and well-documented in the literature[12,13].

MAb was adsorbed on NC with retention of its binding ability to ^{125}IhCG. Binding ability of several ligands is well documented in the blot techniques routinely used in biological research; our studies showed that not only was binding ability retained, but quantification of the binding parameters was possible. The MAb binding to NC was nonreversible at neutral pH and repeated washing of the bound NC–MAb did not result in significant reduction of binding to the NC disc. Infact, iodinated IgG adsorbed on NC did not release any radioactivity on repeated washing (unpublished data).

NC is a good support for such investigations. This is borne by the fact that the MAb bound to the NC consistently. The specific binding to NC was found to be within ±5% in a batch of coated discs (Table 4). The NC as a solid matrix has several advantages. It has large capacity for adsorption of IgGs (as much as microgram/sqcm) compared to a few ng/sq cm on plastic[5]. Thus a lower affinity ligand–ligate system can be investigated using the NC as a solid support. NC can bind to oligonucleotides well, while their binding to virgin plastic is minimal. Thus extending the studies to other systems is feasible. In routine experiments NC has been extensively used as a means for adsorption of proteins and oligonucleotides in several blotting approaches. To our knowledge this is the first instance where it was used as a support which can be utilized for quantitative investigations on the kinetics of the ligand–ligate interactions. NC is an easily accessible and inexpensive support, and binding of the ligand/ligate does not involve any sophisticated technology and chemistry. These advantages could well make NC one of the best solid supports for studying real-time kinetics of ligand–ligate interactions.

The LS approach improved the experimental accuracy. During dissociation, the contents of the tubes were not transferred and hence errors arising out of experimental manipulations were eliminated. Thus the precision of the experiment was significantly increased, resulting in accurate determination of rate constants. The results of dissociation were consistent (Table 4). The rate constants obtained were comparable in all the three methods (Table 3). The LS method has a disadvantage in that the recovery of radioactivity is only partial (30%). However this does not affect the quality of the results as the dissociation is concentration-independent. Thus the parameters derived are as accurate as those obtained from other methods where quantitative recovery of the release is achieved. The LS method is amenable for automation as the number of manual operations involved in measurement of the radioactivity is minimum.

While dissociation analysis provided consistent data, there was always some residual ^{125}IhCG which remained bound to the NC even after several days of dissociation. The extent of dissociation observed ranged between 50 and 80%. However, the rate constants obtained were independent of the extent of the dissociation. It was generally observed that when the ^{125}IhCG was freshly prepared, or had high specific activity the dissociation was much better (70–80%), and reduced to 50% with storage. The reason for this lack of dissociation of a part of the ^{125}IhCG is now known. Infact very limited (Ag–Ab) dissociation of macromolecular complex is frequently observed in the BIAcore experiments also[10–12], and this apparent nonreversibility is not unique for the radiolabel.

In summary, we successfully adopted NC as an alternate support for adsorption of MAbs in real-time kinetic investigations. The experimental approach for the study of dissociation of NC_{com} was simplified using a LS and this improvement makes it amenable for automation. The use of NC would also make the method more versatile extending its range to other types of ligands other than proteins.

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Mitogenic and melanogenic activity of human placental protein/peptides on melanoma cell

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An alcoholic extract of the term human placenta, when resolved into a protein/peptide component was found to show pigment and growth-inducing activity on B16F10 mouse melanoma cell *in vitro*. The protein/peptide component was studied in the dose range 0.01–100 µg/ml. At a concentration of 0.1 µg/ml, the growth of mouse melanoma was maximum, while the melanin formation was optimum at 50 µg/ml. Furthermore, the fraction markedly changes cellular morphology. Presence of ET-1 (endothelin-1) and ACTH (adrenocorticotropic hormone), two important melanocyte response modifiers was detected in the fraction. The activity of the protein/peptide fraction, in respect of morphology, pigmentation and growth, when compared with a mixture of pure ET-1 and ACTH reconstituted, at a level that is present in it, was found to be significantly higher.

MELANOGENESIS is a phenomenon associated with melanin pigment formation primarily in hair and skin of mammals[1]. Melanin, the dark brown pigment, is synthesized within membrane-bound organelles called melanosomes of the epidermal melanocyte cells. Among different bioactive proteins and peptides known to regulate melanogenesis, adrenocorticotropic hormone (ACTH)[2,3] and endothelin (ET)[4,5] have been reported in human placenta. ET though originally identified as a vasoconstrictive peptide, plays an important and regulatory role in mammalian pigmentation. Four mammalian ETs, namely ET-1, ET-2, ET-3 and big ET have been reported[6–8], though little information is available about their biological relevance, except ET-1. These peptides, produced and secreted by keratinocytes, act as strong mitogen[9,10], migration and dendricity inducer[11,12], and melanogen[13] for human melanocytes. ET also stimulates mitogenesis *in Swiss 3T3 fibroblast cell line*[14], renal mesangial cell[15] and vascular smooth muscle cells[16]. Mitogenic and melanogenic effects of ACTH on human melanocytes[17–19] and also in B16 melanoma cells[20] have also been reported in recent studies.

One prototype alcoholic extract prepared from human placenta of HIV and Hepatitis B negative mothers[21]

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