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Separation of Antibodies by Liquid-Liquid Aqueous Partition and by Liquid-Liquid Partition Chromatography

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SEPARATION OF ANTIBODIES BY LIQUID-LIQUID AQUEOUS PARTITION AND BY LIQUID-LIQUID PARTITION CHROMATOGRAPHY

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Abstract

In this review, we describe the use of liquid-liquid aqueous partition as a method for the separation of antibodies. Water-based two-phase systems made up of polyethylene glycol and dextran have, by far, been the most frequently used systems. The distribution of a molecule in these systems depends on its exposed surface properties and is described by its partition coefficient. The separation may be performed in a single step in a batch experiment or in several steps using various forms of automated counter-current extraction methods, referred to in this review as liquid-liquid partition (LLP). The sensitivity and selectivity of the two-phase technique can be considerably improved by employing a column chromatographic approach, liquid-liquid partition chromatography (LLPC). In LLPC, the bottom-phase of the two-phase system is adsorbed onto a support and packed into a column which is eluted with the corresponding top-phase. In the first part of this review, the methodology behind LLP and LLPC is described and outlined in broader terms, before the properties and prestanda of the two approaches are compared. In the second part, the results obtained by LLP and LLPC on antibodies are described in more detail. This review shows, that

liquid-liquid aqueous partition is a powerful tool for antibody analysis, that is for purification and fractionation, detection and separation of conformational isomeric forms, examination of surface properties related to antigen specificities and for providing new interesting information about the events upon antigen-antibody complexation and about possible ligand-induced conformational changes.

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1) Introduction

Partitioning in water-based two-phase systems can be used to separate antibodies as well as proteins in general, DNA, subcellular particles and whole cells[1-7]. The separation can be based on either differences in overall surface properties[8-11] or in a single physico-chemical property, such as size[12,13], hydrophobicity[14,15] and affinity[16-18], depending on how the experiments are designed. Aqueous two-phase systems are generally composed of water solutions of two structurally distinct hydrophilic polymers or of one polymer and salts such as ammonium sulphate, manganese sulfphate and potassium phosphate[1]. Above critical concentrations of these components, spontaneous phase separation takes place with each of the two resulting phases enriched with respect to one of the components. In the case of antibodies, buffered two-phase systems formed by polyethylene glycol (PEG) and dextran are, by far, the most frequently applied systems[7]. The way in which a protein molecule is distributed between the two phases in a given PEG/dextran system depends on its three-dimensional structure and general surface properties[8-11] and is described by its partition coefficient, K (the ratio of the concentration of the molecule in the top phase to that in the bottom phase). Thus, partitioning in aqueous PEG/dextran two-phase systems offers a unique means of separating intact antibodies in solution with respect to their overall exposed surface properties[7].

There are two methodological approaches of the two-phase technique, referred to in this review as liquid-liquid partition (LLP) and liquid-liquid partition chromatography (LLPC), that can be used. In LLP, the separation may be performed in a batch experiment in a single step or in a few repeated steps by optimizing the composition of the phases. However, finding such optimal systems may pose

difficulties in order to obtain adequate separation, and various froms of automated counter-current extraction methods (≤ 180 steps) have therefore been developed[19-21]. Still, LLP has only been used very little for the separation of antibodies[22-24], the limited resolution being a major impediment.

The usefulness of aqueous two-phase partitioning for the separation of antibodies has been increased considerably by adopting a column chromatographic approach, LLPC. In LLPC, the bottom phase of the two-phase system is adsorbed onto a support and packed into a column which is eluted with the corresponding top phase[7,25-27]. LLPC adds the advantages of column chroma-tography in terms of an increased sensitivity and selectivity, and a reduced consumption of time and material. Finding materials suitable as the support for LLPC was for a long time a major problem, however. Several attempts were made to adsorb the bottom phase onto supports made of agarose beads[28], polyethers immobilized on Sepharose[29], silicates[30,31] and cellulose[32,33]. The problem was finally solved by combining the affinity of polyacrylamide for the dextran-rich bottom phase of the most frequently used phase system, the PEG-dextran system, with the mechanical strength of hydrophilic vinyl (LiParGel) or silica (LiChrospher Diol) particles[34]. It has recently been shown that also dextran-grafted agarose beads (Superdex) can be used as a support for LLPC[35]. The stationary phase is considered to be adsorbed mainly inside the pores of both LiParGel and LiChrospher[34,36], while it may be more evenly distributed on Superdex[35]. Interestingly, it has been suggested that the entire Superdex matrix forms an immobilised stationary phase[35]. The coating of both LiParGel and LiChrospher is considered to be due to the incompatibility of the polyacrylamide chains of the supports with PEG in the mobile phase rather than to an attraction of the dextran-rich phase to the matrix[34]. LLPC has been shown to be a unique tool for antibody analysis in that it can be used for both purification and fractionation[26,34,37-40], detection and separation of conformational isomeric forms[38], examination of surface properties related to antigen specificities[37,41-44] and for studying the events upon binding of antigen[38,41,45,46].

Taken together, partitioning in aqueous two-phase systems offers new possibilites to separate antibodies in a single step and/or to obtain a fractionation that is not readily achieved by other techniques. In order to examine the overall surface properties of intact antibody molecules in solution, this technique has several

advantages compared with direct (NMR and X-ray crystallography) and other indirect (spectroscopic, hydrodynamic and immunogenic) methods commonly used. X-ray crystallography requires the molecule to be in the crystalline state and is, like NMR, usually restricted to the analysis of fragments of individual monoclonal antibodies by the size, flexibility and heterogeneity of immunoglobulins[47,48]. Although several indirect methods can be used to examine the surface properties of intact antibodies, most of these techniques detect only a single property such as shape, charge or hydrophobicity. Furthermore, the two-phase technique is a mild method, so the conformation of a partitioned antibody molecule is not likely to be disturbed[1,11]. In addition, the method is rapid (< 2 hours), sensitive (≥0.1 μg protein is required), highly reproducible (the relative standard deviation of K is ≤ 3%), simple and inexpensive[7]. Unfortunately, the K values can not, as yet, be interpreted in structural terms. Such information is still not available although the PEG-dextran two-phase systems have been successfully used for many years to separate and analyse biomolecules. Rather than being seen merely as a source of frustration, the complexity of the parameters determining the K value may be regarded as the beauty of the method in providing a selectivity which may not readily be obtained by any other method or combination of methods.

2) Methodology

The aim of this chapter is to describe and outline the methodology behind LLP and LLPC in broad and general terms. For a more detailed and practical description, the following publications on LLP[1,2] and LLPC[7,25,49] are recommended. Finally, the properties and prestanda of the two approaches are also compared and discussed.

2.1) Liquid-Liquid Partition (LLP)

2.1.1) System Set-Up

The LLP set-up is schematically illustrated in Figure 1. LLP is a quick and simple method to set up. The sample is added to the two-phase system which is then mixed and allowed to settle at the same constant temperature at which the phase system was made. Sample that exists in a dry, salt-free state may in some cases be directly

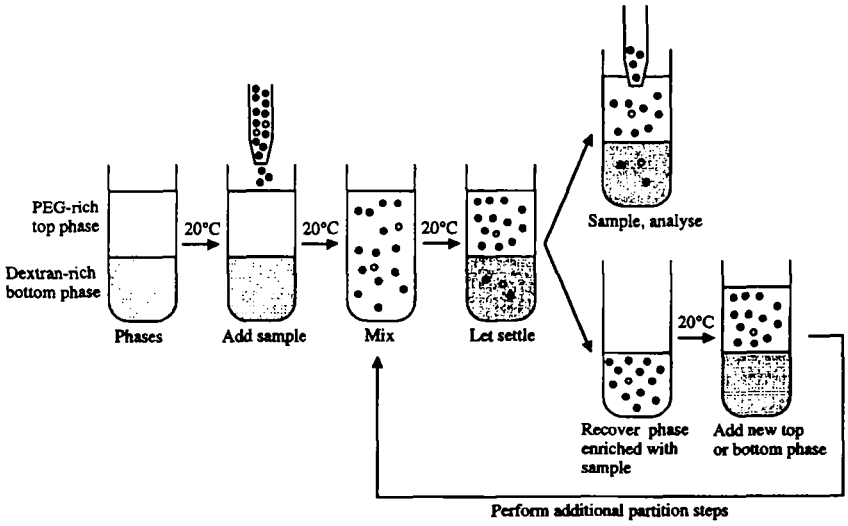


FIGURE 1
Schematic illustration of the LLP set up (batch experiment).

dissolved in the system, while sample dissolved in aqueous solutions are recommended to be dialysed against the buffer of the applied two-phase system. No pre-purification is required as long as all proteins present in the sample are soluble and the component(s) of interest are detectable. An aliqout of each phase is then withdrawn and the sample concentration determined by measuring the absorbancy or by using methods such as ELISA and enzymatic analysis etc. The partition coefficient, K (defined as the ratio of the concentration of the sample in the top phase to that in the bottom phase), is measured. In order to perform additional partition steps, the phase toward which the sample partitioned is recovered and new top or bottom phase is added. This can be done in a batch experiment in several repeated steps (Fig. 1) or by using various automated counter-current distribution (CCD) machines .

2.1.2) Choice of Two-Phase System

Batch experiments are performed in order to find the appropriate system with respect to the size and the concentration of the phase-forming polymers, the pH of the

system, the salt to be added, the total ionic strength and the temperature. By stepwise variation of the listed parameters, the system is ad-justed until the solubility and partitioning (separation) of the sample are satisfactory. When a suitable system has been found, the separation is performed in a single step (batch experiment) or in several repeated steps (batch experiment or CCD) depending on the resolution that is required. In spite of the fact that the theory behind partitioning has been extensively studied[1,11], it is still not possible to predict the K value of a molecule in a given two-phase system. It is, however, known how changes in the composition of the phase system effects the K value[1,11,50]. The distribution of a molecule may thus readily be adjusted to a suitable K value by altering the composition of the phase system.

Some of the two-phase systems and buffer/salt compositions used in LLP are shown in Table I. So far, mainly two-phase systems made up of PEG/dextran have been applied. A serious drawback of these systems is, however, the incompatibility between PEG and immunoglobulins. It is well-known that concentrations of PEG greater than 3% can be used to precipitate antibodies and antigen-antibody complexes[55,56]. Still, polymeric systems with PEG concentrations higher than 5% have been used to analyse antibodies and antigen-antibody complexes, but only at low concentrations (≤ 0.3 mg/ml)[23,24]. However, the incompatibility can be partly overcome by adding appropriate salts. IgG can, for example, be solubilized in PEG-dextran systems at pH 7.0, i.e. near its isoelectric point, in the presence of 100 mM betaine (≤ 0.8 mg/ml)[34] or 0.1 M glycine and 0.1 M sodium chloride (≤ 2 mg/ml)[37], while IgA and IgM, which are less soluble than IgG, can be dissolved in adequate amounts in such systems at pH 9[37]. In addition, even antigen-antibody complexes can be analysed in milligram quantities in PEG-dextran systems containing less than 10% PEG at pH 7 by adding glycine and sodium chloride[38,41,45]. Taken together, the PEG/dextran systems are, for the moment, the obvious choice of two-phase system for LLP of antibodies, whereas the usefulness of other phase systems remains to be elucidated.

2.1.3) Detection and Evaluation

The distribution of an analyte is determined by measuring the absorbence or by using methods such as ELISA and enzymatic analysis, and is expressed as a partition coefficient, K.

TABLE 1

Aqueous two-phase systems and buffer/salt compositions used in LLP and LLPC of antibodies.

System or buffer/salt	References	
	LLP	LLPC
PEG - dextran	23, 44, 51, 51	21, 31, 36-45, 57
PEG - dextransulphate	22, 53	-
PVP - dextran	-	58
PVA - dextran	-	59
PEG - salt	54	60
50 mM Na-phosphate, 100-350 mM NaCl, pH 7.5	21, 24, 51, 53	36, 57
10-50 mM Na-phosphate, 0.1 M NaCl, 0.1-0.2 M glycine, pH 7.0	-	7, 35, 37-46, 49
0.1 M glycine, 75 mM Tris, pH 9.0	-	37
60 mM KBr, 5 mM Li_3-citrate, 10 mM Na-phosphate, pH 7.2 [a)]	-	34
10 mM KBr, 0.1 M NaCl, 10 mM Na-tetraborate, pH 9.5 [a)]	-	34
75 mM KBr, 10 mM NaoAc-AcOH, pH 4.3 [a)]	-	34

[a)] The addition of 100 mM betaine increases the solubility of immunoglobulins.

PEG = polyethylene glycol

2.2) Liquid-Liquid Partition Chromatography (LLPC)

2.2.1) System Set-Up

The LLPC set-up is schematically illustrated in Fig. 2. The two-phase system, exemplified by a PEG/dextran system, is prepared at the same (constant) temperature at which the column is to be run. The matrix is coated with bottom (stationary) phase and packed into a thermostated column which is eluted with top (mobile) phase. The sample, prepared in a similar manner as for LLP analysis, is applied to the column. The analyte is then detected by continuously measuring the absorbancy of the eluates and/or by analysing collected fractions using methods such as ELISA and EIA etc., and its retention volume (V_R) is determined and expressed as a partition coefficient, K_C (the ratio of the concentration of the sample in the stationary phase to that in the mobile phase).

It is preferable, but not a requirement, to use a chromatographic equipment that can operate under high pressure (< 25 MPa) as this allows the packing procedure to be optimized. Prepacked LLPC columns (glass or steel) are not, at least for the moment, commercially available. As the experiments are performed at a constant temperature, the ideal situation is when a thermostated room is available. The incubation of the two-phase system and of the matrix (the coating step) can, however, be performed in a cooled precision incubator while a column thermostate or a thermostatic jacket allows the column to be run in non-thermostated rooms.

2.2.2) Choice of Two-Phase System

As in the case of LLP, batch experiments are performed in order to find a suitable two-phase system. Phase systems formed by polyvinylpyrrolidone (PVP)-dextran, polyvinyl alcohol (PVA)-dextran and PEG-salt solutions have been used for LLPC, but the PEG-dextran systems are, by far, the most frequently used (Table I). Compared with the PEG-dextran systems, there is a big decrease in selectivity of PVP-dextran systems and the viscosity of PVA-dextran systems is fairly high[58,59]. Furthermore, the incompatibility between PEG and proteins is a major problem in the PEG-salt systems, and the large amount of salt present alters the chromatographic mode from one of LLPC to that of hydrophobic interaction chromatography[60]. Hence, the PEG-dextran systems are, so far, the obvious choice of two-phase systems for LLPC of immunoglobulins.

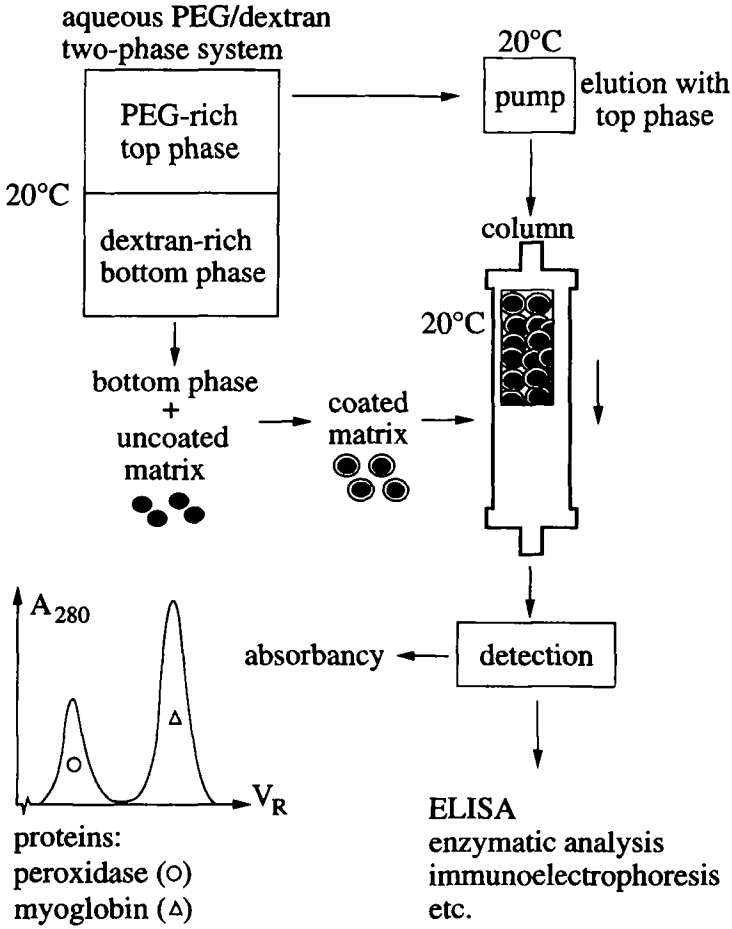


FIGURE 2
Schematic illustration of the LLPC set-up. A typical chromatogram for the reference proteins, peroxidase and myoglobin, obtained on LiParGel 650 (300 x 8 mm I.D.) in a 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0 at a flow rate of 0.12 ml/min (20°C) is shown.

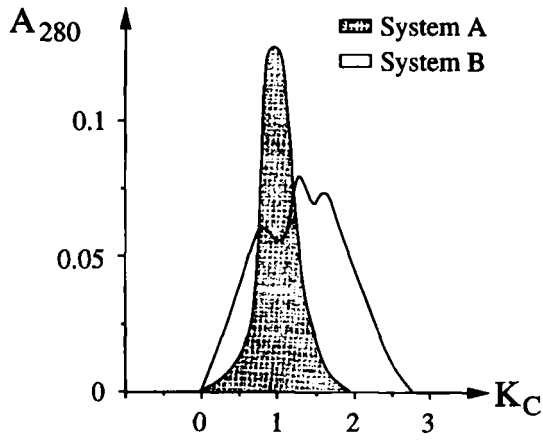


FIGURE 3
LLPC of rabbit IgG antibodies in PEG/dextran two-phase systems with A) 4.4% PEG 8000/5.2% (w/w) dextran T 500, 10 mM sodium phosphate, 0.1 M NaCl, 0.2 M glycine, pH 7.0 or B) 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0. The retention volumes are expressed as K_C. The relative standard deviation of K_C was ≤ 5%. Sample, 0.3-0.5 mg antibody; sample volume, 1 ml ; column, LiParGel 650, 350 x 10 mm I.D., (A) V_S/V_M=0.9, N=205, R_S=1.3, (B) V_S/V_M=0.9, N=240, R_S=1.6; flow rate, 0.2 ml/min; temperature, 20°C. Data adopted from[41].

The phase composition will, of course, affect the prestanda of the LLPC column. The selectivity can, for example, be improved by changing the concentration of one or of both the phase-forming polymers. This is illustrated in Figure 3 by the LLPC chromatograms obtained for polyclonal IgG antibodies. When a 4.4% PEG/5.2% dextran two-phase system is used, the antibodies are eluted as a single component, while several components are detected using a 4.4% PEG/6.2% dextran system. In general, a higher polymer concentration will not only increase the selectivity, but also the phase viscosity. The latter reduces the mass transfer rate in the chromatographic process, which is manifested by a broadening of the peak. Increasing the molecular weight of the polymers also increases phase viscosity. This increase is, however, partly offset by the fact that lower concentrations of polymers (with larger molecular weights) are required for phase separation.

Some of the salt/buffer compositions that have been used in the PEG/dextran systems are given in Table I. A wide range of ionic strengths, buffer compositions and pHs have been used, depending on the separation problem at hand, with sodium phosphate buffers (pH 7.0) containing glycine and sodium chloride beeing the most frequent choice[35-49,57]. As in the case of LLP, the incompatability between PEG and immunoglobulins is overcame by adding betaine[34] or glycine and sodium chloride[37].

In most cases, the LLPC analysis are performed as isocratic runs. A gradient can, however, be applied in order to reduce the elution time for molecules that are partitioned towards the stationary phase[34]. A ligand, as for an example procion red or a metal ion, specific for the protein of interest can also be added to the mobile phase[36] or be immobilized to one of the polymers[61], but the usefulness of such experiments remains to be unravelled.

Most of the LLPC experiments have been performed at 20-25°C. At temperatures above 30°C, the mobile phase should be flushed with a slow stream of nitrogen or argon in order to avoid oxidative degradation of PEG[34].

2.2.3) Choice and Influence of the Gel

Three groups of gels have so far, with respect to phase-adsorbing properties and chromatographic performances, been found suitable to act as supports for LLPC (Table II). The particles of LiParGel (polyvinyl) and of LiChrospher (silica) are grafted with polyacrylamide while those of Superdex (agarose) are grafted with dextran. The particle size and pore size (exclusion limit) of the matrices differ.

Typical parameters obtained for LLPC columns prepared in the same PEG/dextran system using LiParGel, LiChrospher and Superdex as supports are shown in Table III. A ratio of V_S/V_M of 0.6-1.5, a plate number of 200-725 and a resolution of 1.5-3.6 were obtained and the volume of one plate was only 6-78 µl. It should be noted that the flow rates used were those required to elute the reference proteins within 2 hours and lower flow rates would improve column performance even further. In this context it is also of interest to point out that about 60-180 transfers (equilibriums) can be obtained in CCD experiments, with each transfer occurring in a volume of about 2 ml[1].

LiParGel 650 has been used for the analysis of small as well as of large proteins[7,34,37,49] while LiParGel 750 mostly has been used for the analysis of nucleic

TABLE II

Supports for LLPC.

Gel Name	Matrix (particle/graft)	d_P (μm)	Pore size (nm)	Acrylamide content (g/ml wet gel)	Manufacturer	Reference
LiParGel 650	Polyvinyl/polyacrylamide	25-40	n.a.[a)]	0.026	Merck[d)]	62
LiParGel 750	Polyvinyl/polyacrylamide	25-40	n.a.[b)]	0.031		62
LiChrospher-Diol 100	Silica/polyacrylamide	10	10	0.062	Merck	62
LiChrospher-Diol 1000	Silica/polyacrylamide	10	100	0.019		62
LiChrospher-Diol 4000	Silica/polyacrylamide	10	400	0.007		62
Superdex 200 prep grade	Agarose/dextran	24-44	n.a.[c)]	-	Pharmacia	63

d_P = particle size; n.a. = data not available.

a) exclusion limit for globular proteins of 5×10^6 d and for dextrans of 10^6 d.

b) exclusion limit for globular proteins of 5×10^7 d and for dextrans of 10^7 d.

c) exclusion limit for globular proteins of 10^6 d and for dextran of 10^5 d.

d) no longer produced by Merck. Can be prepared by polyacrylation of Toyopearl HW65 and HW75 (Tosohaas)[62].

TABLE III

Typical parameters for Superdex 200, LiParGel 650 and LiChrospher Diol 4000 columns prepared in a 4.4% (w/w) PEG 8000 / 6.2% (w/w) dextran 500 two-phase system containing 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine at pH 7.0 (20°C) using conventional [a)] or HPLC chromatographic equipment [b)]. Peroxidase and myoglobin are used as reference proteins. Data adapted from[35,49].

support	pressure MPa	I.D. cm	L cm	V_S/V_M	V_S/V_C	V_M/V_C	N	V_N µl	R_S	flow rate[c)] ml/min
Superdex	n.d.[a)]	1.0	35	0.6	0.44	0.71	595	46	1.9	0.2
LiParGel	n.d.[a)]	1.0	35	0.8	0.37	0.46	350	78	1.5	0.2
LiParGel	0.7 [b)]	0.80	30	1.0	0.36	0.38	595	25	2.6	0.12
LiParGel	25 [b)]	0.80	30	1.5	0.41	0.27	725	21	3.6	0.12
LiParGel	25 [b)]	0.45	20	1.5	0.41	0.27	200	16	1.9	0.025
LiChrospher	2.6 [b)]	0.45	20	1.0	0.45	0.44	200	16	1.6	0.025
LiChospher	2.6 [b)]	0.45	20	1.5	0.53	0.35	500	6	3.0	0.025

pressure = maximum packing (back) pressure; I.D.= column internal diameter; L= column length; V_S = volume of the stationary phase; V_M = volume of the mobile phase; V_C = column volume; N = plate number for myoglobin; V_N = volume of one plate; R_S = resolution of peroxidase and myoglobin; n.d.=not determined. [c)] flow rate required to elute the reference proteins within two hours.

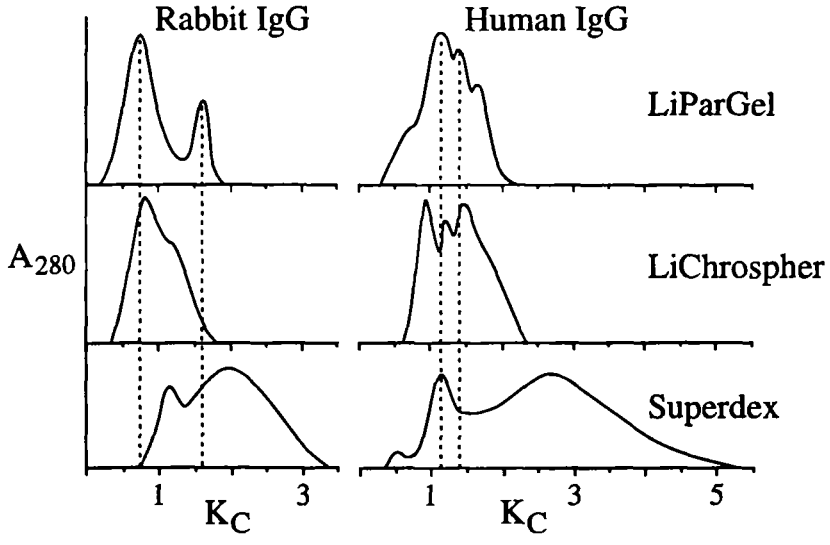


FIGURE 4
The influence of the supports on the partition properties of immunoglobulins. The LLPC chromatograms for rabbit IgG and human IgG obtained on LiParGel 650, LiChrospher-Diol 4000 or Superdex 200 prep grade are given. The retention volumes are expressed as K_C. The relative standard deviation of K_C was ≤ 5%. Sample, 50-150 µg IgG; sample volume, 100 µl (LiParGel and LiChrospher) or 1 ml (Superdex); column, LiParGel (300 x 8 mm I.D., V_S/V_M=1.5, N=725, R_S=3.6), LiChrospher (300 x 8 mm I.D., V_S/V_M=1.1, N=470, R_S=2.6) and Superdex (350 x 10 mm I.D., V_S/V_M=0.6, N=580, R_S=1.9); system, 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0; flow rate, 0.12 (LiParGel), 0.025 (LiChrospher) or 0.2 (Superdex) ml/min; temperature, 20°C. Data adopted from[35,49].

acids[34]. LiCrospher-Diol 100 has been used for the analysis of small proteins (< 100 000 d) while proteins of a wide range of sizes have been analysed on LiChrospher-Diol 1000 or 4000 columns[34,49]. Superdex is suitable for the analysis of small proteins (< 100 000 d)[35].

One condition that must be fulfilled if the LLPC analyses are to be successful is that ideal partitioning is obtained or the separation is enhanced by the interaction between the solute and the support. Partition coefficients determined in batch experiments, i.e. in the absence of any matrix, can, in general, be expected to describe

the ideal partitioning of the molecules. Partition coefficients determined by LLPC on LiParGel, LiChrospher and Superdex have, however, been found to differ (signficinatly) both from each other and from those determined in batch experiments[35,49,57]. As illustrated in Figure 4, LLPC on these supports resulted in different elution profiles for polyclonal IgG, i.e. the supports influenced the partitioning of immunoglobulins in different ways.

The possibility that electrostatic interactions (all the supports are negatively charged at pH > 5) and/or size-exclusion phenomena might explain the non-ideal partition behaviour observed has been thoroughly examined[35,49,57]. In these experiments, the properties for a large set of proteins with different physico-chemical properties were determined by LLPC on all three supports and compared with those obtained by LLP. These studies showed that the deviations from ideal partitioning could neither be explained by an electrostatic interaction between the solute and the support nor could they be ascribed merely to the size of the molecules. However, molecules larger than 100 kDa have been found to be retained (trapped) to a considerable extent on Superdex columns[35], i.e. Superdex is not a suitable support for LLPC of antibodies.

In the case of LiParGel and LiChrospher, it has been suggested that the non-ideal partitioning may depend on the conformation of the applied solute together with the properties of the support with respect to pore size and polyacrylamide coating[49]. Although the influence of these supports on antibodies is obvious, their applicability is not invalidated, as the interactions can be exploited as an additional parameter for separation.

Hence, LLPC of immunoglobulins on both LiParGel and LiChrospher is likely to reflect a combination of their overall surface properties, although the two matrices influence the partitioning in different ways[49]. Accordingly, no relationship between the partition properties of IgG, determined by LLPC on LiParGel, and a single physico-chemical property such as net charge, size or "shape" has been found[41,42]. Thus, both LiParGel and LiChrospher could well be used as the support for LLPC of antibodies.

The performance and capacity of LiParGel and LiChrospher columns are similar (Table III), but LiParGel columns are considerably more easy to pack and, in the long term, much more stable than LiChrospher columns [Wingren and Hansson,

unpublished observations]. Hence, LiParGel is, at least for the moment, the choice of support for LLPC of immunoglobulins.

2.2.4) Column Evaluation

The performance and capacity of a LLPC column are determined by the daily (in order to minimize the experimental error) application of two reference proteins. Peroxidase and myoglobin (or tRNA and 5S RNA) are often used. The batch partition coefficient of a reference protein, K_{batch}, is determined. Assuming ideal partitioning of the reference proteins also on the column, $1/K_{batch}$ is then used as K_C:

$$K_C = C_{stationary\ phase} / C_{mobile\ phase} \qquad \text{Eq. (1)}$$

The volumes of the stationary and mobile phases, V_S and V_M, are calculated from the retention volumes for the reference proteins, V_R, using the relationship

$$V_R = V_M + K_C V_S \qquad \text{Eq. (2)}$$

The plate number, N, is calculated from the peak width at half height (w_h) of the myoglobin (or tRNA) peak according to

$$N = 5.54 (V_R / w_h)^2 \qquad \text{Eq. (3)}$$

The resolution of two reference proteins, as for an example peroxidase and myoglobin peaks, R_S, is calculated as

$$R_S = (N/4)(k/(1+k)) (\alpha - 1) \qquad \text{Eq. (4)}$$

where k is the capacity factor ($k = (V_S / V_M) K_C$) and α is the ratio of the partition coefficients of the references ($\alpha = K_{batch,\ peroxidase} / K_{batch,\ myoglobin}$).

The LLPC column can be stored at the pre-selected and constant temperature. The variation in column parameters with repeated use/storage is exemplified by the ratio of V_S/V_M in Figure 5. V_S/V_M was found to be constant and the same column could be used for at least one year.

2.2.5) Detection and Evaluation

The eluates are continuously monitored by measuring the absorbance. The distribution of an analyte may also be determined in collected fractions using techniques such as ELISA, enzymatic analysis or immunoelectrophoresis. The retention volume (V_R) for each sample (peak) is determined and expressed as a partition coefficient, K_C. K_C is calculated from the relation

$$K_C = (V_R - V_M) / V_S$$

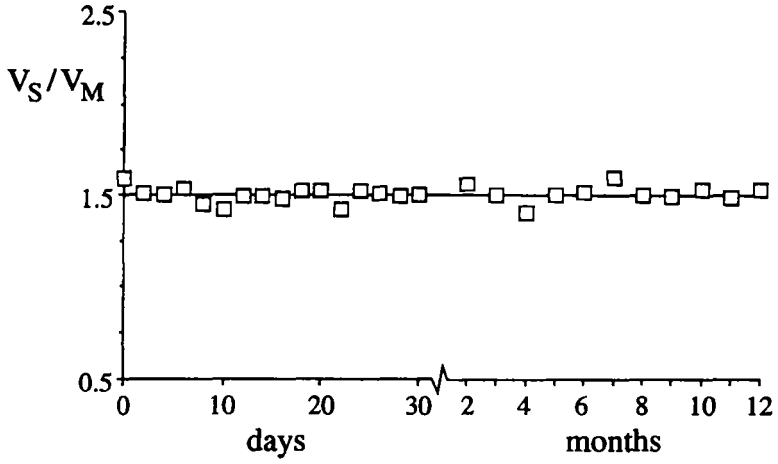


FIGURE 5
Variations in column parameters, exemplified by the volume ratio of stationary phase (V_S) to mobile phase (V_M) for a LiParGel 650 column during a period of 12 months. Sample, 20-40 µg reference proteins, peroxidase and myoglobin; sample volume, 100 µl; column, LiParGel, 300 x 8 mm I.D. or LiChrospher, 200 x 4.5 mm I.D.; system, 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0; flow rate, 0.12 (LiParGel) or 0.025 (LiChrospher) ml/min; temperature, 20°C. Data adopted from[40].

where V_S and V_M, the volume of the stationary and mobile phases, respectively, are calculated from the retention of two reference proteins. The relative standard deviation of K_C is $\leq 3\%$[7]. The use of K_C values, instead of retention volumes, allows the comparison of results obtained from LLPC columns with different parameters. Hence, the surface properties of a biomolecule, determined by LLPC, are described by its partition properties and expressed as a K_C value.

2.3) LLP vs. LLPC

Some of the properties and prestanda of LLP and LLPC are compared in Table IV. LLPC is clearly superior to LLP with respect to the methodological prestanda. In particular, the LLPC approach will provide a substantially higher plate number (i.e. the number of equilibriums), a smaller volume of one plate (i.e. the volume in which each partition step takes place) and, subsequently, a significantly higher resolution.

TABLE IV

Comparison of LLP and LLPC with respect to methodological properties and prestanda. Data adopted from[1,7].

Prestanda/property	LLP			LLPC
	Batch, single step	Batch, ≥ 2 steps	CCD	
Plate number (no. of equilibriums)	1	2 [a]	≤ 180	≤ 1000 [b]
Volume of one plate (µl) [c]	≥ 800	≥ 800	≥ 1400	6 - 78
Resolution	(+)	+	+(+)	++++
Reproducability [d]	≤ 5%	≤ 5%	n.a.	≤ 3%
Time required for one analysis (h) [e]	≥ 1	≥ 2	≥ 3	2
Sample volume	+++	+++	++	+
Susceptibility to sample precipitation	no	no	no	yes
Equipment /cost	+	+	++++	++(+)

[a] the plate number equals the actual number of repeated partition steps, i.e. may be more than 2.

[b] plate number/per column.

[c] the volume in which each partition step occurs.

[d] given as the relative standard deviation of the partition coefficient.

[e] assuming that the set-ups are up and ready to go.

n.a. = data not available

Further, the reproducability of the two approaches are about the same (no data available for CCD). Hence, LLPC will provide a considerably higher sensitivity and selectivity.

The time required for one analysis is about the same for LLP and LLPC. A significantly larger sample volume can be loaded in LLP than in LLPC. While batch experiments can be scaled to handle almost any sample volume, the recommended sample volume in LLPC is only ≤ 5% of the column volume. Further, the LLP method is not affected by any sample precipitation during a run, while this may damage the LLPC column. Precipitates may cause the LLPC column to bleed, i.e. cause the stationary phase to elute. Moreover, the LLP approach (except for CCD) require less equipment and is less expensive to set-up compared with the LLPC method.

Taken together, the properties/prestanda of LLP and LLPC show, as expected, that the choice of approach depend on the separation problem at hand. LLP may be preferred when a large amount/volume of sample is to be applied (in a preparative manner) and a low resolution is acceptable. In contrast, LLPC is the choice of approach when a small amount/volume of sample is to be applied (in an analytical or preparative manner) and the demands on the sensitivity and selectivity are high. It should be noted that LLP and LLPC may be used in a complimentary fashion, especially for preparative purposes. LLP can then be used as a first step to reduce the volume and contaminants of the liquid phase which is to be processed by LLPC in the second (final) purification step to achieve the desired purity of the product.

3) Results

While section 3.2 and 3.3 describes the results obtained by LLP and LLPC on antibodies in great detail, the aim of section 3.1 is to briefly summarize and compare these results in order to give a broad and illustrative overview of the usefulness of LLP and LLPC for the separation and analysis of antibodies.

3.1) LLP vs. LLPC

The usefulness of LLP and LLPC for the separation and analysis of antibodies are compared in Table V. One important use of LLP has been to screen for suitable two-

TABLE V

Comparison of the usefulness of LLP and LLPC for the separation and analysis of immunoglobulins.

	LLP	LLPC
Screening of two-phase system	+++	+
Preparative runs	++(+)	++
Analytical runs	+	+++
Purification and fractionation	+(+)	+++
Detection of conformational isomeric forms	(+)	+++
Examination of surface properties related to specificity	+	+++
Determination of apparent binding constants	+	(+)
Separation of free ag/ab and ag-ab complex	+(+)	+++
Studying the events upon antigen-binding	+	+++

ag = antigen; ab = antibody

phase systems which then have been applied in LLPC. However, LLP has to some extent been used for the separation of free and antibody-bound antigen, to determine apparent binding constants and the approach also have a potential for the use in preparative runs (if a low resolution is acceptable). In contrast, LLPC has proven to be a unique tool for antibody analysis in that it can be used for purification and fractionation (i.e. for both preparative and analytical runs), detection and separation of conformational isomeric forms, examination of surface properties related to antigen specificities, separation of free antigen/antibody and antigen-antibody complex, and for providing us with new, interesting information about the events upon antigen-antibody complexation and about possible ligand-induced conformational changes.

3.2) LLP of Antibodies

Early work showed that binding to antibody markedly affected the partition of certain antigens, such as phycoerythrin and albumin, in aqueous two-phase systems, while

free antibody partitioned much like the formed antigen-antibody complex[64,65]. These observations have been exploited by several investigators to detect and study the interaction between antibodies and antigens such as poliovirus[22,53] and somatommatropin[52], to develop an assay for antibodies against alkaline phosphates[66], to measure binding of digoxin and a variety of hormones to antibody under radioimmunoassay conditions[23,54], as well as for the immunoaffinity partitioning of proteins as well as cells[51,67]. Furthermore, LLP has also been used to determine the apparent binding constant of monoclonal antibody to antigen[24,52,68]. Still, LLP has only been used very little for the separation of antibodies, the limited resolution being a major impediment.

3.3) LLPC of Antibodies

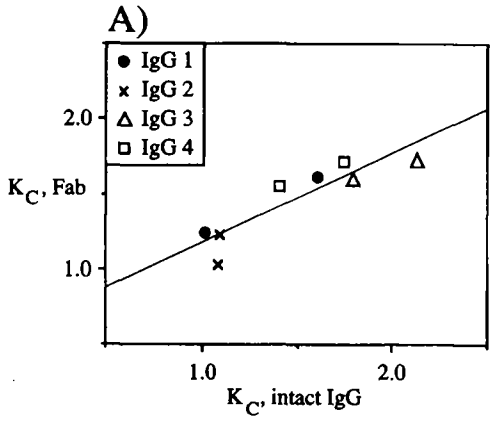
The first LLPC experiments on immunoglobulins indicated a great potential of LLPC for the separation and analysis of antibodies[34,37]. These experiments indicated, for example, that LLPC could be used to fractionate immunoglobulins[37] and to isolate monoclonal antibodies from ascites fluids and tissue culture supernatents[34]. Notwithstanding these promising results, the basis for the observed fractionation was not clear and the usefulness of the method for the separation of antibodies had yet to be explored. Major methodological improvements in terms of an increased sensitivity, selectivity and reproducability were at this point accomplished[7,49], allowing these issues to be directed.

3.3.1) Specificity

In general, polyclonal IgG antibodies were found to be eluted as heterogeneous populations over a broad range of K_C values, while monoclonal IgGs were eluted mainly as single, homogeneous peaks by LLPC[7]. Early LLPC experiments had indicated that functional differences (antigen specificities) might be reflected in the chromatographic behaviour of IgGs[37]. Hence, the question was posed: could LLPC provide us with the means to compare the surface properties of unliganded antibodies related to their antigen specificity ?

Does the Antigen-Binding Sites Dominate in IgG ?

In the first set of experiments, polyclonal rabbit IgG antibodies against three human serum proteins were found to be eluted in overlapping but different K_C regions[37,41].



B)

subclass	K_C, F
IgG1	1.22 ±
-	1.23 ±
IgG 2	1.22 ±
-	1.22 ±
IgG 3	0.20 ±
-	1.33 ±
IgG 4	1.22 ±
-	1.22 ±

FIGURE 6
Comparison of the partition properties of (A) intact human myeloma IgG with those of the corresponding Fab fragments and (B) Fc fragments from one IgG subclass with those of the other subclasses. The retention volumes are expressed as K_C. The relative standard deviation of K_C was ≤ 3% and the 95% confidence limits of K_C are given. Fc parts with identical K_C values are shaded. Sample, 30-170 µg IgG, Fab or Fc; sample volume, 100 µl; column, LiParGel 650, 300 x 8 mm I.D., V_S/V_M=1.6, N=720, R_S=3.6; system, 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0; flow rate, 0.12 ml/min; temperature, 20°C. Data adopted from[42].

No IgG subclasses have, so far, been reported for rabbit IgG, indicating that the properties of their constant domains, i.e. Fc parts, are likely to be the same. Thus, already these results indicated that LLPC had detected differences in surface properties between IgGs located on structures in their Fab parts.

This exciting finding was followed up by more extensive studies in which a large set of well-characterized human and murine monoclonal IgG antibodies were analysed by LLPC[41-43]. These studies clearly showed that there was no correlation between K_C and either the light chain isotype or the heavy chain isotype of the antibodies. This was the case not only for the intact monoclonal IgG antibodies, but also for Fab and Fc fragments from human IgG of different subclasses[42]. A significant ($P < 0.05$) linear correlation between the partition properties of intact human IgG1, 2, 3 and 4 and their corresponding Fab fragments was, however, detected[42] (Fig. 6A). In

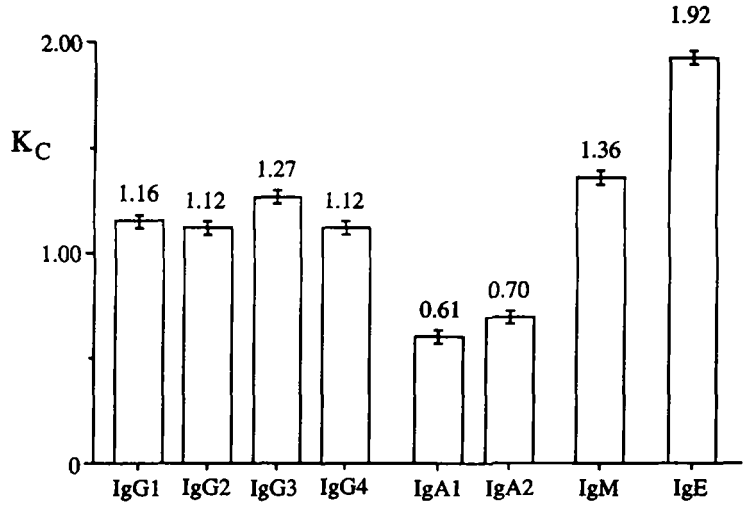


FIGURE 7
Comparison of partition coefficients for chimeric mouse/human anti-NIP antibodies, with identical specificities, corresponding to the human IgA1, IgA2, IgE, IgG1, IgG2, IgG3, IgG4 and IgM isotypes. Except for IgM(pentamer), the antibodies employed were monomers as determined by size-exclusion chromatography (data not shown). The retention volumes are expressed as K_C. The relative standard deviation of K_C was ≤ 3% and the 95% confidence limit of K_C was ± 0.02. Sample, 0.1-20 μg; sample volume, 100 or 200 μl; column, LiParGel 650, 300 x 8 mm I.D., V_S/V_M=1.5, N=700, R_S=3.5; system, 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0; flow rate, 0.12 ml/min; temperature, 20°C. Reproduced, by permission of the publisher, from[43].

contrast to the Fabs, Fc fragments from IgG1, 2 and 4 displayed almost identical surface properties[42] (Fig. 6B).

Further studies revealed a remarkable relationship between the partition properties of an IgG molecule and the structure of its combining site (specificity)[41-43]. In particular, monoclonal IgG antibodies with different affinities for the same antigen or directed against different antigens were eluted with different K_C values, while monoclonal IgGs with identical affinity constants for the same hapten or the same epitope on a protein were found to have identical K_Cs[41,42]. Moreover, chimeric anti-NIP antibodies with identical variable regions, corresponding to the human IgG1, 2

and 4 subclasses, were found to display identical surface properties, i.e. differences in the constant part of the heavy chains did not affect the partitioning[42,43] (Fig. 7). Hence, the surface properties of IgG antibodies are, as detected by LLPC, dominated by those of their antigen-binding sites. It may also be of interest to note that indications of a dominances of ligand-binding sites for other specific proteins, such as enzymes, have been reported[40].

As the interacting surfaces between antibody and antigen are characterised by a high degree of complementarity, similarities in K_C may thus reflect similarities (with respect to conformation and exposed properties) in the epitope against which the antibodies are directed. It was in this connection of great interest to note that three monoclonal antibodies against two structurally similar haptens, T3 and T4, which were eluted with similar K_C values also have been found to be cross-reactive[41].

The domains of IgG all share the same structural feature and they are folded into compact globular structures[69]. The structural differences between different IgGs, with the exception of the hinge, are mainly confined to the complementarity determining regions (CDRs)[69]. Hence, the results imply that the constant parts of IgG form similar scaffolding, onto which CDRs of variable shape and size are interspaced and constitute the major, dominant differences in surfaces properties of IgGs and Fabs that are detected by LLPC. Taken together, LLPC may thus be used to separate IgGs due to differences in their antigen-binding sites.

IgG3 - Does it hinge on the Hinge ?

In the case of IgG3, it was reported that the K_C value of chimeric IgG3 differed significantly from those of the corresponding IgG1, 2 and 4 chimers[42] (Fig. 7). Moreover, the K_C values of Fc fragments from IgG3 differed not only from those of the other IgG subclasses, but also from each other[42,43]. The results thus indicated that Fc had another conformation and was more dominant in human IgG3 than in the other IgG subclasses.

The hinge region of IgG3 may be responsible for the differences observed, since its conformation varies and is different from that of the other IgG isotypes[70-73]. Interestingly, the K_C value of chimeric IgG3 was also affected when its hinge length was made similar to those of the IgG1, 2 and 4 chimers, but the partition properties of IgG3 still differed from those of the other IgGs[43]. The surfaces of Fc may be

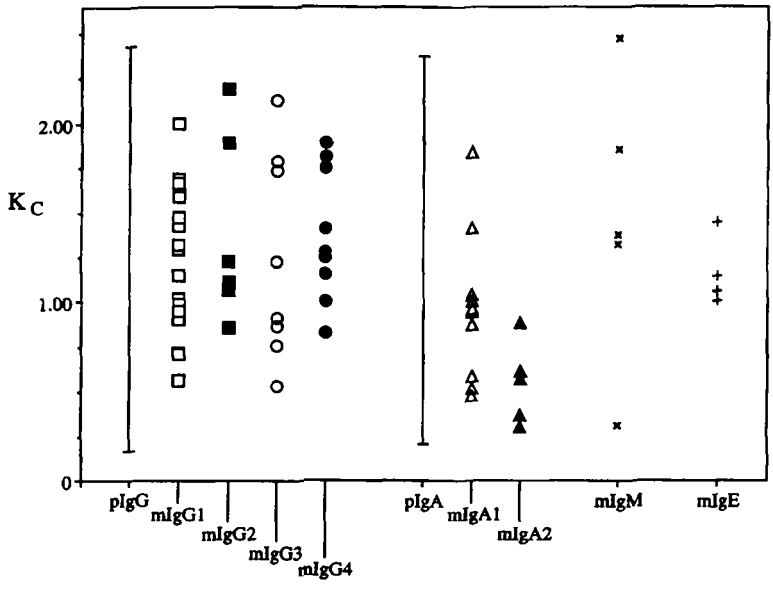


FIGURE 8
Comparison of partition coefficients for human polyclonal and monoclonal immunoglobulins of different classes and subclasses. p=polyclonal; m=monoclonal; number of samples, pIgG (1) mIgG1 (17), mIgG2 (8), mIgG3 (8), mIgG4 (9), pIgA (1), mIgA1 (10), mIgA2 (5), mIgM (5) and mIgE (4). The retention volumes are expressed as K_C. The relative standard deviation of K_C was $\leq$ 3%. Sample, 10-80 µg; sample volume, 100 or 200 µl; column, LiParGel 650, 300 x 8 mm I.D., V_S/V_M=1.5, N=700, R_S=3.5; system, 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0; flow rate, 0.12 ml/min; temperature, 20°C. Reproduced, by permission of the publisher, from[43].

differently exposed, since the structurally heterogeneous hinge region provides antibodies with their flexibility[74]. It is noteworthy that, among the human IgG isotypes, IgG3 is the most flexible. It was therefore interesting to note that the surface properties of chimeric IgG3 were "normalized", i.e. changed and became similar to those of chimeric IgG1, 2 and 4, when the hinge region was shortened by 17 or 30 amino acids IgGs[43]. Provided that their variable regions are identical, LLPC may be used to separate IgG3 from the other IgG subclasses due to differences in their hinge and/or Fc parts.

Antigen-Binding Site dominance - Valid for all Antibodies ?

In comparison with IgG, early LLPC experiments had indicated that the dominant surfaces of IgA and IgM were not only related to their antigen-binding sites, but also to a structure, or structures, present on their heavy chains[37]. Nonetheless, a recent study showed that the surface properties of monoclonal antibodies of different classes and subclasses (IgA1, IgA2, IgE, IgG1, IgG2, IgG3, IgG4 and IgM) fell within the same broad range as that observed for polyclonal antibodies (IgA and IgG) and that no relationship between their partition properties and heavy chain isotype could be found[43] (Fig. 8). However, the K_C values of chimeric anti-NIP antibodies, with identical specificity, belonging to the human IgA1, IgA2, IgE, IgG3 and IgM isotypes, revealed a difference both from each other and from those of the IgG1, 2 and 4 chimers[43] (Fig. 7). Taken together, the results showed that LLPC could be used to detect differences/similarities in the surface properties of both the antigen-binding regions and the Fc parts of IgA1, IgA2, IgE, IgG3 and IgM. Further, LLPC may also be used to separate these isotypes from each other due to differences in their hinge and Fc parts (provided that their variable regions are identical).

It should, however, be emphasized that when antibodies within a given class or subclass only were compared with respect to exposed surfaces, no relationship with the heavy chain isotype could be observed[43]. Hence, the results indicated that the surface properties of antibodies within the IgA1, IgA2, IgE, IgG3 and IgM isotype may also be dominated by those of their antigen-binding sites.

Unique Possibilities to Screen the Repertoire of Antigen Specificities

Autoimmune diseases are characterized by provoking an immune response, often including antibody production, against auto-antigens. In several of these diseases, such as rheumatoid arthritis and primary Sjögren´s syndrom (1°SS), the initiating antigen causing the disease is still unknown[75,76]. New approaches which could provide us with the means to isolate these specific antibodies (of unknown specificities) and/or generate new, additional information about their reactivity would thus be of biological importance.

It has recently been shown that the LLPC profiles for polyclonal IgG antibodies, isolated from the sera of 1°SS patients, differed significantly from those of polyclonal IgG isolated from the sera of healthy individuals[43] (Fig. 9). These antibodies with

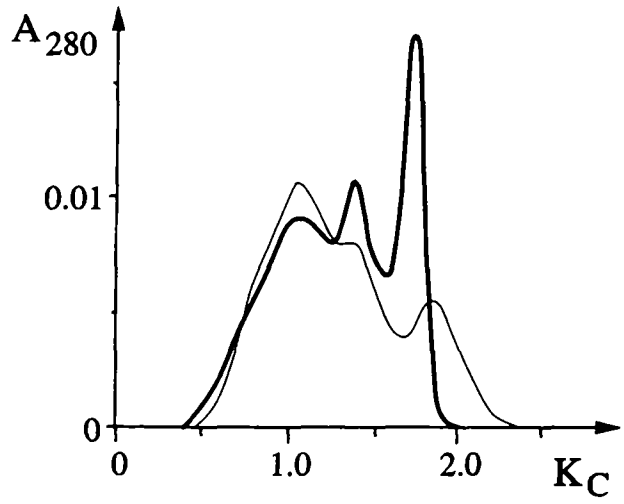


FIGURE 9
Comparison of surface properties of polyclonal IgG isolated from patients with an autoimmune disease (primary Sjögren's syndrome) (thick line) with those of polyclonal IgG isolated from the sera of healthy individuals (thin line). The retention volumes are expressed as K_C. The relative standard deviation of K_C was ≤ 3%. Sample, 70 µg IgG; sample volume, 200 µl; column, LiParGel 650, 300 x 8 mm I.D., V_S/V_M=1.6, N=700, R_S=3.5; system, 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0; flow rate, 0.12 ml/min; temperature, 20°C. Data adopted from[44].

"unique and deviating" specificities could also be isolated by LLPC, and work is in progress to characterize these antibodies with respect to their physico-chemical as well as immunochemical properties. Thus, LLPC can indeed provide us with the means to fractionate and isolate specific antibodies "of unknown specificities".

3.3.2) Conformational Isomerism

Several reports, employing kinetic techniques, have indicated that immunoglobulins may, as enzymes, exist in different conformational (isomeric) forms[77-82]. Based on these studies, it has been suggested that a tenth of all antibodies may display conformational isomerism, but since the phenomenon cannot always be detected by kinetic techniques, the isomerism detected by kinetic data may represent only the tip of an iceberg.

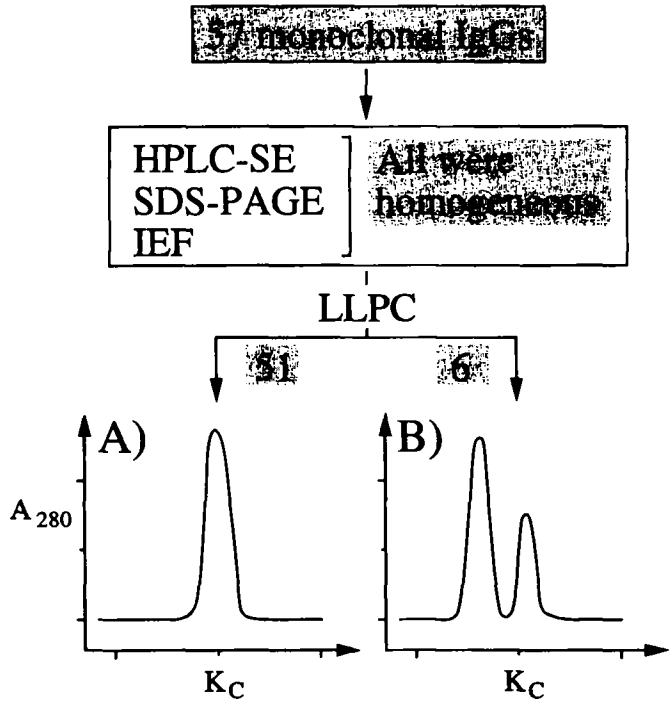


FIGURE 10
LLPC of 57 monoclonal IgG antibodies that all contained > 95% IgG and were homogeneous with respect to their physico-chemical properties as determined by HPLC-SE, SDS-PAGE and IEF. The LLPC chromatograms are schematically illustrated in A) which represents 51 of the IgGs, and B) which represents 6 of the IgGs. The retention volumes are expressed as K_C. The relative standard deviation of K_C was ≤ 3% and the 95% confidence limit of K_C was ± 0.03. Sample, 1-40 µg IgG; sample volume, 200 µl; column, LiParGel 650, 300 x 8 mm I.D., V_S/V_M=1.6, N=650, R_S=3.5; system, 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0; flow rate, 0.12 ml/min; temperature, 20°C. Data adopted from[38].

Interestingly, recent studies have shown that some monoclonal antibodies were eluted as at least two components by LLPC, in spite of the fact that they were homogeneous with respect to their immunochemical and physico-chemical properties[38,41-43]. This phenomenon was observed not only for IgG but also for IgA and IgM myeloma proteins[43]. Consequently, the data gave rise to the question of

whether these antibodies occurred in different conformational forms detectable by LLPC. It should here be noted that the capacity of LLPC to detect conformational isomeric forms of a protein has been demonstrated in a recent study where a large set of well-characterized enzymes known to exist in equilibrium between an open and closed conformation were analysed[38,39].

In the case of the immunoglobulins, six of fifty-seven apparently homogeneous monoclonal IgGs were found to be fractionated into at least two components by LLPC[38] (Fig. 10). The four IgGs in which the minor component constituted 20% or more were selected for further analysis. Rechromatographing experiments showed that there was an equilibrium between these two or three components with different surface properties in all four cases[38].

Since LLPC detects mainly differences within the antigen-binding sites of unliganded IgG[41-43], it was concluded that the conformational isomers differed with respect to their combining sites[38]. The idea of conformational heterogeneity in the antibody combining sites is also supported by NMR and X-ray crystallographic experiments on Fab fragments from monoclonal IgGs[83-85].

Moreover, the study performed by Hansson and co-workers did also reveal that LLPC could be used to study the events upon binding of antigens by these isomeric antibodies[38]. In accordance with kinetic data obtained by Lancet and Pecht[78], the LLPC data showed both isomers of one antibody were able to bind antigen and form different complexes[38]. Taken together, LLPC proved to be very a useful method for detection, isolation and characterization of conformational isomeric forms of IgG.

3.4) LLPC of Antigen-Antibody Complexes

In many cases, LLPC can be used to separate antigen-antibody complexes from either of the free components[38,39,41,45]. Several investigations have, however, demonstrated that the strength of the LLPC method for the analysis of antigen-antibody complexes lies, as discussed below, in the fact that several interesting questions of great biological importance may be addressed by examining the parameters that govern the partition properties of the complexes[7,86].

3.4.1) Binding of Antigen to IgG Antibodies

A large set of antigen-antibody complexes, formed by mainly monoclonal IgG

antibodies and either haptens, hapten-carrier or proteins have been analysed by LLPC[39,41,45]. Interestingly, almost all the antigen-antibody pair analysed in these studies formed complexes which were eluted as single homogeneous peaks by LLPC. Moreover, the complexes were eluted with the same value of K_C irrespective of the molar ratio of antigen to antibody at which they had been formed (ranging from antigen to antibody excess)[39,45]. The contention, that LLPC is an insensitive method could not explain the fact that only one type of complexes could be detected by LLPC in all antigen-IgG mixtures[7]. Thus, the results implied that each antigen-IgG antibody pair formed one type of complex with respect to exposed dominant surfaces. To the best of our knowledge, this is the first study reporting such a feature for antigen-antibody complexes.

Remarkably, a linear relationship between the surface properties of unliganded IgGs and their corresponding hapten-IgG complexes has been reported (Fig. 11), and it was concluded that the surface properties of IgG were dominated by those of its antigen-binding sites even after the specific binding of hapten or hapten-carrier at the combining sites[45]. By contrast, the surface properties of protein-antibody complexes were not related to those of the unliganded antibodies[41,45]. Instead, the surface properties of protein-IgG complexes were found to be related mainly to those of the antigens[41,45]. Depending on the type of antigen (hapten or protein), LLPC may thus be used to separate antigen-IgG complexes due to differences in exposed surfaces of either the antibody combining sites or the antigen.

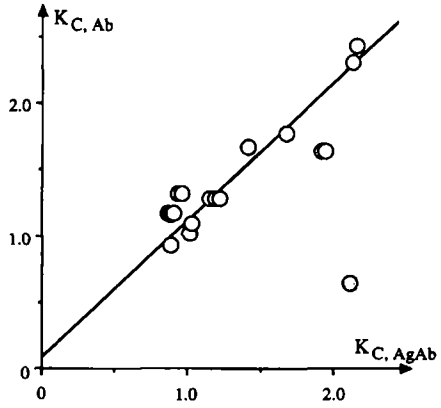
Based on the LLPC data at hand, it has been suggested that the Fc parts of IgG expose similar surface properties before and after binding of antigen[45]. This would imply that no conformational changes, at least detectable by LLPC, occurred in the Fc part of IgG upon antigen-binding. In agreement with this, current opinion favours the view that no ligand-induced conformational changes occur in this part of the IgG molecule[87]. However, further experiments are required before this disputed matter is finally resolved.

Taken together, LLPC is a sensitive method that can be used to provide us with new, interesting information about the events upon antigen-antibody complexation.

3.4.2) Binding of Antigen to Antibodies of Different Ig Classes and Subclasses

In a recent study, hapten-antibody complexes formed by chimeric anti-NIP antibodies with identical variable regions, corresponding to the human IgA1, IgA2, IgE, IgG1,

AbAg system	$K_{C, Ab}$	$K_{C, AgAb}$	The correlation between $K_{C,Ab}$ and $K_{C, AgAb}$
a-NBP – NBP	1.77	1.68	
	2.44	2.14	
	2.30	2.14	
a-DNP – DNP [1]	1.02	1.02	
	1.10	1.04	
	0.93	0.88	
a-T3 – T3 [1]	1.64 [2]	1.92	
	1.64	1.92	
	0.64	2.12	
a-T4 – T4 [1]	1.28	1.19	
	1.28	1.22	
	1.67 [2]	1.41	
	1.28	1.15	
a-NIP – NIP	1.18	0.89	
	1.16	0.87	
	1.31	0.96	
	1.30	0.95	
	1.17	0.86	



$K_{C, Ag}$: NBP = 1.37; DNP = 1.67; T3 = 0.38; T4 = 0.69; NIP = 1.60

[1] Data adopted from [27].

[2] The Fab fragments of these antibodies are shown in Figure 3.

FIGURE 11

The correlation between surface properties (K_C values), determined by LLPC, of free and ligand-bound monoclonal IgG anti-hapten antibodies. Three mouse monoclonal IgG antibodies against nitrobenzylphosphate (NBP) (1 IgG1, 1 IgG2 and 1 IgG2a), three against 2,4-dinitrophenol (DNP) (1 IgG1, 1 IgG2a and 1 IgG2b), three against tri-iodothyronine (T3) (2 IgG1s and 1 IgG2a) and four against thyroxine (T4) (3 IgG1s and 1 IgG2b) were analysed, as were five mouse/human chimeric monoclonal IgG (1 IgG1, 1 IgG2, 2 IgG3s and 1 IgG4) antibodies against 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP). The affinity constants of the antibodies were in the range $5x10^6$ - $1x10^{10}$ M-1. The antibody which formed hapten-antibody complexes with "deviating" partition behaviour is shaded. With the exception of a-NBP – NBP (10:1), the hapten-antibody complexes (AgAb) were prepared at a molar ratio of hapten (Ag) to antibody (Ab) of 5:1. The retention volumes are expressed as K_C. The relative standard deviation of K_C was ≤ 3% and the 95% confidence limit of K_C was ± 0.02 . Sample, 0.2-50 µg; sample volume, 200 µl; column, LiParGel 650, 300 x 8 mm I.D., V_S/V_M=1.6, N=720, R_S=3.4; system, 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0; flow rate, 0.12 ml/min; temperature, 20°C. Reproduced, by permission of the publisher, from[45].

IgG2, IgG3, IgG4 and IgM isotypes, were analysed[45]. Interestingly, the authors found the hapten-antibody complexes to be eluted in a considerably narrower range of K_C values than were the free antibodies[45] (Fig. 12). Hence, the results indicated that conformational changes occurred in either IgA1, IgA2, IgE and/or IgM, but not in the IgGs (see above), making the surfaces of the constant regions of the heavy chains of the Ig classes and subclasses more similar. Hence, LLPC may provide us with the means to examine whether ligand-binding induces conformational changes in the antibody when in solution.

3.4.3) Relationship Between Exposed Surfaces and Effector Functions ?

The Fc part of an antibody mediates the effector functions and different effector functions are attributed to antibodies from different classes and subclasses[87]. However, in the case of the IgG subclasses, all the LLPC data present at hand, suggest the structutal differences related to their different capacity to exert effector functions are small or not exposed in unliganded IgG[42,43]. The observed differences in surface properties of uncomplexed chimeric antibodies of different isotypes[43] (Fig. 7), may thus reflect structural and thereby functional differences of Fc which do not require the antigen to be bound, e.g. compartmentalization of antibodies. Interestingly, IgA, IgG and IgM are also differently distributed between intra- and extra-vascular pools and seromucous secretions, while IgE is frequently found bound to cell surfaces[88,89].

After binding of antigen, antibodies from different classes and subclasses are expected to expose different surfaces, as they are attributed different effector functions[87]. However, LLPC analyses of antigen-antibody complexes formed by hapten or protein and monoclonal indicated that no dominace of effector sites, similar to that observed for antigen-binding sites, could be detected[41,45]. In a recent study, the exposed surfaces, as detected by LLPC, of antigen-antibody complexes formed by monoclonal IgGs of different/identical subclasses and albumin or NIP-BSA, were compared with their ability to bind C1q, protein A and protein G[46]. No relationship between the exposed surfaces of the complexes and their effector functions could be detected. Moreover, it is also known that the oligosaccharide chains in Fc may affect the structural and functional properties of the antibody[90]. However, the LLPC

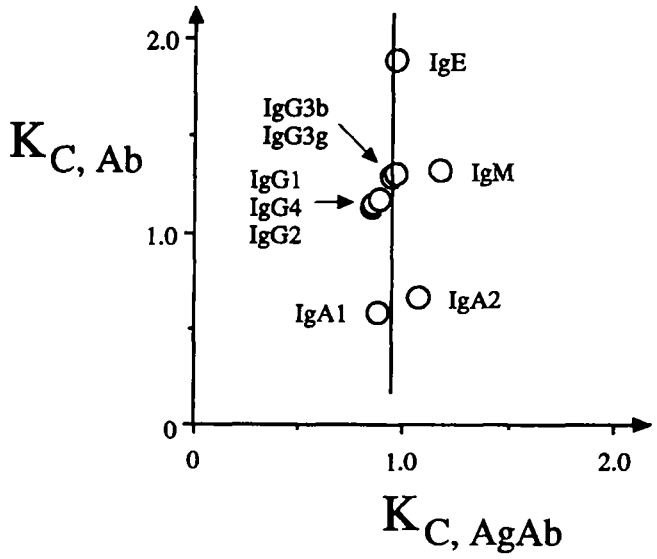


FIGURE 12
Comparison of surface properties (KC values) of mouse/human monoclonal chimeric antibodies, with identical variable regions, corresponding to the human IgA1, IgA2, IgG1, IgG2, IgG3b, IgG3g, IgG4, IgE and IgM isotypes, before and after binding of 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) (free or conjugated to BSA). Only the results obtained for NIP, representative for both the antigens, are shown. The median Kc value of the complexes is shown by the vertical line. Except for IgM (pentamer), the antibodies employed were monomers as determined by size-exclusion chromatography (data not shown). The antigen-antibody complexes (AgAb) were prepared at a molar ratio of antigen (Ag) to antibody (Ab) of 5:1. The retention volumes are expressed as K_C. The relative standard deviation of K_C was $\leq$ 3% and the 95% confidence limit of K_C was $\pm$ 0.03 . Sample, 10.2-20 μg; sample volume, 200 μl; column, LiParGel 650, 300 x 8 mm I.D., V_S/V_M=1.5, N=700, R_S=3.5; system, 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 mM sodium phosphate, 0.1 M NaCl, 0.1 M glycine, pH 7.0; flow rate, 0.12 ml/min; temperature, 20°C. Reproduced, by permission of the publisher, from[45].

analyses showed that the exposed surfaces of the free, as well as ligand-bound IgGs were the same before and after the elimination of these sugar residues[46]. Future experiments will reveal whether a relationship between exposed surfaces of Fc, as detected by LLPC, and effector functions can be established.

4) Concluding Remarks and Future Prospects

This review shows that liquid-liquid aqueous partition offers unique possibilities for the separation and analysis of intact antibodies and antigen-antibody complexes in solution. In particular, LLPC, and to some extent also LLP, is a powerful tool for antibody analysis in that it can be used for purification and fractionation, detection and separation of conformational isomeric forms, examination of surface properties related to antigen specificities and for providing us with new, interesting information about the events upon antigen-antibody complexation and about possible ligand-induced conformational changes. Although the K_C values cannot, as yet, be interpreted in structural terms (LLPC being an indirect method), the combination of the parameters determining the K_C value provides a selectivity which may not readily be obtained by any other method or combination of methods.

By using this approach, several problems of great biological importance may thus be adressed in a new, different manner, which otherwise might be difficult to achieve. For example, LLPC may prove to be an extremely valuable tool for the fractionation and isolation of specific antibodies from sera of patients suffering from (autoimmune) disease in which the initiating antigen causing the disease is still unknown. Further, the concept of conformational isomerism among antibodies may be further highlighted and we may also gain further insight into the events upon binding of ligand by antibody, or by other specific biomolecules, by using this approach. Finally, further development of the method with respect to selection of suitable two-phase system and support may further increase the usefulness, sensitivity and selectivity of the technique.

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6) Nomenclature and Abbreviations

CDR	Complementarity determining regions
DNP	2.4-dinitrophenol
Fab	Fragment of antigen binding
Fc	Fragment crystallizable
Ig	Immunoglobulin
K	Partition coefficient determined in LLP experiment (synonymous to K_{batch})
K_C	Partition coefficient determined in LLPC experiment
LLP	Liquid-liquid partition
LLPC	Liquid-liquid partition chromatography
N	Plate number
NBP	Nitrobenzylphosphate
NIP	5-Iodo-4-hydroxy-3-nitrophenacetyl
PEG	Polyethylene glycol
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
R_S	Resolution
T3	Tri-iodothyronine
T4	Thyroxine
V_M	Volume of mobile phase
V_R	Retention volume
V_S	Volume of stationary phase

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