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Review

Cellulose as a (bio)affinity carrier: properties, design and applications

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Abstract

This contribution presents a framework for the rational design of affinity sorbents based on cellulose materials as a support. A three-level evaluation procedure, utilizing the knowledge of physical, chemical and engineering theories, is discussed, which integrates the design of support, affinity sorbent and chromatographic contactor. The principal support properties, such as morphological, diffusional, hydrodynamic, mechanical or ligand-binding properties, are presented and literature data on them are surveyed. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Over recent years, there has been growing interest in the use of cellulose materials in a broad range of bioprocessing technologies, particularly in diverse affinity bioseparations, including chromatographic techniques. This interest was motivated by the combination of three main factors:

- Cellulose provides an interesting spectrum of geometrical and morphological forms,
- in spite of this diversity of forms, the chemistry of support activation is the same, so that, even when the form is changed, previous know-how can be adapted automatically to the new situation and,
- the support is relatively inexpensive and the raw material is renewable.

The conclusions have already been discussed in our previous review articles [1–3]. In the late 1980s, the importance of cellulose-based affinity media grew concomitantly with the commercial success of pharmaceutical products derived from monoclonal antibody and recombinant DNA technologies (recombinant insulin, human growth hormone, interferon and hepatitis B vaccine), which can largely be attributed to the development and improvement of downstream processing techniques at the same time. Mammalian cell cultures, ascites fluid, filamentous fungi, *Bacillus subtilis* and *Escherichia coli* have all been processed to yield proteins in the desired form and, in all cases, chromatography was there to save the day when substantial purification was needed. Among the chromatographic methods, affinity chromatography has been by far the most powerful one and is being used increasingly for large-scale purifications [4]. Areas of application include, but are not limited to, the processing of human plasma proteins, removing trace amounts of contaminants from therapeutic substances, and preparing interferons, other recombinant proteins, diagnostic enzymes, monoclonal antibodies and restriction endonucleases. Furthermore, it is possible that the future introduction of purpose-designed biomimetic dye-ligands may prove to be important in further increasing the applications of large-scale affinity chromatography [4].

Furthermore, purification and recovery of recombinant protein products can account for as much as 80% of total manufacturing costs for the biopharmaceutical industry [5,6]. Because the majority of isolation and purification procedures involve large-scale liquid chromatography, the availability of cost-effective and efficient chromatographic support materials are both of critical importance for advancements in bioprocessing technology. A general consensus for an ideal matrix is that it should exhibit the following requirements: A high specificity (implying a surface charge content that approaches zero), the absence of hydrophobic binding sites, good chemical stability, good mechanical rigidity, high binding capacity, good recoverability, high reproducibility and low cost [4,7,8].

The frequency of utilization of cellulose-based media in (bio)affinity separations in recent years has confirmed the anticipated tendencies. Regarding the huge amount of papers concerning the characterization and application of cellulosic materials in (bio)affinity separations in the period after 1989 [1], it is not surprising that conclusions taken from different papers are often contradictory. Moreover, the information is spread over different papers and needs to be regrouped. Therefore, the motivation of this paper is:

- To collect and systematize all useful information about (i) the physical and morphological properties of different types of cellulose materials and (ii) chemical activation;
- to provide a critical view of the applications of affinity supports based on cellulose media and
- to discuss the validity of conventional wisdom about the properties of cellulose as an affinity carrier.

All of these aspects in relation to (bio)affinity separations are assessed from an engineering point of view. Due to this approach, morphological properties, transport properties, pressure drops and the mechanical properties of cellulose materials in different geometrical and morphological forms of both non-regenerated and regenerated cellulose could be compared.

The increased demand for cellulose-based media for (bio)affinity separations stimulated effort to improve both of the principal types of cellulose affinity media derived from regenerated and non-regenerated cellulose.

Innovated non-regenerated products, i.e. the fibrous or microgranular media, were developed and manufactured mostly by Whatman Specialty Products Division using proprietary technology [9]. These materials, derived from cotton linters, exhibit significant inherent mechanical strength, which is attributable to a high degree of endogenous crystallinity derived from the native cellulose and arising from intermolecular hydrogen bonds [9].

Cellulose in its non-regenerated form offers a very high protein capacity [10,11] and fast adsorption–desorption kinetics; features that directly influence the dynamic binding capacity and chromatographic resolution. In spite of this, these characteristics favour a high throughput: These materials are operated at low flow-rates in axial-flow columns, which adversely affects the process time [10–12].

Microgranular cellulose provides some advantages (resolving power, higher degree of substitution on derivatization) in comparison with both fibrous and bead celluloses, since its matrix rigidity and porosity are increased by chemical crosslinking and partial hydrolysis; however, for the time being, advantages have been observed in particular at ion-exchangers testing [13]. Microcrystalline cellulose has been recommended also for the preparation of stationary phases for chiral chromatography. In powder form, one must consider higher pressure drops during operation of the column [14].

The fibrous matrix has good hydrodynamic properties and is recommended for use in the early purification steps. Bead cellulose is a further improvement in rigidity and chemical stability, giving the best hydrodynamic properties [15–17].

One of the main objections against affinity adsorbents prepared from cellulose is that they may exhibit nonspecific adsorption, and purification of the desired substance may be difficult to achieve [18].

The regenerated products do not possess such crystallinity and, consequently, they tend to be rigidized by covalent crosslinks. Recent applications of regenerated chromatography media based on beaded cellulose have been reviewed [1–3,9]. Like the non-regenerated particle cellulose, the regenerated one is also manufactured and supplied by several companies, e.g. Amicon/Grace, Chisso, Pierce, Daicel, Lovochemie and Sigma.

The research carried out by our groups and coworkers focused on cellulose as a carrier/chromatographic medium and on the group of so-called general ligands as affinity ligands. As far as cellulose is concerned, our interest has been focused mainly onto the bead cellulose that has recently been made commercially available by Lovochemie Lovosice (Czech Republic) under the name Perloza [1,19,20]. Our approach to general ligands exceeded the standard limit of affinity chromatography and, therefore, in this review article, we would also like to present our experience in the area of covalent affinity chromatography (CAC) and hydrophobic interaction chromatography (HIC). Our contribution to affinity chromatography was concerned with synthetic dye-ligands [1,21] and lectins in particular and, as a consequence, with the affinity chromatography of nucleotide-dependent enzymes and the lectin chromatography of glyco-enzymes/glycoproteins. Thiophilic ligands/adsorbents were used mainly for covalent affinity chromatography and are referred to in our previous review [1]. The second part of our review article dealing with relevant achievements in the field of bio- and pseudobio-affinity chromatog-

raphy using beaded cellulose-based media, is divided into sections on affinity, covalent affinity and hydrophobic (interaction) chromatographies.

2. Rational design of cellulose-based affinity chromatography media

2.1. Procedures and objectives in the design of affinity sorbents

The requirements from an ideal affinity chromatography matrix (support) have been specified briefly in Section 1. A single support will hardly meet all of the criteria, so its choice is a multi-objective procedure where a three-level evaluation is performed. At the bottom level, the matrix must provide the minimum quality requirements of an affinity support. Then, the design of the affinity sorbent (support+ immobilized ligand) is considered, evaluating the properties of the ligand used, the kinetics of interaction between the ligand and the purified molecule, and the conditions required for its use. The top-level of evaluation represents the performance of an affinity chromatographic contactor where efficient, long-term and economic separation is to be achieved. The interrelations between different process design objectives, process characteristics, and affinity support or sorbent properties are illustrated in Table 1.

As follows from Table 1, some sorbent properties, such as high mechanical strength, high affinity sorbent binding capacity, stability or fast kinetics have always positive effect on the chromatographic process. There are, however, properties whose influence cannot be assessed unambiguously. For example, the particle diameter should be small, on the one hand, to minimize the internal mass transfer resistance, but, on the other hand, it should be large, to minimize the pressure drop in the bed. Similarly, a high ligand loading improves the capacity of the sorbent but also increases its price and decreases its binding effectiveness. High ligand loading is achieved by increasing the specific surface area of the support, but not without the risk of increasing the extent of nonspecific interactions.

The design of an affinity sorbent is thus a complex task where the principal questions to be answered are as follows:

- What is the most suitable form of cellulose that should be used?
- What support geometry and dimensions should be used?
- What should its morphological characteristics be?
- What quantity and distribution of the ligand on the support should be used?
- How are the properties of the sorbent related to the whole chromatography process?

Evidently, the optimization of an affinity sorbent requires a systematic approach where the principal options are generated from knowledge of the physics and chemistry of the support and the biochemistry of the ligand–ligate interaction. However, final selection of the best alternative primarily uses engineering knowledge of chromatography theory.

2.2. Design parameters of cellulose affinity sorbents

The engineering assessment utilizes mathematical models that are primarily dependent on the support geometry and configuration of the chromatographic contactor. Table 2 shows that cellulose has been used, in particular, in the fibrous or membrane form, and several combinations of the support geometry and contactor configurations have been studied. The mathematical models describe the mass or momentum balances governing the chromatography process. Such models were used, for example, for evaluation of the kinetics of the one-component adsorption of albumin on a crosslinked cellulose ion exchanger [31] or for studies of a cellulose acetate membrane contactor with pore diameters in the range of 0.65–3 μm [28]. Further levels of classification complementing those given in Table 2 could include the direction of flow in the contactor (axial, radial), feed and elution mode (zonal, displacement or frontal), ligate–ligand interaction (equilibrium or kinetic). The number of resulting alternatives is rather high and it is beyond the objectives of this review to present them in detail. An interested reader can find them easily in monographs dealing with chromatographic theory [32,33].

Table 3 illustrates the extent of quantities needed for building rigorous models of chromatographic

Table 1
Relationship between the chromatography process design objectives, process characteristics and affinity support or sorbent properties

Design objective	Related process characteristics	Required affinity support and sorbent properties
High throughput	Maximum flow-rate	High mechanical strength Large particles Thin membranes
	Affinity sorbent binding capacity	High specific surface area High ligand density High binding capacity
	Ligate–ligand binding reactions	High equilibrium and rate constants of adsorption High dissociation and rate constants of desorption under the elution conditions
	External and pore mass transfer rates	High porosity and pore diameters Small characteristic dimension
	Axial dispersion	Small particles Thick membranes Narrow size distribution and regular shape
	Nonspecific interactions	No support surface charge and hydrophilic character Low specific area
Long life time	Operational stability	High mechanical strength High stability of the ligand and ligand support bonds
Low costs	Sorbent preparation costs	Low support price Ligand reusability High ligand binding capacity Cheap, easy and mild ligand immobilization
	Contactor dimension	High binding capacity
	Ligand quantity	Optimal distribution of ligand

Table 2
Applications of different cellulose supports in affinity chromatography

Support geometry				Contactor configuration		Reference
Particles		Membrane	Fibers	Packed bed	Filtration module	
Regular	Irregular					
+				+		[22–25]
	+			+		[26]
		+			+	[27–29]
			+	+		[30]

Table 3
Illustration of the quantities used in the mathematical models of affinity chromatography contactors

Affinity sorbent-related quantities	
Morphology and pore mass transfer	D_e, ε_p, d_p
Binding capacity and ligand distribution	$c_{pm}(r)$
Adsorption–desorption kinetics	k_1, k_2, K
Contactor operation related quantities	
Hydrodynamics and residence time	w, L, d_c, ε, k_L, D_z, Δp
Component concentrations	Initial and boundary conditions

D_e, ligate effective diffusivity; ε_p, support porosity; d_p, characteristic dimensions of support (diameter for spherical particles); c_{pm}, saturation concentration of ligate; r, radial co-ordinate; k_1, adsorption rate constant; k_2, desorption rate constant; K_d, ligate–ligand dissociation constant; w, superficial flow velocity; L and d_c, column length and diameter, respectively; ε, interparticle void fraction; k_L, mass transfer coefficient; D_z, axial dispersion coefficient; Δp, pressure drop for column.

processes. The number of parameters required is obviously rather high and, in general, they are not easily obtainable, which often prevents the use of rigorous models. Nevertheless, knowledge of any of these quantities is inappreciable in the evaluation of sorbent or contactor performance, even if a rigorous model cannot be employed. The following subsections review the available information on cellulose related supports and sorbent quantities as well on the hydrodynamic properties of cellulose supports. Specific information on adsorption equilibria on bead cellulose affinity supports is given in Section 3.

2.2.1. Morphological properties

The morphology of chromatographic supports directly influences several properties that are important for their separation performance. It determines the ability of the ligand to penetrate the support, the capacity and, eventually, the intraparticle distribution of ligand. The penetrability of supports for ligate influences not only the adsorption isotherm but also the dynamics of the intraparticle process and, consequently, the dynamics of the whole contactor. The size, shape and distribution of the pores are thus the sorbent characteristics that are fundamental in a rational design of chromatographic sorbents.

Cellulose is a material that is used in different areas and, so, its pore morphology has been investigated frequently. The standard methodology used, especially with less porous cellulose materials, has been inverse size-exclusion chromatography (SEC), which is also called gel permeation chromatography. The inherent problem of this technique is the interpretation of experimental results. As is well known, SEC is based on measuring the particle's partition coefficient for solutes of varying molecular sizes. The determination of pore size distribution requires a knowledge of the accessibility of pores in relation to the solute vs. the pore size ratio. Various theories have been developed for this purpose [34–38]. Unfortunately, an elaborate mathematical treatment of SEC data is necessary to obtain the pore size distributions using these theories, so there are only a few examples of their rigorous application [39–41].

Most authors have satisfied themselves, for this reason, with presenting the relationship between partition coefficient and solute size as an apparent cumulative pore size distribution. However, too many authors have made erroneous interpretations by identifying the experimental relationship with the true cumulative function of pore size distribution. It is therefore not useless to emphasize that this identification implicitly introduces an unrealistic assumption, i.e., that solute molecules penetrate equally into each pore that is larger than themselves. This oversimplification leads to overestimation of the solute pore concentration and, consequently, an underestimation of the pore dimensions. As has been shown recently for a Ca–pectate gel, the true pore sizes are two–three times larger than the apparent sizes estimated from SEC data [42].

Broek et al. [41] were the only investigators who employed a rigorous approach for the characterisation of the pore size distribution of cellulose materials. They investigated the morphology of three different hemodialysis membranes that were prepared from regenerated cellulose. They found mean

pore diameters of 4 nm (for low-flux membranes) and 8 nm (for a high-flux membrane), whereas the SEC data pointed to apparent mean pore sizes of about 1 and 2.5 nm, respectively. The apparent mean pore size was estimated as the value of solute diameter at which the particle's partition coefficient was half of its maximum value, determined for small solutes. In this simple way, the apparent pore sizes presented in Tables 4 and 5 were estimated from other SEC data published on cellulose materials. The ratio of true to apparent pore sizes, mentioned above, is generally valid for unimodal pore size distributions, therefore, the apparent mean pore sizes should be multiplied by a factor of two–three to obtain the true pore size.

Cellulose sorbents that can be used for chromatographic separations consist of morphologically very different materials. Some examples of such cellulose materials (including its derivatives) are reverse osmosis and hemodialysis membranes, with pore sizes of a few nanometers, ultrafiltration membranes, with typical pore sizes of 3–30 nm, and microfiltration membranes, where the smallest pores have a size of 20 nm and the largest are several micrometers [2]. As the membrane pores are formed in a controlled process of void formation [43], it can be expected that a second porous structure, closer to that of solid cellulose, will be found in cellulose membranes. Such a bidisperse character has been reported for reverse osmosis membranes made from cellulose and different cellulose acetate materials [44,45]. The reason that a bidisperse structure has not been reported very often is that the process rate is fully controlled by larger pores.

Particulate cellulose materials are used in chromatography in solid or gel forms. The morphological properties of solid celluloses were investigated mostly by researchers dealing with cellulose hydrolysis. Table 4 shows that cellulose in its solid form is a material with a low overall porosity and small pores. Although the morphological properties of the materials listed in Table 4 vary slightly, their similarity, especially from the point of view of chromatographic separations, is prevalent. The internal void fraction (porosity) is typically very low, between 0.3–0.5, or, in the form that is almost always given, from 0.4 to 1 cm^3 per gram of dry mass. Table 4 further shows that the apparent mean pore sizes are in the range

Table 4
Morphological properties of solid celluloses

Cellulose	Total pore volume (cm^3 g^{-1})	Apparent mean pore size (nm)	Apparent exclusion limit (nm)	Reference
Solka Floc BW-300	1.5	1–1.3	2	[47]
Solka Floc BW-300[a]	2.0	2–2.5	10	[47]
Sigma milled cotton	0.35	2–3	4	[48]
Sigmacell 20	0.5	3–5	10	[48]
Sigmacell 50	0.4	4–6	9	[48]
Sigmacell 100	0.5	3–4	9	[48]
Solka Floc SW-40	0.5	3	9	[48]
Solka Floc BW-100	0.5	3	8	[48]
Avicel	0.45	2	50	[49]
Avicel PH 102	0.35	2–2.5	n.a.	[46]
G.J.S.45–60 mesh	0.35	2	n.a.	[46]
G.J.S. 200–3000 mesh	0.4	2	n.a.	[46]
Solka Floc BW-300	0.4	4	n.a.	[46]
Avicel PH101	0.7	1.5	5	[50]
Sigmacell 100	1.1	1	3–4	[50]
Competencia cotton	0.9	2	3–4	[50]
Amorphous cotton[b]	1.35	1.5–2	5–6	[50]
Whatman CF-11	0.4	1.5	4–5	[50]

[a] NaOH treated.
[b] Prepared from the cotton, Competencia.
n.a., not available.

Table 5
Morphological properties of cellulose gels

Cellulose gel	Total pore volume (cm^3 g^{-1})	Apparent mean pore size (nm)	Apparent exclusion limit (nm)	Reference
CF-1, 3%	n.a.	100	n.a.	[51]
CF-1, 6%	n.a.	30–40	150–200	[51]
CF-1, 9%	n.a.	10	35	[51]
Cotton linter, 1%	n.a.	100	n.a.	[51]
Cotton linter, 1.5%	n.a.	50	n.a.	[51]
Cotton linter, 3%	n.a.	30	150	[51]
DEAE Sephacel	n.a.	5	30	[51]
DE 52	n.a.	2	7–8	[51]
HP-Regcell 16–50	2.4	2	6	[52]
HP-Regcell 8–50	6.0	2.5	10	[52]
HP-Regcell 4–50	10.0	3.5	15	[52]
HP-Regcell 2–50	15.0	5	20	[52]
HP-Regcell 1–50	14.5	6	50–60	[52]
HP-Regcell 4–30	5.6	3.5	10	[52]
HP-Regcell 4–100	21.0	4	10	[52]
2.6%	n.a.	12	20	[53]
4.5%	n.a.	12	18	[53]
6.5%	n.a.	11	16–17	[53]
8.9%	n.a.	5	9	[53]
10.4%	n.a.	4	6	[53]
33.7%	n.a.	2	4	[53]

HP-Regcell, regenerated, crosslinked hydroxypropylated cellulase gel.
n.a., not available.

from 1 to 5 nm. This means that solid cellulose materials are impenetrable for solutes with a molecular mass of the order of $1 \cdot 10^4$. In spite of the low porosity, solid celluloses have a relatively large specific surface area. For example, Neuman and Walker [46] have reported values ranging from 150 to 400 m^2/g for four types of cellulose. The use of solid celluloses in affinity chromatographic separations is based solely on the large specific surface area, when binding occurs predominantly at the outer surface of particles and the contribution of the matrix size exclusion effect to the separation is negligible.

Table 5 gives the morphological properties of some cellulose gels. Apparently, cellulose gels also demonstrate a broad spectrum of morphological characteristics, ranging from rather rigid gels, with internal void fractions of 0.6–0.7, up to soft gels, where more than 90% of the particle volume is occupied by pores. Rigid gels have very small pores (several nanometers), like those the solid cellulose, whereas the size of the pores of soft gels can reach 100 or more nanometers. The cellulose gels with large pores are thus morphologically comparable with other conventional affinity chromatography sorbents, such as agarose or silica. It is also necessary to underscore that the theories of gel and polymer networks suggest that the vacancies of the size of several nanometers represent the intrinsic gel structure (swollen polymer network), not the conventional pores (defects or spaces in the particle morphology free of building constituents of solid phase).

The importance of the support's morphology and its relation to the process performance have already been stressed. In fields such as heterogeneous catalysis or membrane technology, knowledge of the support's morphology and its influence on process performance is often rigorously applied in the design of catalysts or separation membranes. Unfortunately, this does not occur in affinity chromatography, which is a limitation in the design of chromatographic sorbents. Here, a reasonable simulation and modelling could be especially beneficial as expensive media are used and the extent of experimentation is limited.

2.2.2. Transport properties

Transport properties of chromatographic sorbents significantly influence the dynamics of the whole separation process. As there is a strong correlation between particle morphology and transport properties, cellulose membranes can easily be distinguished from the solid celluloses or cellulose gels by their transport properties. The transport of solutes in membranes proceeds via both diffusion and convection. Hence, the transport behaviour of membranes is a superposition of hydrodynamics and material physical properties. This section deals only with diffusional transport, which is common to all three geometries of cellulose sorbents.

The quantity characterizing the diffusion of ligate in the chromatographic sorbent is the effective diffusivity, D_e. This quantity is primarily related to the liquid diffusivity of the ligate, D_0, which is a function of state conditions of the liquid phase (temperature, pH, ligate concentration, etc.). It is well known that the effective diffusivity of low-molecular-mass solutes that do not exhibit an interaction with the sorbent is smaller than D_0 and can be expressed through the familiar relationship

$$D_e = \frac{\varepsilon_p D_0}{\tau} \qquad (1)$$

where ε_p is the intraparticle void fraction (porosity) and τ is the tortuosity, a factor expressing the variation of pore size and direction. In the case of ligates separated by affinity chromatography, the effects of partitioning and hindered (restricted) diffusion in pores have to be taken into account, due to the significant size of diffusing molecules compared to the pore size. As it would be difficult to distinguish the effect of tortuosity from other effects, it is usually not used in the interpretation of effective diffusivities in the field of affinity chromatography.

The effective diffusivity in a particle with a uniform pore size is usually expressed as follows,

$$D_e = \varepsilon_p K_p K_d D_0 = \varepsilon_p K_p D_p \qquad (2)$$

where two more quantities, the diffusive hindrance factor, K_d, and the pore partition coefficient, K_p, are contained in this definition of effective diffusivity. K_d, or the solute pore diffusivity, D_p, can be evaluated using different equations, derived from hydrodynamic theories, which have been reviewed by Deen [54]. These equations are specific for different pore and solute geometries and the key parameter is the ratio of the sizes of solute and pore, ϕ. One example is the Renkin equation [55], one of the first equations of this kind, which describes the hindered diffusion quite well, except for very low ϕ values. In the case of the distribution of pore sizes, Eq. (2) has to be modified by replacing the product of $K_p K_d$ with its mean value, obtained by integrating over the pore size distribution [56].

There have not been many studies dealing with hindered diffusion in general and, so, it is difficult to find enough representative data for cellulose supports. Table 6 shows the results of a study on the diffusional properties of solid cellulose [57]. These results confirm the very restrictive character of a cellulose matrix. Even for the smallest polyethyleneglycol (PEG), with a molecular mass of 200, the effective diffusivity was only 15% of the value in free solution. This result is consistent with the values obtained for the effective diffusivities of low-molecular-mass solutes in cellulose membranes (cellophane and cuprophane) reviewed in the paper of Gemeiner et al. [2], where the effective diffusivities were typically in the range of 10–20% of the liquid diffusivities. Table 6 further shows that the effective diffusivity decreases strongly with the molecular mass of the solute, therefore, study of the diffusion of solutes with molecular masses above 10 000 did not seem of interest, because of the very low rate of transport.

No data on hindered diffusion in cellulose gels have been found in the literature. In order to

Table 6
Diffusional properties of cellulose Avicel PH 102, investigated with polyethyleneglycols of varying molecular mass [57]

M_r	$D_0 \times 10^{11}$ (m^2 s^{-1})	$D_e \times 10^{11}$ (m^2 s^{-1})	D_e/D_0
200	63.3	9.56	0.151
400	41.2	5.30	0.129
600	32.1	3.61	0.112
1000	23.6	2.21	0.094
1450	18.8	1.49	0.079
3350	11.8	0.638	0.054
8000	6.73	0.277	0.041

The temperature was not specified in the source paper, but 20°C is the most probable value.

demonstrate the expected diffusional properties of these materials, the results of the study of Boyer and Hsu [58] on the hindered diffusion in a similar material (agarose gel) are presented in Table 7. A significant difference compared to the values presented in Table 6 is apparent. The molecular mass of the smallest protein investigated is twice as large as that of the largest PEG from Table 6, but its effective diffusivity is still 34% of the corresponding liquid diffusivity. Even for the largest proteins, with molecular masses of $1–2\cdot10^5$, the effective diffusivities are about 10–15% of the liquid diffusivities, which are values comparable with the behaviour of low-molecular-mass solutes in a solid cellulose matrix. The diffusive hindrance factor, K_d, varied for the probe proteins, from 0.5 to 0.25, which is a favourable value for chromatographic separations.

2.2.3. Hydrodynamic and mechanical properties

The hydrodynamic properties of the chromatographic beds are primarily determined by the size and shape of the support. The higher the hydrodynamic resistance, the larger are the process and equipment costs. Moreover, increased pressure imposes mechanical strength, which can deform the support, or even damage it irreversibly. Deformation of the support leads to a decrease in interparticle voids and, consequently, to larger pressure drops per unit column length. The mechanical and hydrodynamic properties of affinity chromatography supports are thus closely related. As follows from the Ergun equation, the pressure drop in a column packed with non-compressible sorbent particles and under conditions of laminar flow is a linear function of liquid velocity. Thus, the linearity of pressure drop–flow-rate dependencies can by used to test the rigidity of chromatographic supports.

The hydrodynamic properties of chromatographic packed-bed columns of rigid particles have been studied in detail. For solid celluloses in microgranular or fibrous forms, such studies showed that the pressure drops were about 5–7 kPa/cm at typical flow velocities, up to 100 cm/min [10,11,30,59]. Many small agarose- or cellulose-beaded gel supports, however, begin to deform at about 4 kPa/cm, or even less, and the deformation is then followed by an exponential increase in the pressure drop across the column [60]. The structure of the porous network of such materials may change as a consequence of bed compression. This will influence the permeability and porosity of the gel and at the same time will affect the accessibility of the adsorbate to the immobilized ligand [23].

Peška et al. [61] compared the properties of bead cellulose and dextran supports and showed that dextran gel has an inferior rigidity to that of bead cellulose. Kang et al. [24] compared hydrodynamic properties of packed beds from a beaded cellulose with those of other gel supports used in affinity purification. All of the matrices appeared to have a linear relationship between particle diameter and pressure drop, except for one type of crosslinked agarose that exhibited increased compressibility. It was interesting that the lowest pressure drop was achieved in the bed of cellulose gel, although it is necessary to emphasize that the cellulose beads had the largest diameter (of all supports tested). The mechanical properties of cellulose beads were studied by Kaster et al. [25]. They showed that the solid content of particles in the range of 0.9–3.2% strongly influenced the rigidity of the support. The

Table 7
Diffusional properties of agarose Sepharose CL-6B at 4°C, probed using proteins of varying molecular mass [58]

Protein	M_r (Da)	$\varepsilon_p K$	$D_0\times10^{11}$ ($m^2\ s^{-1}$)	$D_e\times10^{11}$ ($m^2\ s^{-1}$)	D_e/D_0
Myoglobin	16 890	0.734	6.9	2.35	0.343
β-Lactoglobulin	35 400	0.625	4.7	1.25	0.265
Ovalbumin	45 000	0.610	4.4	0.82	0.184
Albumin	67 000	0.550	3.6	0.56	0.155
Hexokinase	102 000	0.549	3.6	0.52	0.146
Immunoglobulin G	161 000	0.457	2.3	0.25	0.102
Catalase	225 000	0.474	2.6	0.30	0.119

acceptable pressure increased by a factor of ten in this range. In a subsequent paper from this laboratory, a solid concentration range of 4.5–11.2% was examined [53]. A further increase in the acceptable pressure was observed, but, even more impressive was that the superficial velocities could be increased up to 300 cm/min. The mechanical stability of the gel was somewhat decreased if mixtures of cellulose and cellulose derivatives were prepared [62]. It is also noteworthy that several authors found that bead cellulose supports had superior rigidity compared to that of more common crosslinked agarose- [4,24,25,63] or dextran supports [53].

2.2.4. Binding capacity, ligand distribution and stability

The quantity of ligand bound to the support is the main parameter that can be adjusted, depending upon the requirements of the affinity separation process. The ligand density is usually defined as the quantity of ligand coupled per unit volume or mass of the support. The binding capacity (loading capacity) is the amount of protein that can be adsorbed specifically by the affinity ligand. The binding capacity is the product of the effective concentration of ligand (the quantity of ligand in the pores that is accessible for the protein per unit volume or mass of the support) and its valence for the protein (the number of protein molecules bound per molecule of ligand). The relationship between the sorbent ligand density and its binding capacity is thus ambiguous. A high ligand density can lead to pore blocking and decrease their accessibility for the protein [8], which results in a decrease in the effective ligand concentration. Similarly, the valence also usually decreases with high ligand density, so the existence of an optimum ligand density giving the maximum binding capacity can be expected.

The effect of ligand density on the binding capacity for a cellulose support was studied by Ramirez et al. [26]. They used protein A as a ligand and immunoglobulin G (IgG) as a ligate. They found that, at relatively low concentrations of ligand, the molar ratio of bound IgG to the total amount of protein A was 2:1, which is the same value as that found with soluble proteins. On the other hand, when the protein A concentration was increased to saturation values, this ratio decreased to 0.3 mol of IgG per mol of protein A.

The relationship between binding capacities and support morphology has been studied by Horstmann et al. [64]. They observed that the maximum binding capacities of their sorbents increased with decreasing particle size, but the factor of change differed for various ligates. In the case of lysozyme, the increase in the binding capacity was smaller than the increase in the external surface area of particles, whereas it was more than four times larger for bovine serum albumin (BSA). It was likely that the change in particle size influenced the pore morphology and/or the ligand distribution in the support.

It has been shown that proteins adsorbed on more highly substituted gels are more difficult to elute [65]. This tighter binding has been attributed to multivalent interactions between the protein and immobilized ligand [66]. Hammond et al. [67] found that enzyme bound on either lightly or highly substituted adsorbents was difficult to elute, suggesting that an intermediate range of dye concentrations was optimal. It is apparent that ligand concentration can affect various systems differently and, hence, should be considered early in the development of a chromatographic process.

The operational stability of affinity sorbent strongly influences the economics of the process. Therefore, the aim is to prepare cellulose–ligand conjugates that are as stable as possible. Generally, there are three reasons why a cellulose–ligand conjugate can lose its binding capacity:

- Progressive irreversible inactivation of the ligand by binding with impurities that cannot be removed by washing, and microbial attack,
- leakage of ligand from the support,
- loss of specific binding capacity of the protein ligand due to the denaturation of its molecule.

The latter two causes can be more easily prevented by the proper design of the affinity sorbent. For example, the leakage of ligand could be diminished by activation of the support [68]. The stability of glycine immobilized to Cl-CO-ONBr-activated supports depended strongly on the matrix and was larger for a noncrosslinked cellulose support than for

different agaroses. The existence of crystalline structures in the cellulose matrix should explain the higher stability of this type of affinity sorbent.

3. Macroporous cellulose hydrogel in beaded form: Material predestined for column affinity chromatography?

3.1. Preparation and properties

The most frequently appearing form of cellulose in regenerated form is the bead or spherical cellulose. The data published [1,3] and information brochures from distributors [1] highlight numerous advantages following on from the use of bead cellulose in column liquid chromatography, including affinity chromatography, the most favoured points being:

- An enhanced ability to activate the subsequent chemical reactions (due to a lowered proportion of the crystalline phase),
- an enhanced reactivity in chemical reactions required for derivatization (increased accessibility of hydroxyl groups),
- it is supplied in two forms, i.e. as a ''ready for use'' material that has been pre-swollen in water and in the form of dried beads that are intended for chromatography in non-aqueous or water-immiscible solvents,
- its excellent flow characteristics under pressure are substantially better than those of the commercially available polysaccharide gels (mainly agarose) that are used for affinity chromatography,
- superior strength under dynamic flow conditions;
- it is stable within the 1–14 pH range and at high salt concentrations,
- its volume change is less than +1% over the entire pH/ionic strength range,
- on autoclaving, it is stable at 120°C and pH 7 for 1 h
- the regular geometric shape and good mechanical properties of beaded cellulose ensure reliable packing of columns,
- its porosity is regular and defined, making it possible to control its size-exclusion properties,
- it comes in a wide range of solid contents (2–12 wt.%), bead sizes (100–1500 μm) and pore sizes (8–2000 Å);
- a well-defined geometry of particles facilitates a rational (based on theory) design for optimal performance,
- crosslinking of bead cellulose for affinity chromatography is, in some cases, not necessary and
- cellulose beads can be activated under mild conditions for immobilizing the affinity ligands [1–3,24,54,69].

Bead cellulose is available from several distributors, e.g. Amicon–Grace, Chisso, Pierce (USA) [70,71], Daicel Chemical Industries (Japan) [72] and North-Bohemian Chemical Works, Lovochemie Lovosice (Czech Republic) [73]. Amicon–Grace and Chisso recommend their Matrex Cellufine Media as medium-pressure gels that are ideally suited for a wide range of chromatographic separations. For the bead celluloses from these manufactures, a similar statement holds as for the literature [14,51,61,74–78]. The methods used for preparation are obviously different and comparison of the results achieved under similar conditions is not available.

Macroporous reconstituted cellulose in regular form produced under the trade mark Perloza by Lovochemie is manufactured by a special technology that is based on the dispersion of a solution of cellulose xanthate in an inert liquid with subsequent thermal solidification of the xanthate, followed by regeneration [79,80]. The bead cellulose has regularly arranged layers with optimum through-flow properties when used in columns. Good mechanical strength is preserved, in spite of the high porosity. Due to its chemical composition, it is highly hydrophilic and is well tolerated by biosystem. Its insolubility in water and a number of others solvents allows it to be used for interactions in liquid media. Perloza is a suitable material for a wide range of derivatizations and for subsequent applications in affinity chromatography; these have been summarized by Gemeiner et al. [1]. In contrast to Czech manufactures, foreign manufacturers probably use a procedure via cellulose triacetate, which was previously known only by Japanese manufacturers [72]. This was also the case for a less well-known producer and distributor of bead cellulose [81], who later designed a granulate cellulose matrix, Granocel

[82]. The bead cellulose Divicell [68,83] is produced by Leipziger Arzneimittelwerk (Leipzig, Germany), whereas the cellulose (Divicell) ONB-Carbonate A (oxy-5-norborene-2,3-dicarboximidyl residue) is distributed by Eurochrom-Knauer (Berlin, Germany) [84].

Medium-pressure Matrex Cellufine gels, supplied by Amicon (Danvers, MA, USA) are composed of spherical, cellulose beads. They offer low nonspecific adsorption, outstanding physical strength and high pressure resistance capabilities, all at an economic cost. The use of Cellufine in bioaffinity chromatography has been described by Anspach et al. [23].

The major problem with bead production was a lack of neutral cellulose solvents. Cellulose insolubility was overcome by using cellulose derivatives, such as cellulose acetate or xanthate (i.e. viscose), as the starting material. The advent of novel solvent systems, such as *N*,*N*-dimethylacetamide (DMAc) and lithium chloride (LiCl), for cellulose allowed the exploration of hydrogel production from unsubstituted cellulose. This made it possible to take advantage of the exceptional rigidity of the cellulose backbone, which is the primary basis of hydrogel strength. Cellulosic hydrogels in bead form were prepared by the dropwise addition of cellulose solutions in DMAc and LiCl to azeotropic methanol or isopropanol as nonsolvent [53].

3.2. Size-exclusion chromatography

Cellulose beads are a suitable support for chromatography techniques. Individual examples have demonstrated outstanding flow-rates using several column sizes and thus have lead to fast separations, high resolution, due to small particle sizes, high capacity, allowing large samples loads, and high throughput, due to fast flow capabilities [70]. They also report minimal generation of fines, even with rough handling, with minimal changes in swelling and no shrinkage during operational changes, compatibility with all commonly used chromatographic solvents and buffers, autoclavability and low cost per unit separation capacity [70]. Pierce supplies cross-linked bead cellulose, the so-called Excellulose, which has a relative molecular mass exclusion limit of $5 \cdot 10^3$. Desalting of macromolecules and fractionation of small molecules ($400 < M_r < 500$) have been successful [71]. Daicel Chemical Industries provide cellulose gels that are characterized by relative molecular mass exclusion limits ranging between $1 \cdot 10^4$ and $1 \cdot 10^6$ and differing in compressibility by the eluent flow [72]. The particle sizes that are typical of beads produced by all of the manufactures mentioned above are 40–100 and 100–200 μm [70–72]. The data characterizing Perloza are continuously updated [73], however, complete data on Perloza as a column packing medium for SEC have not been published to date.

Tests on the size-exclusion low-pressure chromatographic properties of Perloza samples of different pore sizes (Perloza MT 100 M, 200 M, 500 M and low-porous MT 50) showed that, in contrast with the declared low adsorptivity of cellulose beads, the retention time for elution of proteins was considerably longer in the absence of a detergent in the mobile phase and, in some cases, the sample remained on the top of the column [85]. Increasing the content of sodium dodecyl sulphate (SDS) present in the mobile phase lowered the retention volume. At $C_{SDS} =$ 0.1%, retention volumes could be easily obtained for all of the proteins tested. For Perloza MT 100 M, 200 M and 500 M, the relationship between K_{av} and the relative molecular mass of proteins could be described by the equation $K_{av} = a' + b' M_r^{1/3}$ [85].

In the latest issue of the Sigma catalogue [69], it is noted that regenerated cellulose beads have very favourable properties for gel filtration, with fractionation ranges (globular proteins) of 10 000–1 000 000, 100 000–3 000 000 and 2 000 000–5 000 000.

3.3. Affinity chromatography

A wide range of derivatized celluloses have been described and use of them is generally widespread throughout protein purification methodology. The several limitations of cellulose in affinity chromatography were reduced by the introduction of the beaded form, which is porous and spherical. Numerous examples of the use of bead cellulose conjugates as affinity adsorbents are known from the literature. As follows from Table 8, the majority of the affinity ligands used are natural ones.

Table 8
Isolation and purification of biopolymers using derivatized bead cellulose as the affinity adsorbent

Covalently coupled affinity ligand	Biopolymer purified	Reference
Trypsin inhibitor	Chymotrypsin	[86]
Aprotinin	Kallikreins	[87]
Bacitracin	Proteinases	[88]
Peptides	Chymosin	[89]
Acetate	Proteinases	[90]
Anhydrotetracycline	Anhydrotetracycline oxygenase	[91]
Anti-human IgG	Immunoglobulin(s)	[92,93]
Immunoglobulin(s)	anti-IgG	[83]
Anti-IgG	Immunoglobulins	[83]
IgG	Protein C	[25]
Monoclonal antibody	Atrazine	[84]
Saccharides	Lectins	[83,94]
Concanavalin A	Ovalbumin	[83]
Concanavalin A	Invertase	[95,96]
Concanavalin A	Polygalacturonase	[97]
DNA	DNase	[98]
Histamine	Methaemoglobin	[99]
Hydroxamic acids	Urease	[20]
Reactive dyes	Lactate dehydrogenase	[22,100–105]
Reactive dyes	Glucose-6-phosphate dehydrogenase	[106]
Reactive dyes	7 Various enzymes (oxidoreductases, kinases)	[107]
Reactive dyes	Albumin(s), serum	[83,107,108]
Reactive dyes	Glycerolkinase	[109]

3.3.1. Dye-ligand affinity chromatography

The synthetic ligands, presented in Table 8, resemble natural ligands either in their structures or action (or in both). It is customary to describe their interaction with proteins (biopolymers) as being biomimetic [110], with various degrees of biorecognition. The most extensive and significant group of synthetic ligands are textile reactive dyes [111–114], called ''universal pseudoaffinity ligands'' [115]. The immobilized dyes, in many cases, mimic the properties of natural biological molecules and bind with high specificity to the ligand-binding sites of proteins [116]. For example, the anthraquinone textile dyes, especially Cibacron Blue 3G-A, are known to interact with representatives of almost every conceivable class of enzyme, and with a host of other, seemingly unrelated proteins [110].

The dye-linked adsorbents prepared from cellulose beads using other dyes of the triazine and vinylsulfone type, e.g. Procion Red HE-3 B [117] and Remazol Yellow GGL [118], exhibit group specificity towards proteins. Blue adsorbents (e.g. Cibacron Blue 3G-A and its Czech analogues) function on the basis of specific interaction with serum albumin (isolation and removal of albumin from blood serum) and nucleotide-dependent enzymes (lactate dehydrogenase). Also, the red adsorbent has an increased affinity for nucleotide-dependent enzymes. Yellow cellulose has the ability to bind immunoglobulins [118] whereas the blue diethylaminoethyl (DEAE)–cellulose has not [108]. This was utilized in their isolation and purification.

Rational design of affinity chromatography processes involves both mathematical modelling and experimental measurements of several parameters. Any model for the separation of enzymes by affinity chromatography must consider the equilibrium relationships between the respective enzyme and the immobilized ligand [116,119]. The foregoing relationship may be easily characterized by the enzyme–ligand dissociation constant.

Determination of dissociation constants of enzyme–dye complexes immobilized on cellulose beads (immobilized ligand; K_{I-L}) and of those

immobilized on a water-soluble polysaccharide (mobile ligand; K_{M-L}) revealed information about the cellulose beads as an affinity matrix. The methods applied are batch-wise adsorption and zonal analytical chromatography, and the enzyme often used for these purposes [111–113,115,120] is the well-known enzyme, lactate dehydrogenase (LDH) [121,122].

Besides determining the dissociation constant of the enzyme–ligand complex, batch-wise adsorption makes it possible to establish the nature of the interaction between the enzyme (LDH) and the immobilized ligand (dyed cellulose beads), when using the time–concentration model of adsorption [101,123,124]. It was assumed that the interaction of LDH with Cibacron Blue immobilized on bead cellulose is a boundary one, composed of biospecific and hydrophobic interactions, whereas the interaction of LDH with immobilized Remazol Blue is unambiguously hydrophobic, i.e. non-stoichiometric [101,123,124]. These results were compared with those achieved by zonal analytical chromatography [101]. In the treatment of the experimental results, the molecular retention model, describing a competitive monovalent interaction, was used [125]. Competition between the immobilized and mobile Cibacron Blue (Cibacron Blue bound to dextran T 10) for the binding site on LDH was employed and dissociation constants during zonal elution were estimated. Values of the dissociation constant of the LDH-immobilized Cibacron Blue complex ($0.6 \leq K_{I-L} \leq 2.6$ μmol l^{-1}), established by both methods, indicated the presence of biospecific interactions. On the other hand, the differences in the values of dissociation constants K_{I-L} and K_{M-L} ($K_{I-L} < K_{M-L}$) were ascribed to nonspecific interactions with bead cellulose [101]. In Table 9, values of the concentration of accessible immobilized Cibacron Blue and of the dissociation constants of LDH–immobilized Cibacron Blue on Sepharose CL-6B and on bead cellulose are compared.

The interaction of LDH with triazine dyes, spacer-linked to beaded cellulose, was also studied [22,105]. The length of the spacer does not influence either the binding capacity of the dye–affinity adsorbent or elution of the enzyme by NAD–sulphite. However, the length of the extension arm strongly affects the elution of LDH by salt [22]. The results with beaded cellulose carrying butyl, hexyl and decyl residues point to pure hydrophobic interactions with LDH. The introduction of a terminal amino or carboxyl group in the aliphatic chain significantly changes the binding and elution behaviours of the enzyme. It is assumed that, in addition to specific interactions realized by the dye chromophore and specific domains on the surface of the enzyme, the spacer arm generates a second type of binding force, which points to hydrophobic interactions. The latter might be the result of specific orientation governed by the dye-ligand–protein interaction [105].

In addition, the thermodynamic constants, associated with the interaction of three proteins [lysozyme, alcohol dehydrogenase (ADH) and serum albumin (HSA)] with triazine dye affinity sorbents, have been

Table 9
Interaction of CB support–LDH

Adsorbent	Dye content (μmol/ml)	LDH	Method	pH	Accessible dye (μM) (μmol/ml)	K_{I-L} (μM)	K_{M-L} (μM)	Reference
CB-Sepharose 6B			Frontal analysis	7.5	$1.7 \cdot 10^{-3}$	0.23	2.4	
CB-Sepharose 6B	0.12	Rabbit muscle	Static analysis	7.5	$2.3 \cdot 10^{-3}$	0.17	–	[126]
CB-Sepharose 6B			Zonal analysis	7.5	–	0.19	3.7	
CB-bead cellulose	0.46	Bovine muscle	Zonal analysis	7.0	–	1.92	11.1	[101]
CB-bead cellulose			Static analysis	7.0	$2.2 \cdot 10^{-2}$	2.40	–	

derived from bath and frontal analysis experiments [23]. In cases where mass-transfer restrictions are very high, calculation of the thermodynamic constants directly from frontal analysis experiments could not be achieved. In such cases, a portion of the adsorbate was always present in the effluent, a situation that has its effect as the split peak phenomenon. With Fractogel-based triazine dye affinity sorbents, none of the test proteins applied in frontal analysis were adsorbed. A similar behaviour was observed for a dyed Cellufine GC 700 (bead cellulose from Amicon) sorbent during the adsorption of HSA and for a Blue Sepharose CL-6B sorbent during the adsorption of ADH, which displayed much slower apparent adsorption kinetics than observed in the bath experiments. These phenomena were shown to be associated with changes in the gel structure, caused, in part, by the column packing procedure [23]. Anspach et al. [23] observed that the mechanism of interaction between lysozyme and CB-Cellufine beads varied with increasing protein concentration, resulting in non-linear Scatchard plots (batch procedure analysis in 0.05 *M* Tris–HCl, pH 7.8). Dividing the Scatchard plot into two parts, high protein concentrations and low protein concentrations, two sets of parameter values of the adsorption isotherm were obtained. The high-affinity sites were attributed to the immobilized triazine dye ligand, whereas the low affinity sites were attributed to binding sites on the chromatographic support itself (protein–support and protein–protein interactions). However, the values of the dissociation constants, associated with these nonspecific interactions, were in the same range as those found for the interaction of protein with immobilized ligand ($1 \cdot 10^{-5}$ mol/l).

Crude LDH was purified by preparative affinity chromatography on the dyed bead cellulose column by using NADH as an elution agent [100] or competitive dyed dextran [102,127]. The purity of LDH after ultrafiltration and dialysis was controlled by means of a fast-protein liquid chromatography (FPLC) procedure [102]. Comparison of purification results for LDH from various sources and with various adsorbents (bead cellulose) are introduced in Table 10.

The competitive elution of an enzyme from the dye-ligand cellulose by mobile ligands can be substituted by gradient elution with a solution of salts. However, the efficiency of this elution process is generally lower [107].

Bead cellulose (Perloza, Divicell), depending on the type, the amount of immobilized dye and the pH during the sorption procedure, can bind 2.4–40 mg of BSA or 6–24 mg of HSA per ml of dyed gel. Elution of bound albumin was without problems. It was achieved with an eluent of pH 8.5 containing KSCN (or NaSCN) within the range 0.2–1.0 *M* [83]. Pokorný et al. [128] took out patent on the isolation of HSA from blood plasma. The procedure included the sorption of albumin on Cibacron Blue 36-A bead cellulose in 0.05 M NaCl with subsequent elution with 3.5 *M* NaCl. Furthermore, the dyed derivatives of bead cellulose were successfully used for purification of other dehydrogenases, such as glucose-6-phosphate dehydrogenase [106], kinases, such as glycerokinase [107], and other enzymes, such as glutathione reductase [107] and lysozyme [23]. All of these were specifically bound to Cibacron Blue 3G-A (CB) or Remazol Brilliant Blue R (RBB) derivatives of bead cellulose and subsequently displaced with eluent of high ionic strength (NaCl, KCl) or using a specific eluent of NADH, NADP or ATP.

Cascade chromatographies, i.e. combinations of

Table 10
Purification of LDH by dye–bead celluloses

Adsorbent	Source	Specific activity (U/mg)	Yield (%)	Purification (*n*-fold)	Reference
RBB-bead cellulose	Rat liver	33	60	23.4	[100]
CB-bead cellulose	Bovine muscle	328	80	39.0	[102]
CB-Divicell	Bovine heart muscle	–	60	3–4	[22]
Procion Scarlet MX-G (diaminohexyl)–Divicell	Bovine heart muscle	200	52	34	[22]

dye-ligand affinity, hydrophobic and ion-exchange chromatographies on bead cellulose derivatives, were found to be effective for the isolation of glycerokinase [109] and alkaline proteinase [129]. The most effective isolation procedures remained, as anticipated, the dye-ligand [109] and ion-exchange chromatographies [129].

The beaded form of cellulose with enhanced mechanical strength and lowered porosity was simulated by silica-coated cellulose that was derivatized subsequently with dye-ligands (CB) [130]. Zonal affinity elution with NADH and CB-dextran showed, in addition to the already anticipated advantages, a noticeable decrease in the nonspecific adsorption of proteins, which was typical of silica media.

A paper on the synthesis of novel dye-like molecules designed specifically for affinity chromatography [131] should also be taken into consideration when determining a rational design for affinity chromatography processes. This approach was chosen to design the dye–ligand affinity chromatography of Na^+, K^+-ATPases [132,133] and is preferable to the conventional approach of empirical testing of textile dye analogues [22].

An overview summarizing our experience with the design of affinity elution procedures for dye-ligand chromatography was presented in condensed form in ref. [134].

3.3.2. Coenzyme (general ligand) affinity chromatography

Regarding coenzymes, ATP analogues have been synthesized with a reactive tail represented by phenylisothiocyanates [135] or asymmetric disulfides [136]. These ATP analogues are able to react selectively with natural enzymes, e.g. Na^+, K^+-ATPase [137] as well as with engineered enzymes such as polycysteine galactokinase [138].

3.3.3. Lectin chromatography

Cellulose beads were investigated also for the immobilization of lectins and, subsequently, in lectin affinity chromatography of glycoproteins. Lectin affinity chromatography is a considerably extended type of group-specific affinity chromatography, and concanavalin A (Con A) remains the best investigated lectin to date [139]. Divicell Con A and Perloza Con A were used in the column chromatography of ovalbumin from hen eggs [83] and of invertase from yeast [95,96] as the models. Elution of adsorbed protein with buffer containing D-glucose led to electrophoretically pure ovalbumin (recovery 66%) and was similar to results that were obtained using Con A–Sepharose [83]. A different situation appeared following the column chromatography of invertase (glyco-enzyme that is extremely rich in gluco-manno residues) on Con A–bead cellulose (Perloza), where an extraordinarily strong binding glycoenzyme with high storage and operational stability was involved [140,141]. In spite of the extraordinarily strong binding of invertase to this Con A conjugate ($K_D = 5 \cdot 10^{-9}$ M), conditions have been found to use Con A–cellulose beads (Perloza) as an affinity chromatography medium [95]. The critical factor in the release of the bound invertase by the counter-ligand (α-methyl-D-mannoside) is the time of incubation. This phenomenon was demonstrated in both batch and flow-through (frontal) experiments. A concentration of 1.5 mg of Con A per ml of gel was found to be suitable with regard to the maximal invertase–Con A binding ratio and for optimal invertase recovery (94%). As a result of the strong biospecific interaction, the purification of invertase was very effective (above tenfold). Verification by FPLC (Fig. 1) and polyacrylamide gel electrophoresis (PAGE) of the product purity revealed only one significant protein band [95].

The prepared Con A bead cellulose was used in the affinity purification of further glycoenzymes (polygalacturonase from Rohament P and exopolygalacturonase from carrot juice) as well as glycoproteins (extracellular mannan-glycoproteins from the yeast *Cryptococcus laurentii*) [97]. The nonspecifically adsorbed part of the sample was eluted with equilibrium buffer and glycoproteins were eluted with a solution of the corresponding counter-ligand α-methyl-D-mannopyranoside. The purification of all glycoproteins hereby examined was remarkable. The yeast mannanprotein was isolated from the extracellular heteroglycoprotein fraction. The monosaccharide component of purified mannanprotein was mainly mannose, with traces of glucose. The glycoenzymes and the yeast mannanprotein were homogeneous on FPLC chromatography [97]. The results of purifications on Con A–bead cellulose are summarized in Table 11.

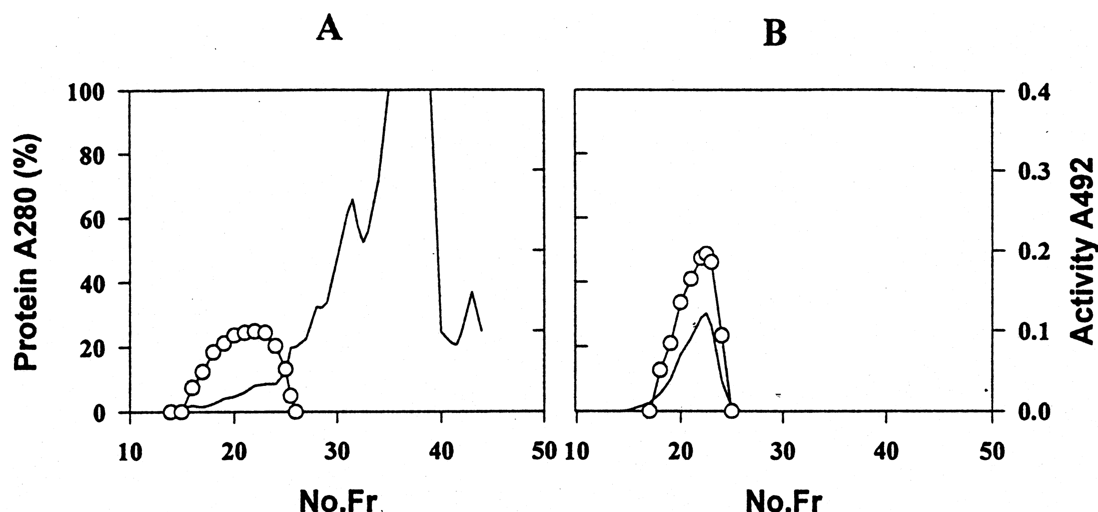

Fig. 1. FPLC of invertase on Superose 6. (A) Original sample and (B) sample after purification; —, proteins; ○, activity.

The adsorption of invertase on Con A–bead cellulose was found to be influenced by several parameters, such as pH, ionic strength and hydrophobic interaction. Quantitative parameters of the invertase adsorption on Con A–bead cellulose under optimal sorption conditions (50 m*M* acetate buffer, pH 4.7, containing 0.1 *M* NaCl) were investigated. Batch experiments were used for the determination of dissociation constants. The results for matrices with different concentrations of immobilized are shown in Table 12 [95].

The strong biospecific interaction between Con A and invertase was employed to determine the bound enzyme and this principle was used for the investigation of an alternative direct method for monitoring the lectin affinity chromatography of glycoenzymes. The results obtained by flow microcalorimetry showed that the ability of the affinity matrix decreased with the number of consecutive chromatographic runs, although its storage stability was satisfactory. [96]. Con A–bead cellulose was also used for the simple and rapid determination of lectin–saccharide interactions [143]. The inhibition effect of the saccharides on the formation of Con A–glycoenzyme was determined with the help of the glycoenzyme sorption on a conjugate Con A–bead cellulose support.

The affinity of many proteins to carbohydrates can be used for the chromatographic separation and

Table 12
Quantitative parameters of invertase affinity sorption

Adsorbent (mg Con A/ml gel)	q_m (mol/ml gel)	K_D (M)
0.92	$0.83 \cdot 10^{-9}$	$8.60 \cdot 10^{-9}$
1.14	$0.96 \cdot 10^{-9}$	$5.93 \cdot 10^{-9}$
3.50	$1.03 \cdot 10^{-9}$	$4.60 \cdot 10^{-9}$
5.17	$1.20 \cdot 10^{-9}$	$5.01 \cdot 10^{-9}$

Table 11
Results from Con A affinity purification

Glycoprotein purified	Specific activity (U/mg)	Yield (%)	Purification fold	Reference
Invertase	2154	71.4	14.5	[95,97]
Polygalacturonase	268	68.6	93.1	[97]
exo-Polygalactouronase	2.9	90.7	49.0	[97]
Ovalbumin	–	50.0	–	[142]

purification of lectins [83,94,144]. For this purpose, GlcNAc and GalNAc, immobilized to Divicell Epoxy [83] and Perloza, were utilized for the purification of Con A [94], crude wheat germ- (WGA), soybean- (SBA) and peanut (PNA) agglutinins [83] as well as lectin of *Pinus nigra Arn.* [144] from the corresponding plant seed extracts. The isolated amount of WGA was 420 mg from 5 g of total protein in the crude extract, obtained from 1 kg of wheat germ. The binding capacity of WGA is comparable to that described for Sepharose affinity sorbents [145,146]. The eluted WGA was electrophoretically pure (SDS–PAGE) and showed a high biological activity. The titre in the haemagglutination assay was about 2000 in a dilution series starting with 10 mg WGA/ml.

On reusing Divicell GlcNAc, the amount of WGA eluted decreased after eight cycles to 0.2 mg/ml of gel. It is probable that deacetylation of bound GlcNAc by formic acid was partly responsible for the diminished efficiency, because, after reacetylation and further use of the sorbent, the WGA eluted increased to 2.4 mg/ml of gel [83]. The purification of SBA and PNA on Divicell GalNAc was carried out under similar conditions to those described for WGA. When Divicell GalN prepared from Divicell ONB-Carbonate was applied, a binding capacity for SBA of 2.6 mg/ml of gel was obtained [83]. Binding capacities of used sorbents for purified proteins are summarized in Table 13.

3.3.4. Calmodulin affinity chromatography of pharmaceuticals

Interaction of various medicaments, mainly neuroleptics and local anaesthetics, with the calmodulin–agarose gel (CALA) was characterized by batch-wise adsorption [124]. Employment of the method made it possible to establish the dissociation constant, K_D value, not only for the case of the adsorption directed by stoichiometry but also for the case of the adsorption directed by the partition law. Medicaments active in adsorption on CALA directed by stoichiometry [124] should have been isolated by competitive affinity elution [124]. Such an analogy holds with dye-ligand chromatography of LDH on dyed bead cellulose [101,102,124,127]; this hypothesis can actually be verified because calmodulin–bead cellulose is available [147].

3.3.5. Bioaffinity adsorbents/chromatography of glycanohydrolases

In addition, nonderivatized cellulose may serve as a ligand, namely for β-glucanases, when suppressing its substrate properties and offering it to the enzyme as an imitation of the substrate. This is achieved mostly by crosslinking. This was done in the case of crosslinked bead cellulose that was used as an affinity adsorbent for β-1,3-glucanases [148]. Only weak interactions were observed between the enzyme and the adsorbent.

3.3.6. Immobilized metal (chelate) affinity chromatography

The hydrophilic skeleton of bead cellulose has a favourable effect on the kinetics of adsorption on chelating ion exchangers, i.e. on those with bonded 8-oxyquinoline, salicylic-, chromotropic- and ethylenediaminetetraacetic acids, and diethylenetriamine. The specificities of these and other ligands bonded on bead cellulose are known in analytical chemistry. These adsorbents, after adsorption of metal ions, were used in affinity chromatography, the so-called immobilized metal affinity chromatography (IMAC) method, e.g. of subtilisin [149].

Table 13
Protein purification on Divicell-cellulose-based sorbents [83]

Divicell sorbent	Content of ligand (mg/ml gel)	Applied material	Purified protein	Bound protein (mg/ml gel)
Con A	8.8	Crude ovalbumin	Ovalbumin	4.4
GIcNAc	7[a]	Plant seed extract	WGA	7.2
GalNAc	9[a]	Plant seed extract	SBA	2.6
GalNAc	9[a]	Plant seed extract	PNA	10.0

[a] μmol/ml gel.

3.3.7. Immunoaffinity chromatography

Cellulose was considered to be a perspective solid support at the beginning of imunnoaffinity chromatography [150]. This optimism was later partly corrected [151] and, at present, it is again admitted that cellulose may be a perspective solid support, especially for applications in immunoaffinity chromatography for the isolation of organelles [152]. This is mainly for cases where only unpurified polyclonal primary antibodies are available and, consequently, a high capacity immunoadsorbent has to be used [152].

3.3.8. Mixed types of affinity ligands

Experience with this group of affinity ligands involves mainly those acting selectively against proteases. These are either antibiotics [88] or hormones, e.g. 3,5-diiodo-L-tyrosine [153].

3.4. Covalent affinity chromatography on thiophilic sorbents

Chemisorbents of thiols and disulfides, so called thiophilic sorbents, occupy a dominant position among chemisorbents in biochemistry and biotechnology. Derivatives of bead cellulose that function as chemisorbents of thiols and disulfides are presented in Table 14 and are referred to in a review by Gemeiner et al. [1]. Regarding their use in biochemistry and biotechnology, the above-mentioned functional groups may be classified as synthetic ligands. Moderate conditions of adsorption and desorption, selectivity and reactivity are some of their typical properties. This is valid, for example, in the case of pyridyl disulfide derivatives XII and XIII [154], which have been known for a long time to be ligands in the covalent chromatography of thiol proteins, including enzymes.

Table 14
Review of bead cellulose derivatives that have been used as thiophilic sorbents

Derivative	Schematic structure of thiophilic sorbent	Abbreviation	Adsorptive	Reference
I	Cel-$O(CH_2)_2NCSNHC_6H_4CHO$	FP-cellulose	Phenylmethane thiol, Cys, GSH	[155,156]
II	Cel-$NHCSNHC_6H_4CHO$	FP-cellulose	id.	[155,156]
III	Cel-$N(CH_2)_2NHCSNHC_6H_4CHO$	FP-cellulose	id.	[155,156]
IV	Cel-OCH_2CH_2SH	ME-cellulose	5,5′-Dithiobis-(2-nitrobenzoic acid)	[157–159]
V	Cel-$OCH_2CH(OH)CH_2SH$	MHP-cellulose	id.	[157–159]
VI	Cel-$OCH_2CH_2SO_2C_6H_4SH$	MP-cellulose	id.	[157–159]
VII	Cel-$OCH_2C_6H_4NHC_6H_4NHN{=}C(CN)_2$	PHPD-cellulose[a]	Phenylmethane thiol, Cys, 2-ME, MAA	[160]
VIII	Cel-$O(CH_2)_2NHCH_2COC_6H_4NHCO$ $C_6H_4NHN{=}C(CN)_2$	id.	id.	[160]
IX	Cel-$O(CH_2)_2NH(CH_2)_2COC_6H_4NHCO$ $C_6H_4NHN{=}C(CN)_2$	id.	id.	[160]
X	Cel-$O(CH_2)_2NH(C_2H_5)_2CH_2COC_6H_4$ $NHCOC_6H_4NHN{=}C(CN)_2$	id.	id.	[160]
XI	Cel-$O(CH_2)_2N(C_2H_5)_2(CH_2)C_6H_4NH$ $COC_6H_4NHN{=}C(CN)_2$	id.	id.	[160]
XII	Cel-SSC_6H_4N	–	Cys, 2-ME, sodium sulfide	[154]
XIII	Cel-$OCH_2CH(OH)CH_2SSC_6H_4N$	–	id.	[154]
XIV	Cel-$OCH_2CH(OH)CH_2S_2O_3^-Na^+$	THP-cellulose	Cys, 2-ME MAA, papain	[161]

[a] Prepared from powdery and fibrous celluloses from Whatman (Maidstone, UK) and Serva (Heidelberg, Germany).
GSH, 2-ME, MAA, reduced glutathione, 2-mercaptoethanol and mercaptoacetic acid, respectively. Cel = cellulose.

Table 14 can be supplemented by the isolation of β-adrenergic receptors from crude membrane fractions of dog myocardium with a 2-pyridyl (or 5-nitrobenzoic acid) disulfide of bead cellulose and, subsequently, with the particular Perloza–alkaprenol conjugate [161].

3.5. Hydrophobic interaction chromatography

Conventional hydrophobic interaction chromatography (HIC) has been carried out mainly on unchanged alkyl and aryl derivatives of agarose. It was found that retention and selectivity depend substantially on the type of ligand being used. Proteins are generally more retained on supports that have more hydrophobic ligands. If the ligand is too hydrophobic, however, it is difficult to elute proteins in native states from the column. In contrast, if the ligand is very hydrophobic, very high concentrations of salt are required to retain proteins. Therefore, the ligand should be moderately hydrophobic [162]. Phenoxyalkyl chains bound to porous cellulose beads might fulfill all requirements.

In a preliminary study of hydrophobic adsorption [156–158], it was pointed out that the ratio between the amount of adsorbate and its concentration in water under equilibrium conditions is constant and independent of bulk concentration. Hence, hydrophobicity of an adsorbent might be adequately characterized by the partition coefficient of the amphiphilic probe in a system of amphiphilic adsorbent–water [134].

The above consideration were verified experimentally using the batch-wise adsorption of phenothiazine derivative (trivial name, perphenazine) and BSA onto bead 3-phenoxy-2-hydroxypropylcellulose (PHPC) [147]. A kinetic study of adsorption revealed that, in equilibrium, the partition law governs the behaviour. The partition coefficient, p, was proved to be dependent on the hydrophobe density, and was independent of the bulk concentration of the adsorptive. On the basis of these results, the mechanism is considered to be that of liquid–liquid partition (LLP). The essential part is the formation of ''high surface coverage'', producing a coherent liquid-like film around the cellulose beads. It was anticipated that the properties of such layers would depend on the chain length of the hydrophobe and its density [163].

The nonstoichiometric model, suggested for adsorption of perphenazine and BSA on bead PHPC and considered to be equal to the partition mechanism, was verified by batch-wise adsorption of six proteins on bead PHPC [164]. It was found that the surface of the hydrophobic segment (PHP groups) of the adsorbent had to be sufficiently large so that the hydrophobic region of the protein surface might come into contact with it through multiple residues. Fulfilment of this criterion should be a necessary prerequisite for predominance of partition of the protein between the hydrophobic segment, in the form of a liquid-like film, and the surrounding solution during adsorption. The absence of displacement phenomena in the zonal chromatography of six proteins on bead PHPC was observed. Displacing agents (water, PHP–PEG) and gradient elution (sodium chloride, ethyleneglycol) had no effect on desorption [164]. With regard to high loading capacity ($B_e \leq 75$ mg IgG/g PHPC), it is recommended PHPC beads be used for preconcentration, purification and preseparation of substances and complex mixtures [165].

This project was extended later with the study of a series of six proteins (trypsin, chymotrypsin, lysozyme, ovalbumin, serum albumin, immunoglobulin) [82,109,166–168] and haemoglobin [82]. Possible predictions of their behaviour in HIC on PHPC were tested [167,168]. These papers also give methodic recommendations for quantitatively determining some of the parameters characterizing the hydrophobicity of proteins by accessible methods (Fig. 2) [168]. Results reported in these papers could even be of practical use when predicting or interpreting data (or rational design) obtained using HIC with further enzymes/proteins, e.g. those obtained with glycerokinase [109] or recombinant α_2-interferon from *Pseudomonas putida* VG-84 [82].

To date, only a few examples of the use of PHPC for the isolation of proteins have been reported in the literature [82,147]. The results have shown that bead PHPC may conveniently be used instead of Phenyl Sepharose for routine isolation, both in the laboratory and in the large scale isolation of calmodulin (CaM) [147] and recombinant α_2-interferon from *P. putida* VG-84 [82]. Calmodulin from nerve tissue of

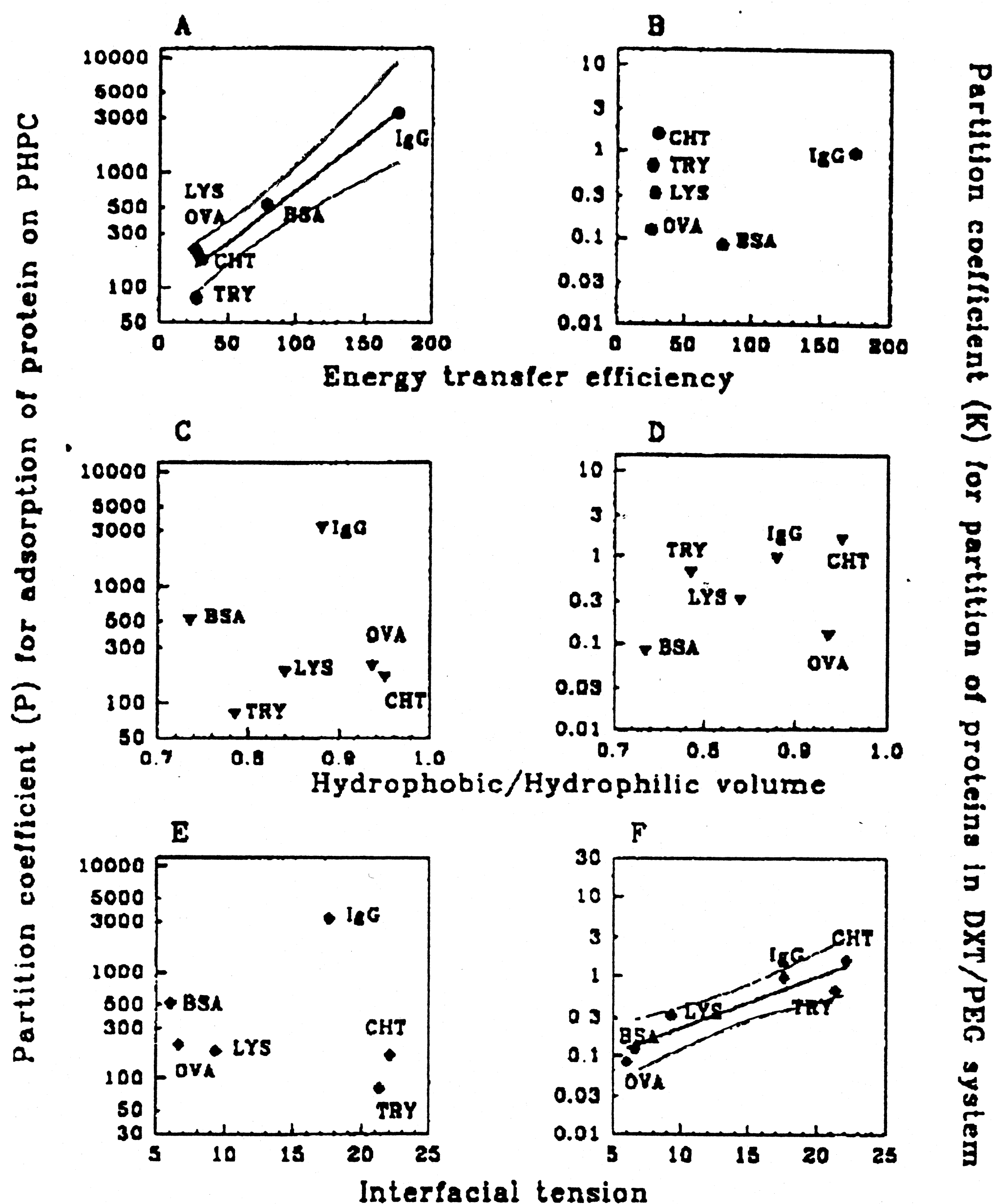

Fig. 2. Correlation of partition coefficients, P (obtained in system form by 3-phenoxy-2-hydroxypropyl derivatives of bead cellulose and water solutions; panels A, C, E), and K (obtained in aqueous polyethyleneglycol–dextran two phase system; panels B, D and F), with efficacy of energy transfer (panels A, B; values of efficacy of energy transfer were taken from Kato and Nakai [169]), hydrophobic ratio (panels C and D; values of hydrophobic ratio were computed according to Bigelow [170]) and interfacial tension (panels E and F; values of interfacial tension were taken from Keshavarz and Nakai [171]). Symbols: LYS, lysozyme; TRY, trypsin; CHT, chymotrypsin; OVA, ovalbumin; BSA, bovine serum albumin; IgG, immunoglobulin G. Relationships on the panels A and F offered significant correlations ($r=0.964$, $p<0.01$ and $r=0.936$, $p<0.05$, respectively).

cattle has been isolated using hydrophobic chromatography on PHPC, and is now commercially available [172]. Using gel electrophoresis, the purity of CaM isolated on this carrier was >95% [172]. A 3.5-fold enrichment of α_2-interferon from mother broth in large-scale production (2–5 l columns) with good recovery (ca. 75%) was also achieved [82].

The effectiveness of HIC can be modulated by varying the length of the hydrophobe (ligand). Very promising results were obtained with model HIC of serum albumin with a series of eight hydrophobized bead celluloses (Table 15) [83]. Reuse of Divicell Pentyl was tested with a series of 22 loading–elution cycles. The support was regenerated using a 1-*M* sodium hydroxide solution after every ten cycles. For each loading–elution cycle, the recovery of BSA was 93±1.5% ($n=20$). Only traces of protein were eluted during regeneration and no significant changes in the binding capacity, recovery or the flow-rate could be observed during these experiments [83].

It seems that even non-derivatized bead cellulose of a certain macroporous structure is capable of binding low density lipoproteins (LDLs) selectively and of simultaneously maintaining the level of high density lipoproteins (HDLs) [173]. However, only partial regeneration was possible using a 0.4-*M* NaCl solution. The LDL-binding capacity after successive regeneration procedures decreased progressively. After treating the cellulose with a 6-*M* urea solution, almost complete restoration of the LDL-binding capacity was possible. The adsorption mechanism of LDL fixation by the bead cellulose is, however, not yet fully understood [173].

4. Conclusion

The optimum design of affinity chromatography devices is a complicated task because a multitude of parameters has to be considered. A chemical engineering approach is required, enabling one to quantify the influence of each significant parameter on the chromatographic device's performance and to predict the behaviour of the chromatographic device. The basic mathematical models of the affinity processes are given. These models can become a powerful tool in three steps of affinity sorbent design:

- In the search for optimum cellulose-based media, based on literature and laboratory data,
- in the search for optimum properties of the ligand–support conjugate, based on literature and laboratory data,
- in the prediction of the optimal conditions for operating the affinity chromatography device.

While experimental investigation of the performances of the affinity sorbents is irreplaceable, mathematical modelling can reduce the number of experiments required and increase the effectiveness of the

Table 15
Binding capacities and recoveries for bovine serum albumin according to Boeden et al. [83]

Divicell derivative	Immobilized ligand (μmol/ml gel)	Capacity for BSA (mg bound/ml gel)	Recovery (%)	Elution medium
Propyl	32.3	23.5	99	0.05 *M* P (pH 7.4)
Butyl	30.6	22.4	99	0.05 *M* P (pH 7.4)
Pentyl	34.0	23.0	95	6 *M* urea
Hexyl	34.0	21.3	95	50% acetonitrile[a]
Octyl	34.0	28.7	85	50% acetonitrile[a]
Decyl	27.1	47.0	90	80% methanol[a]
Dodecyl	27.1	47.2	90	80% methanol[a]
Benzyl	26.7	29.7	90	6 *M* urea

[a] Mixture with water (v/v); P means phosphate.
Preparation of supports was carried out according to the pentyl derivative (see Table V in Ref. [83]).
The coupling yield was 90–100%, except for Divicell Benzyl.
In each instance, 100 mg of BSA were loaded on the column (2.5×1 cm I.D.) at room temperature.
The binding buffer was 0.05 *M* phosphate (pH 7.4) containing 2 *M* $(NH_4)_2SO_4$.

design. Some predictions can be made from partial literary data. There is, however, an enormous lack of systematic experimental work dealing with the optimum design of cellulose-based affinity sorbents. Therefore, the systematic design cannot be decided upon without carrying out extensive experimental work, which is necessary to provide the consistent data that is needed for the mathematical modelling.

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