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Synthesis of silica-based heparin-affinity adsorbents ☆

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Abstract

In these investigations, methods for the synthesis of rigid heparin-affinity adsorbents were developed. An optimal binding-site accessibility of the ligand was achieved by end-point attachment of the heparin through reductive amination onto amino-derivatised silica-based support materials. The rigid LiChroprep Si60 and Fractosil 1000 supports were derivatised by treatment with γ-aminopropyltriethoxysilane, and a range of different heparin contents were immobilised onto these materials through controlled coupling conditions. Thus, heparin-LiChroprep Si60 adsorbents and heparin-Fractosil 1000 adsorbents with heparin contents ranging from 0.6 to 26 mg and 1 to 14 mg heparin/g adsorbent, respectively, were obtained. Due to their radically different pore size and specific surface areas, the heparin-LiChroprep Si60 (pore size 60 Å, 500 m^2/g) and heparin-Fractosil 1000 (pore size 1000 Å, 20 m^2/g) adsorbents possessed markedly different adsorption properties. The small pore size of LiChroprep Si60 largely restricts the immobilisation of heparin to the external surface regions of these silica particles, with interaction with proteins in chromatographic applications mimicking non-porous affinity adsorbents. With the heparin-Fractosil 1000 adsorbents, the larger pore size (1000 Å) enabled both the immobilisation of heparin and the solute interaction to take place within the pores to a much greater extent. Although high ligand densities per unit-accessible-area of the heparin-LiChroprep Si60 adsorbents could be achieved, decreased yield for the amino-group modification due to steric effects was observed. With the heparin-Fractosil 1000 adsorbents, lower ligand densities per unit area were obtained, but the binding-site accessibilities were considerably higher. These differing ligand densities were a function of differences in coupling yields (around 10 to 20% on LiChroprep-NH_2, but only 1 to 3% on NH_2-Fractosil 1000), and the different (accessible) surface areas.

The hydrolytic stability of the aminopropyl-modified supports and heparin adsorbents was also investigated. A new quantitative method, based on absorbance measurements in 96-well microtitre plates, to determine the extent of the aminopropylsilyl group and ligand leakage is described. Although some instability of the heparin adsorbents was observed at pH values below pH 8.0, the ligand loss rate was much lower than the silane leakage on corresponding aminopropylated support materials lacking the heparin coverage. Thus, the heparin-LiChroprep Si60 and heparin-Fractosil 1000 adsorbents typically retained greater than 95% of their initial heparin content when stored in 0.2 *M* sodium acetate, pH 5.0 at 4°C for eight weeks.

Keywords: Affinity adsorbents; Immobilization; Stationary phases, LC; Heparin; Aminopropyltriethoxysilane

1. Introduction

There are two major motives for the development of heparin-immobilisation chemistries. Firstly, the

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therapeutic need exists for chemically stable non-thrombogenic (blood-compatible) surfaces based on immobilised heparin and heparin-like substances. Secondly, alternative synthetic routes to heparin-affinity adsorbents [1–3] required for the purification of proteins and other biopolymers have been sought since the first observations on the biological properties of heparin. The covalent coupling of heparin to an inert matrix for the purpose of generating affinity adsorbents has involved several different approaches over the years. Initially, heparin was immobilised onto cyanogen bromide (CNBr)-activated Sepharose [4–7], or conversely, CNBr-activated heparin was immobilised onto an underivatised support [8]. One drawback with affinity adsorbents based on the CNBr-activation chemistry has been the limited stability of the chemical bonds of the attachment sites. Furthermore, with this procedure the heparin molecules are coupled in a randomly oriented manner [6,9]. Since specific heparin–solute interactions involve functional side groups on the heparin carbohydrate chain, these random modes of immobilisation potentially restrict the accessibility of specific binding sites on the heparin molecule to solutes.

The general trend in recent years has therefore been to use alternative immobilisation chemistries involving end-point-attached heparin. One approach to achieve this outcome involves the coupling of the heparin through reductive amination onto an amino-functionalised support, utilising the aldehyde group present at the reducing end of the heparin molecule [1,10]. Aldehyde groups are well known to react with primary amines to produce labile Schiff bases that can then be converted into stable secondary amines by reduction with sodium cyanoborohydride [1]. Aldehydes in polysaccharides are however largely present in the form of cyclic hemiacetals, and low coupling yields are therefore anticipated with this method. One strategy to circumvent this problem has been the introduction of aldehydes by using partially depolymerised heparin [1]. Since this degradation could result in loss of specific protein binding sites (such as that for antithrombin III (AT III)) on the heparin molecule, the benefits, however, of depolymerisation are in doubt [9].

Immobilisation of non-degraded heparin would most likely result in a better preserved ligand multifunctionality, consequently offering a wider range of possible protein interactions with the affinity adsorbents. Non-degraded heparin has been successfully immobilised onto soft gel support materials by several investigators [9–11]. Sasaki and co-workers furthermore reported higher capacities for AT III with "native" heparin than with nitrous acid-degraded heparin [9]. Soft gel support materials such as Sepharose and other agarose-based materials have often been employed for these purposes. However, the relatively low rigidity of such materials limits the maximum allowable back pressures and flow-rates in liquid chromatographic systems, thus rendering them less suitable than rigid materials such as silica [12] for use in high-speed and higher-performance liquid chromatographic applications. The immobilisation of heparin onto rigid supports has often involved methods where heparin is not coupled directly onto the support, but to a passively coated layer [13–17]. Since the stability of support materials involving passively coated layers may be uncertain, the establishment of conditions for the direct immobilisation of heparin onto pressure and mechanically stable supports would present significant advantages.

A basic requirement when synthesising useful silica-based affinity adsorbents is a complete and stable modification of the silica surface with a silane [18,19] which usually contains the desired functional group. If an amino-functionalised silica is required, γ-aminopropyltriethoxysilane (APS) is the most commonly used reagent. An even silane coverage is desired, since unreacted silanol groups will cause non-specific adsorption with the adsorbent. To obtain a monomeric silane layer free of polymerised silanes, the reaction must be controlled, either in an aqueous system by carefully balancing the molar quantities of the components [20–23], or in an anhydrous environment where water is only present in catalytic amounts [22,24–27]. Silane coverages of 3.5 to 4.0 μmol/m^2 surface area are generally anticipated as a complete modification of the silica surface.

Based on the considerations presented above, a range of rigid heparin adsorbents were synthesised by end-point attachment of non-degraded, unfractionated heparin onto silica through reductive amination. Since the immobilisation of heparin onto silica through reductive amination required aminated support materials, the porous silica materials used in this study (LiChroprep Si60 and Fractosil 1000) were

aminopropyl modified using APS. Although the silylation chemistry in most cases is quite straightforward, APS modification is an exception to this rule. The basic properties of the amino group reduce the stability of this particular silane, and the aminopropylsilyl group has a tendency to leak off the support [19,28]. Nevertheless, as demonstrated in these investigations, the synthesis of rigid heparin adsorbents via APS modification of naked silicas can be successfully carried out, particularly when macroporous silica such as Fractosil 1000 are used.

2. Experimental

2.1. Materials

The silica-based support materials examined in these investigations were the mesoporous LiChroprep Si60 (particle diameters in the range of 25–40 μm and 40–63 μm), and the macroporous Fractosil 1000 (particle diameter in the range 40–63 μm) obtained from E. Merck (Darmstadt, Germany). The LiChroprep Si60 material consists of angular silica particles with a total surface area of 500 m^2/g and a pore diameter of 60 Å. Fractosil 1000 is also an angular silica, with a total surface area of 20 m^2/g and a pore diameter of 1000 Å. Heparin Sepharose was purchased from Pharmacia (Uppsala, Sweden). Aminopropyltriethoxysilane (APS) was purchased from Sigma (St. Louis, MO, USA). Purified heparin used in the synthesis of affinity adsorbents was a porcine mucosal heparin preparation from Diosynth (Oss, Netherlands).

2.2. Qualitative test for amines (TNBS test)

Aliquots of amine-containing adsorbents, or the effluents from washes (100–200 μl) were mixed with saturated sodium tetraborate solution (2 ml) in a 10-ml glass test tube at room temperature. A small amount (typically 2–3 mg) picrysulfonic acid was added and the mixture was vortex-mixed. The presence of unblocked amino groups on adsorbents, or free ammonia in the effluent produced a yellow colour, hydrazines a purple colour. If no colour formed within 10 min, the reaction was considered negative.

2.3. Quantitative picrysulfonic acid (PSA) assay

This method allowed the quantitation of amino-group contents on silicas, as well as free APS in wash effluents. The method relied on the formation of a yellow colour with picrysulfonic acid in the presence of free amines. Samples of amino-derivatised silica were dissolved by heating in a 2 *M* sodium hydroxide solution in 10-ml (75×12 mm) glass test tubes. For standard samples, APS was diluted to between 0.05 m*M* and 1.0 m*M* in 2 *M* NaOH. Samples of the amine-containing effluent from washes of the APS-modified silicas were also diluted in 2 *M* NaOH. Dissolved silica, diluted standards and effluents were further diluted 1:5 in a saturated sodium tetraborate solution to achieve an operating range of 0–0.2 A_{405} units. Aliquots (20 μl) of these dilutions were pipetted into the wells of a 96-well flat-bottom microtitre plate (Greiner Labortechnik, Frickenhausen, Germany), 2 m*M* picrysulfonic acid in saturated sodium tetraborate (50 μl) added, and the plate incubated at room temperature for 1 h. Finally the absorbance at 405 nm was measured using a Titertech Multiskan MCC/340 Elisa plate reader (Flow Laboratories, Helsinki, Finland). A first-order linear correlation coefficient (r) of 0.998 was obtained when assaying APS dilutions in the range 0.05 to 1 m*M*. The detection limit of the assay was 50 nmoles APS.

2.4. HCl-titration method for amines

As an alternative to the picrysulfonic acid assay, a modified titration method for the quantitation of amino-group content of the chromatographic supports (Huisden et al. [27]) was used. The titration method produced numerical values similar to the PSA assay. Briefly, small samples of dry support (0.2 to 0.4 g) were degassed in 4 *M* NaCl (10 ml) containing eight drops of a 1% methyl red-bromocresol green (5:1) indicator solution. The suspensions were then titrated with 0.1 *M* or 0.01 *M* HCl (previously titrated against a 0.05 *M* Na_2-tetraborate solution) for LiChroprep-NH_2 and NH_2-Fractosil

1000, respectively. During the titration, the support was kept in suspension using a magnetic stirrer.

2.5. Indole method for quantitation of heparin content

A spectrophotometric method for the determination of hexosamines [29] was optimised in order to measure the immobilised heparin contents on the synthesised adsorbents. The method involved the deamination of hexosamines (which are a major constituent of heparin) with nitrous acid. The resulting anhydrohexoses produced a characteristic orange colour when reacted with indole in dilute hydrochloric acid. The increase in absorbance at 492 nm upon deamination was proportional to the hexosamine content, and therefore the heparin content in a sample. Hexosamine could thus be detected in samples containing 5–100 μg hexosamine (approx. 10–200 μg heparin). A linear response was observed for heparin concentrations between 0.05 and 1.0 mg/ml (r>0.999). A 30-mg sample of a heparin adsorbent, containing between 1.5 and 25 mg heparin/g dry adsorbent, fell within this range.

Heparin standards were diluted to between 0.05 and 1.0 mg heparin/ml. Samples of dry heparin adsorbent (10 to 60 mg) were suspended in H_2O (500 μl), equalling 20 to 120 mg adsorbent/ml H_2O. For deaminated samples, 5% sodium nitrite (0.5 ml) and 33% acetic acid (0.5 ml) were added to a 0.5-ml sample in glass test tubes, and the tubes were vortex-mixed. After 10 min reaction, 1 M ammonium sulfamate (0.5 ml) was added and the mixtures were vortex-mixed, then reacted for 30 min with shaking. For non-deaminated samples, equal volumes of 5% sodium nitrite, 33% acetic acid and 1 M ammonium sulfamate were mixed, then stirred for 10 min. The pre-mixed solution (1.5 ml) was added to a 0.5-ml sample in glass test tubes. Deaminated and non-deaminated samples were then vortex-mixed, and reacted for 30 min with shaking. Following this step, the indole reaction was performed on the reaction mixtures (deaminated or non-deaminated samples and H_2O blanks). For samples containing heparin immobilised onto a polysaccharide support such as Sepharose, the support material had to be removed from the liquid by spinning the tubes at 4500 g in a Sorvall RT6000 refrigerated centrifuge, and then collecting a certain volume (typically 1.5 ml) of the supernatant for the indole reaction. The determination of subsequent reagent volumes (HCl, indole solution and ethanol), was based on this volume. For other samples (2.0 ml), 5% HCl (2.0 ml) and 1% indole in ethanol (0.2 ml, freshly made for each assay) was added. The test tubes were vortex-mixed until visible liquid layers were completely mixed, then boiled for 5 min, and cooled by immersing in cold water. Ethanol (2.0 ml) was added to each tube, and the mixtures were vortex-mixed. Aliquots (200 μl) were pipetted into the wells of a 96-well flat-bottom microtitre plate (Greiner), placing "H_2O-blank" samples in column 1. A_{492} and A_{520} were then measured on a Titertech Multiskan MCC/340 Elisa plate reader (Flow Laboratories).

Heparin contents were calculated by plotting the absorbance factor A (where $A=\delta A_{\text{deam.}}-\delta A_{\text{non-deam}}$, and $\delta A=A_{492}-A_{520}$) against heparin amount (μg) for the standards and the data analysed using a linear regression program. Linear estimates (LE_x) of unknowns were then made using the linear regression. Heparin contents in adsorbent samples were finally determined as LE_x/C (mg heparin/g adsorbent), where C=mg/ml dry adsorbent in the sample solution.

2.6. Preparation of affinity adsorbents

Aminopropyl-group derivatisation of silica

A method adapted for the aminopropyl-group modification of the preparative silicas using γ-aminopropyltriethoxysilane was employed in this investigation. This chemical reaction proceeds according to the scheme shown in Fig. 1.

Silylation under aqueous conditions

γ-Aminopropyltriethoxysilane (1.13 ml) was dissolved in distilled H_2O (300 ml). The pH of the solution was adjusted to pH 3.5 with 1 M HNO_3, and dry silica (30 g) added. The pH was checked again, and adjusted if necessary. During the pH measurement, a suspension of silica particles was maintained using a suspended impeller to prevent mechanical grinding of the particles. The construction of the equipment used for these experiments has been described elsewhere [23]. The mixture was then degassed for 5 min with manual swirling, and

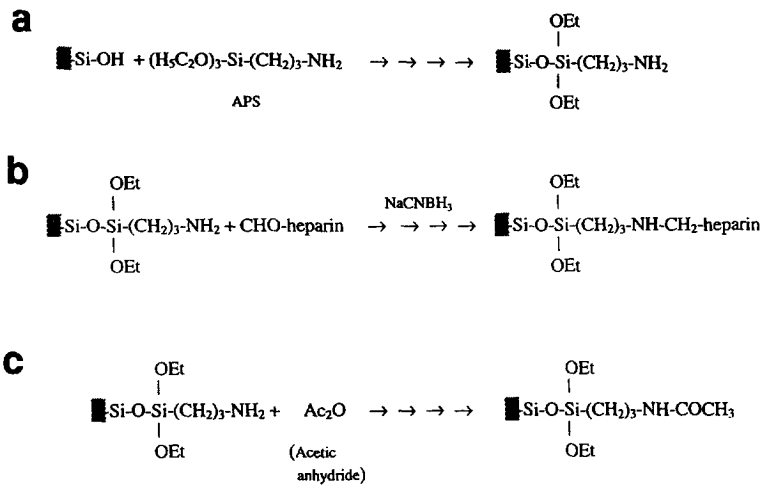

Fig. 1. Aminopropyl group derivatisation of silica. (a) Aminopropyl group modification of the preparative silica using γ-aminopropyltriethoxysilane (APS). (b) Coupling of heparin through reductive amination. (c) Blocking of unreacted amines with acetic anhydride through acetylation.

transferred to a 500-ml air-tight reaction bottle. The mixture was reacted with lateral shaking at 90°C for 3 h. The modified silica was then washed on a sintered filter funnel with distilled H_2O (4×150 ml), followed by 0.1 *M* HCl (6×150 ml) and finally H_2O (4×150 ml), then dried at 110°C overnight.

Silylation under anhydrous conditions

γ-Aminopropyltriethoxysilane (1.88 ml) was added to freshly distilled toluene (300 ml). Fractosil 1000 (40–63 μm particle size, 50 g), pre-dried under high vacuum for 12 h at 180°C was then added. The suspension was sonicated for 1 min, and refluxed for 12 h at 150°C. The aminopropyl-derivatised silica was then washed with toluene (200 ml), followed by isopropanol (150 ml) and finally dried at 110°C.

Immobilisation of heparin onto aminopropyl-derivatised silica

Heparin (approximately 10–200 and 50–1000 mg/g silica for LiChroprep Si60 and Fractosil 1000, respectively) was dissolved in 0.2 *M* potassium-phosphate buffer, pH 7.0 (5 ml/g silica). Dry aminopropyl-derivatised silica and $NaCNBH_3$ were then added (0.1 mg $NaCNBH_3$/mg heparin). The slurry was degassed for 5 min with careful swirling, and then allowed to react at room temperature for 60–70 h with end-over-end mixing using a rotary suspension mixer. The adsorbent was washed on a glass sinter funnel with 0.2 *M* potassium-phosphate buffer pH 7.0, H_2O and 0.2 *M* sodium acetate (40 ml of each per g adsorbent), and then suspended in 0.2 *M* sodium acetate (40 ml/g adsorbent). Unreacted amines were acetylated by sequential addition of acetic anhydride at 2-min intervals over 6 min (4 times 0.25 μl/mg adsorbent) with vigorous stirring using a suspended impeller. The pH in the blocking step was kept between 7 and 8 by titration with 5 *M* NaOH. The reaction was continued for an additional 4 min after the last addition of acetic anhydride. The adsorbent was finally washed with 0.2 *M* sodium acetate solution pH 5 (40 ml/g adsorbent), and stored at +4°C in the same buffer.

2.7. Silane stability

Four different wash sequences with amounts of 0.4 g (dry weight) of the aminopropyl-modified silica supports were investigated: (1) 8×2 ml H_2O wash (no HCl); (2) 8×2 ml 0.1 *M* HCl wash; (3) 4×2 ml H_2O→8×2 ml 0.1 *M* HCl→4×2 ml H_2O wash (same washing procedure as that used for the removal of "uncoupled" silanes in the aqueous silylation method); (4) 4×2 ml H_2O→8×4 ml 0.1 *M* HCl→4×2 ml H_2O wash (double HCl volume).

The loss of silanes in the coupling step was also

studied, by reacting the aminopropyl-modified silica (0.5 g dry weight) in 0.2 *M* potassium-phosphate, pH 7.0 (coupling buffer) with end-over-end mixing at room temperature for 70 h. The supports were then washed on glass sinter funnels with 1.0 *M* NaCl–0.01 *M* Tris pH 7.5 followed by distilled H_2O (2×5 ml each). Washed silica was dried at 110°C overnight, and the amine content measured by the PSA assay and/or titration with HCl.

2.8. Stability of the heparin adsorbents

Samples of the heparin adsorbent (0.5 ml) were stored in duplicate in 0.01 *M* Tris–0.15 *M* NaCl, pH 7.5, or in 0.2 *M* sodium acetate buffer, pH 5.0 at 4°C or 37°C for 2, 4 or 8 weeks. Prior to storage, the adsorbent samples were washed with 0.01 *M* Tris–1.0 *M* NaCl, pH 7.5 followed by storing buffer (2×5 ml each). Following storage, the adsorbent samples were washed immediately with 0.01 *M* Tris–1.0 *M* NaCl, pH 7.5 followed by distilled water (2×5 ml each), then freeze-dried and analysed for heparin content using the indole method.

2.9. Standardisation of adsorbent weights and volumes

Batch adsorption weights

When working with the chemical modification of silica, weights are usually derived in terms of dry mass. In bioaffinity chromatographic applications, such specifications of adsorbent amounts are however impractical, and settled bed volumes are often used instead. In the present investigations, conversion factors between dry adsorbent weight and settled adsorbent volume were required. For dry weight determinations, the total removal of residual liquid was necessary. With native or aminopropyl-derivatised silica, an oven-drying procedure was employed for this purpose. For heparin-affinity adsorbents, freeze-drying was used instead, in order to avoid damaging the ligands.

In batch adsorption studies and column experiments previously performed in this laboratory [30,31], adsorbent amounts were specified in terms of settled adsorbent volume. Since heparin contents were initially expressed in terms of dry adsorbent weight, conversion factors from dry weight to settled adsorbent volumes had to be determined. Furthermore, in the batch adsorption experiments, a determination of the liquid fraction in the settled adsorbent was required. This volume fraction was derived as shown below using values based on the mass per ml of settled adsorbent, namely:

$$f_{liq} = (m_s - m_d)/V_s$$

where f_{liq} is the liquid fraction in settled adsorbent (ml); m_s is the settled adsorbent weight (g); m_d is the dry adsorbent weight (g); and V_s is the settled adsorbent volume (ml).

The ratios of the dry adsorbent weight to settled adsorbent volume were found to be slightly different in the batch adsorption procedure compared to packed bed experiments. Volume standardisations in these two cases are described below. The results are summarised in Table 1.

Table 1
Specific weight–volume relations for LiChroprep Si60, Fractosil 1000 and heparin-Sepharose

Material	Batch adsorption experiments			Column experiments
	m_s/V_s (g/ml)	m_d/V_s (g/ml)	f_{liq} (ml/ml$_s$)	m_d/V_s (g/ml)
LC Si60 (25–40 μm)	1.17±0.03	0.44±0.01	0.73	0.44±0.02
LC Si60 (40–63 μm)	1.15±0.10	0.40±0.03	0.75	0.41±0.01
FS1000 (40–63 μm)	1.28±0.07	0.56±0.05	0.72	0.52±0.10
Commercial hep.-Seph.	1.0	0.036[a]	0.96	0.036[a]

The specific weight–volume relations for different support materials in batch- and column-experiments were determined experimentally as described in Section 2. LC Si60=LiChroprep Si60; FS1000=Fractosil 1000; hep.-Seph.=heparin-Sepharose; m_s/V_s=g settled adsorbent/ml settled adsorbent; m_d/V_s=g dry adsorbent/ml settled adsorbent; f_{liq}=liquid fraction in settled adsorbent; ml$_s$=volume settled adsorbent.
[a] Value based on 27.78 g wet/g dry support.

Batch adsorption volumes

Six aliquots of the heparin-affinity adsorbents with settled volumes in the range 0.050–0.200 ml (determined by a procedure described elsewhere [30]) were transferred into weighing trays and weighed directly (for settled adsorbent weight determinations), or into pre-weighed glass test tubes and dried in an oven at 110°C overnight, which then were weighed again (for dry weight determinations).

Column volumes

Samples of dry support material or heparin adsorbent were weighed and suspended in 0.15 *M* NaCl–0.01 *M* Tris, pH 7.5, then degassed and transferred to a Pharmacia HR 5 column. After settling the material using a flow of 1.0 ml/min for 10 min the settled bed height was measured. Five replicate measurements were made for each adsorbent. The settled support (or heparin adsorbent) volumes were calculated using the column cross-sectional area of 0.196 cm^2.

3. Results and discussion

3.1. Aminopropyl-derivatisation experiments

With a silica-based support material of surface area 500 m^2/g and an anticipated surface coverage of 3.5–4.0 μmol NH_2/m^2, an amino-group content between 1750 and 2000 μmol silane per g dry support was expected to be achieved for the LiChroprep-NH_2. However, as evident from Table 2, the amino-group contents on LiChroprep-NH_2 (25–40 μm and 40–63 μm particle size) were between 727 and 895 μmol NH_2/g dry silica, respectively, determined by HCl titration, or 750 and 914 μmol/g, respectively, as measured with the PSA assay. The amino-group contents were thus considerably lower than expected for a complete modification. The amino-group content on Fractosil 1000, modified under aqueous conditions, (21 μmol NH_2/g support), was, according to the HCl-titration results, similar to the blank values for corresponding non-derivatised support material (18 μmol NH_2/g support). The value for blank LiChroprep Si60, determined by the titration method, was approximately 49 μmol "amino-group" equivalents/g support, and represented only 6.7% of the titrated amine content on the corresponding LiChroprep-NH_2 support material. Since the blank values for the unmodified LiChroprep Si60 and Fractosil 1000 were both zero as determined using the PSA assay, this analytical procedure was more specific than the titration procedure.

Fractosil 1000, surface modified under anhydrous conditions, was found to contain 4.92 and 3.32 μmol NH_2/m^2 as determined with the HCl titration and PSA assay, respectively. This amino-group content was 3 to 4 times higher than that obtained for the support silylated under aqueous conditions (1.05 μmol NH_2/m^2). This result indicated that near maximum possible coverage had been achieved using the anhydrous silylation procedure. As a comparison, Huisden and co-workers [27] reported amino-group contents of 0.13 and 0.10 mmol NH_2/g on APS-modified LiChrospher Si1000 (surface area 30 m^2/g), as determined by HCl titration, and a picric acid assay described by Alpert and Regnier [21]. These values are equivalent to 4.3 and 3.3 μmol NH_2/m^2, respectively, for this 10-μm analytical grade porous silica. As can be seen from the comparative data shown in Table 2, the picric acid assay results of Huisden and co-workers thus corresponded very well with the PSA assay result obtained for the preparative grade Fractosil 1000 aminopropylated under anhydrous conditions in the present investigations (3.32 μmol NH_2/m^2), while titration values for the same material were typically about 50% higher. A similar trend was evident in the studies of Huisden et al. [27]. The titration and PSA-assay values for the NH_2-LiChroprep Si60 and NH_2-Fractosil 1000 modified under aqueous conditions in contrast followed the opposite trend, namely lower values were obtained with the titration procedure. The differences in these results nevertheless fell within the inter-replicate variation of the assays (see Table 2, columns 2 and 4).

As also found by Huisden et al. [27] with LiChrospher Si1000, the amino-group content for the aminopropyl-modified Fractosil 1000 as determined in the present study by HCl titration was actually higher than the theoretical value anticipated for 50% coverage of the available silanols (3.5–4.0 μmol/m^2). This finding, together with the good correlation between the literature data and the PSA-assay result

Table 2
Summary of amino-group contents on LiChroprep-NH_2 and APS-modified Fractosil 1000

Support	HCl titration		PSA assay	
	μmol NH_2/g support	μmol NH_2/m^2	μmol NH_2/g support	μmol NH_2/m^2
LC-NH_2 (25–40 μm)	730±16	1.45	750±30	1.50
LC-NH_2 (40–63 μm)	895±24	1.79	914±62	1.83
APS-FS 1000 (40–63 μm) aqueous silylation	21.0±5.0	1.05	20±2.3	0.98
APS-FS 1000 (40–63 μm) anhydrous silylation	99±3.5	4.92	66.4±7.9	3.32
Blanks:				
LC Si60 (25–40 μm)	48.8	0.1	<0.01	<0.01
FS 1000 (40–63 μm)	17.9	0.9	<0.01	<0.01

The amino-group contents (μmol NH_2 per g support) on commercial LiChroprep-NH_2 and Fractosil 1000 aminopropyl-modified through the aqueous and anhydrous silylation procedures were determined by HCl titration and PSA assay. All measurements were made in duplicate. The μmol NH_2 per g support were converted to μmol NH_2 per m^2 using the specific surface areas 500 m^2/g LiChroprep-NH_2 and 20 m^2/g Fractosil 1000. LC=LiChroprep, FS=Fractosil.

(3.32 μmol NH_2/m^2, or an apparent silylation yield between 83 and 95% of the accessible coverable silanols), suggested that the value obtained with the PSA assay was more reliable.

Determination of amino-group contents by μ-Kjelldahl analysis was also investigated. This analytical procedure was however found to be unsuitable for these investigations, since the values were found to be too close to the lower detection limit.

3.2. Heparin immobilisation

Heparin contents on rigid and soft gel adsorbents are compared in Table 3. Results for two heparin-Sepharose adsorbents, synthesized in this laboratory using analogous methods, are also shown. For these adsorbents, heparin was immobilised through reductive amination onto epoxy-activated Sepharose 4B (HS 4) and CL-4B (HS 5) according to a procedure described by Sasaki et al. [9]. As evident from Table 3, the high heparin content achieved by Sasaki et al. (10 mg/g wet Sepharose) was not reproduced on the soft gel supports using their procedure. The heparin contents on the HS 4 and HS 5 adsorbents were between 20 and 25% of the value reported by Sasaki et al. [9]. Nevertheless, the heparin content on the HS 5 adsorbent was found to be only slightly lower than the heparin content on the commercially available heparin-Sepharose, when the adsorbents were assayed together in the present study. Although the heparin content on the HS 4 adsorbent was actually higher than that of the commercial adsorbent, an adsorbent such as HS 5 would, due to its cross-linked and therefore more rigid nature, be more preferable for chromatographic applications.

Table 3
Heparin contents of the soft gel and rigid adsorbents

Adsorbent	mg H/ml settled adsorbent
Commercial heparin-Sepharose CL-6B	2.5
HS 4 (Sepharose 4B)	3.4
HS 5 (Sepharose CL-4B)	1.8
Heparin-LiChroprep Si60	0.3–8.6
Heparin Fractosil 1000	0.9–7.6
Sasaki et al. [9] (Sepharose 4B)	10

A more detailed summary of heparin contents on the rigid adsorbents is given in Table 4. As evident from this table, the coupling yields (relative to the amount heparin applied) were generally around 10% for the heparin-LiChroprep Si60 adsorbents. Low coupling yields (in the 10% range) have been reported by other investigators for this particular immobilisation chemistry involving non-depolymerised heparin [10]. The coupling yields with the heparin-Fractosil 1000 adsorbents were even lower, typically about one tenth of the coupling yields achieved with the heparin-LiChroprep Si60 adsorbents. The coupling yields based on aminopropyl–ligand densities were in the range 0.01% to 0.21% for the different heparin-LiChroprep Si60 adsorbents, as seen in column 5 of Table 4. These data demon-

Table 4
Heparin contents and coupling yields on heparin-LiChroprep Si60 and heparin-Fractosil 1000 adsorbents

Adsorbent	Heparin applied (mg/g dry adsorbent)	Heparin coupled (mg/g dry adsorbent)	Coupling yield (%)	
			(a)	(b)
HLC 19	60	3.73 (±26%)	6.2	0.04
HLC 20	60	4.21 (±11%)	7.0	0.03
HLC 21a	8.93	0.67 (±22%)	7.5	0.01
HLC 21b	26.8	2.54 (±8%)	9.5	0.03
HLC 21c	53.6	5.83 (±7%)	10.9	0.06
HLC 21d	214	19.5 (±10%)	9.1	0.20
HLC 21e	54.8	5.86	10.7	0.06
HLC 22a	9.09	1.00	11.0	0.01
HLC 22b	27.3	3.49	12.8	0.03
HLC 22c	54.5	7.42	13.6	0.06
HLC 22d	218	23.8	10.9	0.20
HLC 23a	9.09	1.1	12.1	0.01
HLC 23b	27.3	3.40	12.4	0.03
HLC 23c	54.5	6.67	12.2	0.06
HLC 23d	218	25.8	11.8	0.21
HLC 24	31.8	6.18	19.4	0.05
HFS 1	54	1.60 (±2.5%)	3.0	N.D.
HFS 4	222	4.77 (±1.7%)	2.2	
HFS 2	500	8.75 (±1.2%)	1.8	
HFS 3	1000	13.6 (±4.8%)	1.4	
HFS 5	54	1.82	3.4	N.D.
HFS 6	222	4.13	1.9	
HFS 7	500	7.01	1.4	
HFS 8	1000	12.9	1.3	
HFS 13	500	6.98	1.4	N.D.
HFS 14	1000	12.7	1.3	
HFS 27	500	6.52	1.3	
HFS 28	500	6.22	1.2	

Heparin contents were determined through the indole assay. HLC 19 and HLC 21 are adsorbents with 25–40 μm particle size, while HLC 20, HLC 22, HLC 23, HLC 24 and the HFS adsorbents have the particle size 40–63 μm. Deviations (SEM) have been shown in cases where heparin contents have been determined by repetitive indole assays. Other values represent the average of duplicate measurements. Coupling yields are shown based either on the amount of heparin applied (a), or on the amino ligand density (b). Coupling efficiencies based on amino-ligand density have been calculated using the amino contents in Table 1, and heparin's average molecular mass: 13 500 g/mol. HLC=heparin-LiChroprep Si60; HFS=heparin-Fractosil 1000. N.D not determined.

strate how, due to the small pore size, very little of the pore-surface area of the mesoporous LiChroprep Si60 is utilised for immobilisation of heparin.

3.3. Hydrolytic stability of the aminopropylsilicas

The γ-aminopropyltriethoxysilane APS has a tendency to chemisorb to silica surfaces without being covalently bound. The presence of the free γ-aminopropyltriethoxysilane in the wash effluents following modification of the silica could be eliminated by washing the modified support with 0.1 *M* HCl. This free silane was initially believed to be non-covalently adsorbed to the silica (probably through hydrophobic interactions), and released under aqueous conditions. However, aminopropylated silica which initially was entirely devoid of the free aminopropylsilyl groups following the acidic wash sequence, again displayed free aminopropylsilyl groups in wash effluents after being stored in distilled water. This result suggests that covalently bound aminopropylsilyl groups had been released during the storage. The hydrolysis of this aminopropylsilyl-modified support could potentially have a detrimental effect on the chromatographic performance of these heparin-silica adsorbents by facilitating the release of ligands and

causing an increase in free silanols. Hydrolytic instability of APS-modified silica has been observed by other investigators [19]. By functioning as a base, the immobilised amino group is capable of generating near to the silica surface a micro-environment with high pH, leading to the possibility of autohydrolysis of the Si–O–Si bond under aqueous conditions. This possibility was highlighted in the present studies when the heparin contents on the adsorbents were analysed. Fractosil 1000, aminopropyl-modified under anhydrous conditions, had an amino-group content very close to the theoretical silylation optimum for the surface modification. Nevertheless, following coupling of the ligand, the heparin adsorbents based on Fractosil 1000 aminopropyl-modified under anhydrous conditions had heparin contents almost identical to those obtained with the support material amino-modified under aqueous conditions (see Table 2). This result could either imply that a limiting ligand density, independent of aminopropylsilyl content existed, or that the majority of aminopropylsilyl groups attached to the support material under anhydrous conditions had been hydrolytically detached during the coupling and washing steps. This hydrolysis concept was tested by subjecting aminopropyl-modified silica either to combinations of H_2O and HCl washes or to coupling conditions, then drying the APS-modified supports and measuring the amino-group content as described in Section 2. The data generated in this study are summarised in Table 5 in terms of the μmol NH_2/g silica, the μmol NH_2/m^2, and the % NH_2 retained on the adsorbents after the washing procedures.

As evident from these data, the amino-group loss was negligible with Fractosil 1000 aminopropyl-modified under aqueous conditions, whether the surface-derivatised silica was subjected to H_2O and/or HCl washes or to the coupling conditions. However, Fractosil 1000 aminopropyl-modified under anhydrous conditions, and the commercial LiChroprep-NH_2 both displayed significant aminopropylsilyl-group losses on washing or when subjected to the buffer employed as the coupling conditions. Washing the NH_2-Fractosil 1000 with distilled water resulted in a loss of approximately 30% of the initial amino-group content. Since washing with HCl only resulted in a 14% decrease in amino-group content (extended HCl washing did not increase the losses significantly) the larger decrease in amino-group content caused by the H_2O wash conditions was apparently due to greater hydrolysis. Quantitation of the amino-group content using the PSA assay indicated that the H_2O–HCl wash sequence did not release more aminopropylsilyl groups than the wash with distilled water alone. LiChroprep-NH_2 on the other hand, was not significantly affected by either the H_2O or HCl wash, but lost a similar aminopropylsilyl-group proportion as NH_2-Fractosil 1000, modified under anhydrous conditions, when subjected to the mixed H_2O/HCl wash sequence.

The NH_2-Fractosil 1000 supports, prepared by silylation under anhydrous conditions, lost between 42 and 46% of their initial amino-group content when subjected to the heparin coupling conditions. This effect resulted in a final amino-group content similar to that of the NH_2-Fractosil 1000 amino-modified under aqueous conditions. This trend provides an explanation for the similar heparin contents obtained with these two types of adsorbents. Based on PSA-assay values, commercial LiChroprep-NH_2 was not affected by the coupling conditions to the same extent as the NH_2-Fractosil 1000 aminopropyl-modified under anhydrous conditions, since only ca. 20% of the initial amino-group content on the commercial LiChroprep-NH_2 was lost. As noted by other investigators [12,18,19,32], the efficiency of the surface modification is dependent on the composition of vicinal, geminal and isolated silanols on the silica surface. Taken together with the results of the present investigations, the conclusion can be reached that the modification reaction with APS results in the introduction of aminopropylsilyl groups bound to the silica surface with a range of different binding strengths. The experimental results implicate the Fractosil 1000 surface having a lower abundance of binding sites associated with "hard" type A silica coverage, i.e., fewer of the stable Si–O–Si bonds derived from the more readily modified geminal silanols than the LiChroprep Si60. Such behaviour is consistent with the type B silica structure of this support material [32].

3.4. Differences between PSA-assay and HCl-titration values

As is clearly seen in Table 5, differences between the PSA-assay and HCl-titration values for the amino-group contents of the supports were observed

Table 5
Amino-group contents on NH_2-Fractosil 1000 and LiChroprep-NH_2—stability study

Treatment	μmol NH_2/g silica	μmol NH_2/m2	% NH_2 retained
NH_2-Fractosil 1000 (40–63 μm), aqueous silylation conditions			
None	19.7±2.3 (21.0±5.0)	0.98±0.11 (1.05±0.25)	100 (100)
H_2O wash (1)	– (24.1±0.2)	– (1.20±0.01)	– (100)
H_2O/HCl wash (3)	20.4±0.6 (19.1±0.5)	1.02±0.03 (0.96±0.03)	100 (91.4)
Coupling condition	20.4±0.6 (22.3±0.3)	1.02±0.03 (1.12±0.02)	100 (100)
NH_2-Fractosil 1000 (40–63 μm), anhydrous silylation conditions			
None	66.4±7.9 (98.5±3.5)	3.32±0.40(4.92±0.17)	100 (100)
H_2O wash (1)	49.8±3.8 (66.6±2.0)	2.49±0.19 (3.33±0.10)	75.1 (67.7)
HCl wash (2)	57.2±1.9 (–)	2.86±0.10 (–)	86.2 (–)
H_2O/HCl wash (3)	49.9±6.2 (27.2±0.9)	2.50±0.31 (1.36±0.04)	75.2 (27.6)
H_2O/2×HCl (4)	– (26.3±0.2)	– (1.31±0.01)	– (26.6)
Coupling condition	30.6±2.2 (41.2±1.2)	1.53±0.11 (2.06±0.06)	46.1 (41.9)
Commercial LiChroprep-NH_2 (40–63 μm)			
None	914.5±62.3 (895.4±24)	1.83±0.12 (1.79±0.05)	100 (100)
H_2O wash (1)	885.4±49.9 (803.6±4.0)	1.77±0.10 (1.61±0.10)	96.8 (89.9)
HCl wash (2)	897.6±125 (–)	1.80±0.25 (–)	98.1 (–)
H_2O/HCl wash (3)	731.7±75 (284.6±10)	1.46±0.15 (0.57±0.02)	80.0 (31.8)
Coupling condition	724.0±37 (587.9±20)	1.45±0.07 (1.18±0.04)	79.2 (65.7)
Commercial LiChroprep–NH_2 (25–40 μm)			
None	750.0±30.0 (727.0±16)	1.50±0.06 (1.45±0.03)	98.5 (–)
H_2O wash (1)	738.7±22.7 (–)	1.48±0.04 (–)	100 (–)
HCl wash (2)	758.7±68.7 (–)	1.52±0.14 (–)	100 (100)
Non-derivatised silica (blanks)[a]			
Support	μmol NH_2/g silica	μmol NH_2/m^2	
Fractosil 1000	0.0 (17.9±0.03)	0.0 (0.89±0.002)	
LiChroprep Si60	0.0 (48.8±0.4)	0.0 (0.10±0.001)	

The amino-group contents on the aminopropyl-derivatised Fractosil 1000 (aqueous and anhydrous silylation conditions) and commercial LiChroprep-NH_2 were determined using the PSA assay or HCl titration. Values in brackets represent HCl titrations. All measurements were made in duplicate. The μmol NH_2 per g support has been converted to μmol NH_2 per m^2 using the specific surface areas 500 m^2/g LiChroprep-NH_2 and 20 m^2/g Fractosil 1000. The decrease in amino-group contents by submitting the supports to four different wash sequences ((1)→(4)) or incubation under coupling conditions (as described in the text) was studied. The remaining amino-group contents, "% NH_2 retained" are calculated relative to the contents on untreated supports.

[a] Background measurement for the naked silicas expressed as apparent NH_2 equivalents.

following HCl washes. When the aminopropyl-modified support samples are suspended in 4 *M* NaCl used in the HCl-titration procedure, due to the participation of an ion-exchange process involving the protonated amino group and the chloride ion under these conditions, any hydroxide ions thus formed will result in a pH shift, and be titrated with the HCl. If the support has been washed with HCl alone, chloride ions can remain chemisorbed on the support. The outcome of this process will be an overestimate of the amino-group content determined for the HCl-washed relative to the non-acid washed adsorbents. As a consequence, the PSA-assay procedure was considered to be the more reliable assay.

3.5. Stability of blocked aminopropyl-modified silicas

Following the immobilisation of heparin onto an aminopropylated support, a complete and stable blocking of any remaining unreacted amino groups was important in order to maintain a high performance of the affinity adsorbent. Free amino groups would give rise to non-specific adsorption sites, and could result in the earlier-mentioned high-pH microenvironment, which would indirectly lead to the release of immobilised heparin. In the course of these experiments, leakage of acetyl groups introduced to block the unreacted amino groups was

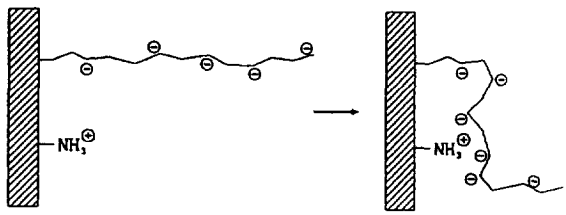

Fig. 2. Schematic illustration of the possible types of ionic interaction between heparin and free amino groups on the surface of an aminpropyl-modified silica support material.

observed using the qualitative TNBS test upon storage of the heparin adsorbents in aqueous buffers. Thus, a heparin-LiChroprep-NH_2 adsorbent, which initially contained only blocked amino groups, tested positive for amino group after four months of storage in 0.01 *M* Tris–0.15 *M* NaCl, pH 7.5 at 4°C. During this time period, the heparin content decreased from 2.08 to 1.54 mg heparin/g moist adsorbent.

This hydrolysis of acetamido bonds would yield exposed amino groups on the support surface. Such exposed amino groups, apart from mediating further hydrolysis of the Si–O–Si bonds, could potentially bind ionically to immobilised heparin molecules, thereby decreasing the accessibility of these ligands for protein interactions. This effect would be of a particular importance in the case of sequence-specific interactions such as the binding of antithrombin III to heparin, but would also occur to a lesser extent with the non-specific ionic heparin–thrombin interaction. A schematic representation of this concept is shown in Fig. 2.

3.6. Stability of heparin adsorbents

The hydrolytic stability of heparin-LiChroprep Si60 adsorbents, and heparin-Fractosil 1000 adsorbents amino-modified under anhydrous or aqueous conditions, were studied as described in Section 2. The stabilities of the LiChroprep Si60 and Fractosil 1000 based adsorbents are shown in Table 6 and Table 7, respectively.

Heparin-LiChroprep Si60 adsorbents

As seen from Table 6, the data indicate that storage in 0.2 *M* sodium acetate buffer, pH 5 is preferred with the heparin silicas. For example, when stored in 0.01 *M* Tris–0.15 *M* NaCl, pH 7.5 at 37°C, the heparin content on the HLC 24 adsorbent decreased by 32% from 6.5 to 4.4 mg heparin/g adsorbent during the first two weeks, whilst only ca. 30% of the original content remained after two months storage (see Table 6, column 2). In contrast, the heparin content of this adsorbent had decreased by 32% after two months when stored in 0.2 *M* sodium acetate at pH 5 (see Table 6b, column 3).

Heparin-Fractosil 1000 adsorbents

As seen from Table 7, the heparin content on HFS 13 (an adsorbent based on Fractosil 1000, aminopropyl-modified under anhydrous conditions) de-

Table 6
Hydrolytic stability of heparin-LiChroprep Si60

Incubation time (weeks)	pH 7.5	pH 5	
	37°C	37°C	4°C
(a) Retained heparin content (mg H/g dry adsorbent)			
2	4.4 (±3.1%)	5.1 (±3.1%)	–
4	3.5 (±1.4%)	4.2 (±2.4%)	5.3 (±3.9%)
8	2.2 (±5.2%)	4.4 (±0.1%)	6.2 (±2.3%)
(b) Percentage of heparin retained			
2	67.7	78.5	–
4	53.8	64.6	81.5
8	33.8	67.7	95.4

Aliquots of the HLC 24 adsorbent (heparin-LiChroprep Si60, 40–63 μm) were stored at pH 5 and 4 or 37°C, or at pH 7.5 and 37°C, then washed, freeze-dried and assayed in duplicate samples for heparin content as described in the text. The initial heparin content on this adsorbent was 6.5 mg H/g dry adsorbent. In (a) the retained heparin contents (mg H/g dry adsorbent) following incubation are shown; (b) shows % heparin retained relative to the initial content.

Table 7
Stability of heparin-Fractosil 1000: anhydrous versus aqueous amino-modified NH_2-Fractosil 1000

Storage time (weeks)	Anhydrous modification		Aqueous modification	
	37°C	4°C	37°C	4°C
(a) Retained heparin content (mg heparin/g dry adsorbent)				
4	5.29	–	4.95	–
8	4.27 (6.38)	7.35	3.79	6.00 (7.95)
(b) % heparin retained				
4	66.5	–	79.6	–
8	53.6[a] (51.0)	92.3	60.9[b]	96.5 (98.7)

Aliquots of "anhydrous" (HFS 13 and HFS 14) and "aqueous" (HFS 26 and HFS 28) adsorbents were stored at 4 or 37°C, then washed, freeze-dried and assayed for heparin content as described in the text. Initial heparin contents on these adsorbents were 7.96, 12.51, 8.05 and 6.22 mg H/g dry adsorbent for HFS 13, HFS 14, HFS 26 and HFS 28 respectively. Values in parentheses represent results with the "anhydrous" HFS 14 and the "aqueous" HFS 26 adsorbents.

[a] 80.6% of value at 4 weeks.

[b] 76.5% of value at 4 weeks.

creased by only 8% to 92% of the initial value over a two-month period at 4°C. Assuming a linear rate of ligand leakage, the decrease to 67% in 1 month at 37°C would, based on this value, simulate storing at 4°C for 8.7 months, whilst the decrease to 54% over 2 months at 37°C would simulate storing at 4°C for 12 months. Moreover, the HFS 26 and HFS 28 adsorbents (based on Fractosil 1000, surface-modified under aqueous conditions), only lost 1.3% and 3.5% of their respective heparin contents over a two-month period of storage at 4°C. The heparin-Fractosil 1000 adsorbents based on aqueous amino-propyl modifications were therefore hydrolytically more stable than adsorbents based on Fractosil 1000 modified under anhydrous conditions. It was possible that this higher stability was achieved through a more extensive cross-linkage (polymerisation) of the aminopropylsilyl groups occurring during the aqueous silylation procedure.

In the present study, a sample of the HFS 28 adsorbent was also stored at 37°C for eight weeks with 0.5% azide added to the buffer. Under these conditions, 64% of the initial heparin content remained at the end of the storage period (equivalent to 81% of the value at four weeks), whereas ca. 60% remained under similar conditions without azide present. The difference in amount of the remaining ligand with/without azide was thus only small compared to the overall decrease in heparin content under these storage conditions. This result indicated that heparin losses were not caused by bacterial degradation.

3.7. Geometric considerations

Pore size versus protein diameter

The choice of chromatographic support material, with regard to pore size, is largely determined by the chromatographic application. Thus, the modified mesoporous LiChroprep Si60 (pore size 60 Å) would be more suitable for small polypeptides capable of binding heparin, such as fibroblast growth factor (FGF), whereas the pores of the modified macroporous Fractosil 1000 (pore size 1000 Å) will accommodate to a greater extent larger molecules such as the heparin binding proteins thrombin or antithrombin III. In order to provide estimates of the geometrical relationship between protein diameters and adsorbent pore sizes, the formula derived by Janin [33] was used, namely, $A=11.1\ M^{2/3}$, where A=accessible surface area in $Å^2$ for monomeric globular proteins, and M=the molecular mass of the protein. Using this formula, and the area of a sphere ($A=4\ \pi r^2$), the following conversion from molecular mass to protein diameter (D) could be derived:

$$D = \left[2\sqrt{\frac{11.1\ M^{2/3}}{4\pi}} \right]$$

The validity of this expression was investigated by comparing the formula-derived diameters with literature data [34] for a number of proteins of known molecular masses. Overall, the predicted values corresponded well for globular proteins up to an approximate molecular mass of 200 000. For example, the formula-derived diameter of human α-thrombin (a globular protein with M_r=36 600 [35]) was 62 Å, which coincided relatively well with the average diameter of 45 Å arrived at through X-ray crystallographic studies [36]. These values suggested that protein molecules of this size would largely span the cross-sectional distances of the 60-Å pores in heparin-LiChroprep Si60 adsorbents, and that consequently the protein–ligand interactions would be

restricted to the outer surface area of the silica particles. The small pore size will furthermore have a considerable influence on the heparin immobilisation. The calculated size proportions on a heparin-LiChroprep Si60 adsorbent suggested that such mesoporous adsorbents would perform virtually in a non-porous manner, with adsorption capacities much lower than for macroporous adsorbents. On a heparin-Fractosil 1000 adsorbent, however, the possibility of accommodating both heparin ligands and proteins within the 1000-Å pores would be much greater, as shown in Fig. 3. Even if the end-point-attached heparin molecules were fully extended, the longest heparin species would not quite reach to the centre of the pores. The access to more deep-lying cavities in the pore network would nevertheless be restricted if the ligand densities are high.

3.8. Ligand densities on heparin-LiChroprep Si60 adsorbents

If it was assumed that the total surface area of the preparative LiChroprep-NH_2 (25–40 μm particle size) was entirely accessible to the heparin molecules, a 1:1 molar heparin/amino-group coupling ratio could theoretically be achieved. This conclusion is based on the following considerations. Firstly, from the X-ray crystallographic data presented by Nieduszynski et al. [37], the cross-sectional area of the heparin molecule was determined to be $1.15 \cdot 10^{-18}$ m^2, which in the present calculation was chosen to represent the area on the silica surface which is shielded by an end-point-attached heparin molecule. Secondly, with a measured amino-group content on LiChroprep-NH_2 (25–40 μm) of 727 μmol NH_2/g support, and the total surface area of 500 m^2/g on this silica, an average surface area of $1.14 \cdot 10^{-18}$ m^2 per amino group can be derived. If every aminopropyl group was accessible to react with the heparin ligands, it follows that theoretically between 3.6 and 21 g heparin/g adsorbent (based on the molecular mass range of heparin, and accessibility to the total surface area) could be immobilised, which clearly are experimentally unachievable values. In reality, due to the small pore size of LiChroprep Si60, it is most unlikely that the heparin ligands would be coupled to any significant extent inside the pores, since the lengths of the heparin molecules used in the present investigations (M_r 5000–30 000) would be in the range 64–420 Å (based on a 0.84-nm disaccharide repeat in the heparin molecule [38]). The measured heparin contents on the heparin-LiChroprep Si60 adsorbents were found to be between 0.6 and 26 mg/g adsorbent, values which are approximately 1000-fold lower than the theoretically derived values for an equimolar heparin–aminopropyl group coupling yield.

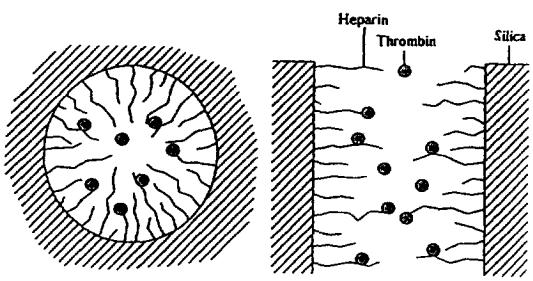

Fig. 3. Relative size proportions on a heparin-Fractosil 1000 adsorbent.

Based on the assumption that only the outer surface area (OSA) of the LiChroprep Si60 particles is accessible for coupling to heparin, predictions of the coupled heparin contents (see Table 9), and the percentile ligand coverages (see Table 10), with the LiChroprep Si60 adsorbents could be made using values calculated for the accessible outer surface areas of the particles. The percentile ligand coverages represent the fraction of the total surface area which is shielded by end-point-attached heparin ligands. As shown in Table 8, the outer surface area of LiChroprep Si60 (approximately 0.1% and 0.05% for the 25–40 μm and 40–63 μm particles, respectively), only constitutes a small fraction of the total surface area of the particle. In Table 9, the predicted heparin contents are given based either on heparin's average molecular mass of 13 500 g/mol [13] or on the molecular mass range of 5000–30 000 g/mol. These values thus represent maximum possible heparin contents, assuming a 1:1 molar ratio of heparin/NH_2 content immobilised onto the outer surface area.

Comparison of the theoretical values (Table 9) with the measured heparin contents (given in column 3, Table 10a) reveal a good correlation for the 25–40

Table 8
Calculation of outer surface area (OSA) on LiChroprep Si60

Particle size (μm)	Average diameter[a] (cm)	Volume per particle[b] (ml)	Particles per ml support[c]	OSA per particle (cm^2)[d]	OSA per ml settled support (cm^2/ml)	ml of settled support per g dry support	OSA per g dry support (m^2/g)
25–40	$25 \cdot 10^{-4}$	$2.97 \cdot 10^{-9}$	$2.02 \cdot 10^{8}$	$12.4 \cdot 10^{-6}$	2507	2.273	0.570
40–63	$62 \cdot 10^{-4}$	$45.6 \cdot 10^{-9}$	$0.13 \cdot 10^{8}$	$76.6 \cdot 10^{-6}$	996	2.475	0.246

Theoretical values for the total accessible outer surface areas on LiChroprep Si60 particles of 25–40 and 40–63 μm particle size relative to the heparin and protein molecules.

[a] Values for average particle diameters were obtained by particle counting.

[b] Due to the irregular shapes of the LiChroprep Si60 particles, the volume estimations have been made by treating the particles as cubes with the space diagonal representing the particle diameter d. The side of a cube$=h$, where $h=d/\sqrt{3}$, and the volume of this "particle"$=h^3$.

[c] The number of particles per ml settled support material were determined as 0.6/(particle volume) based on the approximation that a densely packed bed of irregular non-porous silica particles contains approx. 60% solid silica.

[d] The OSA per particle is given by: $6 \times (d/\sqrt{3})^2 = 2d^2$.

Table 9
Predicted heparin content on heparin-LiChroprep Si60

Particle size (μm)	μmol NH_2/g support	μmol NH_2/m^2	μmol NH2 on OSA per g support[a]	mg heparin/g adsorbent[b]	
				(1)	(2)
25–40	730	1.45	0.83	11.2	4.14–24.9
40–63	895	1.79	0.44	5.9	2.20–13.2

Theoretical values for the heparin content with heparin immobilised on the outer surface area (OSA) of LiChroprep Si60 particles (25–40 and 40–63 μm particle size), based on the measured amine content on LiChroprep-NH_2 and the calculations of total accessible outer surface area as described in Table 8. The numbers used in these calculations have been explained below the table. Values were calculated, firstly based on the average molecular mass 13 500 g/mol for heparin in the 5000–30 000 molecular mass range, secondly using the whole range.
[a] (μmol NH_2/m^2)/(m^2 OSA/g support). If a 1:1 molar ratio heparin/NH_2 is assumed, this equals μmol heparin on OSA/g adsorbent.
[b] (μmol NH_2 on OSA/g support)×($M_{r\ (heparin)}$/1000).
(1) Based on the $M_{r\ (heparin)}$=13 500 g/mol (average value).
(2) Based on the $M_{r\ (heparin)}$=5000–30 000 g/mol (M_r range).

μm particles. Thus, the highest heparin content experimentally obtained was very close to the highest predicted value. In a protein adsorption system, pore diffusion effects with the heparin-immobilised LiChroprep Si60 would therefore not be expected to be involved, not only because most protein molecules would normally be too large to penetrate the pores, but also because the length of the heparin molecules themselves was greater than the 60-Å pore size.

The average distribution of heparin on the silica was determined by calculating the percentile ligand coverage on the outer surface of the heparin-LiChroprep Si60 particles. These computations are presented in Table 10. Obviously, values above 100% are not feasible for monolayer coverage (see for example the values for HLC 21d, 23d, 22c, 22d and 24). Such theoretical values could be expected to deviate from experimental values, since the ligand density and coverage calculations rely on the following assumptions: (a) average values for particle sizes reflect the true size distribution; (b) a simplified geometry used in the calculation of outer surface area; (c) the proportion of outer surface area occupied by pore openings neglected; (d) an even distribution of the molecular mass species of coupled heparin; and (e) the heparin was exclusively immobilised in a directed manner (end-point attached).

Significant deviations from the theoretical model in regard to one or more of these constraints are likely to result in a decreased correlation between the theoretical and experimental data. For example, if the size distribution of the heparin is shifted towards longer heparin chains, i.e. the average molecular mass of heparin is higher than 13 500, the calculated coverage may reach a value higher than 100%. Calculated coverages higher than 100% are also demonstrated by the values for μmol heparin/m^2 on OSA. These values should reasonably not be higher than the amino-group coverages (μmol/m^2) shown in Table 9, assuming the amines are evenly distributed on the silica surface.

Percentile coverages were also calculated using the lower (M_r 5000) and upper (M_r 30 000) limits of the heparin molecular mass range. Although these coverage ranges, as seen in Table 10b, also included values far higher than 100%, the lower end of the ranges may provide more useful information. Thus, in the case of the HLC 24 adsorbent, this calculation suggests that the ligand coverage would under any circumstances be no less than 55%. Still, even with this conservative value, it is apparent that close to the support surface, the ligand density on the HLC 24 adsorbent would be high. The accessibility of solutes for binding sites on the ligands would therefore most likely be sterically restricted. In contrast, the conservative value for heparin coverage on the HLC 21a adsorbent was 3%. This lower coverage would fall below the maximum sterically possible binding of a protein such as thrombin. A

Table 10
Calculated ligand coverage (%) on OSA of LiChroprep Si60

Particle size (μm)	Adsorbent	mg Heparin per g adsorbent (measured)	μmol Heparin per m^2 on OSA[a]	Ligand coverage[b] (%)
(a) Average percentile ligand coverage [$M_{r\ (heparin)}$=13 500 g/mol (average M_r)]				
25–40	HLC 21a	0.67	0.087	6.0
	HLC 21b	2.54	0.330	23
	HLC 21c	5.83	0.757	52
	HLC 21d	19.5	2.54	176
40–63	HLC 22a	1.00	0.301	21
	HLC 22b	3.49	1.05	73
	HLC 22c	7.42	2.23	154
	HLC 22d	23.8	7.17	497
	HLC 23a	1.10	0.331	23
	HLC 23b	3.40	1.02	71
	HLC 23c	6.67	2.02	140
	HLC 23d	25.8	7.77	538
	HLC 24	5.83	1.76	122
(b) Possible ranges of percentile ligand coverages ($M_{r\ (heparin)}$=5000–30 000 g/mol)				
25–40	HLC 21a	0.67	0.039–0.235	3–16
	HLC 21b	2.54	0.148–0.891	10–62
	HLC 21c	5.83	0.341–2.05	24–142
	HLC 21d	19.5	1.14–6.84	176–474
40–63	HLC 22a	1.00	0.134–0.813	9–56
	HLC 22b	3.49	0.476–2.84	33–196
	HLC 22c	7.42	1.00–6.02	69–416
	HLC 22d	23.8	3.22–19.3	223–1334
	HLC 23a	1.10	0.149–0.894	10–62
	HLC 23b	3.40	0.461–2.76	32–191
	HLC 23c	6.67	0.908–5.45	63–377
	HLC 23d	25.8	3.49–21.0	242–1454
	HLC 24	5.83	0.789–4.76	55–330

Theoretical values for the average percentile ligand coverage, using the average molecular mass 13 500 g/mol for heparin in the 5000–30 000 molecular mass range (a), or the possible ranges of percentile ligand coverage, using the whole molecular mass range for heparin (b), were calculated for heparin immobilised on the outer surface area (OSA) of LiChroprep Si60 particles (25–40 and 40–63 μm particle size). The calculations were based on the measured heparin contents on a range of heparin-LiChroprep Si60 adsorbents, and the calculations of total accessible outer surface area as described in Table 8.

[a] (mg heparin/g adsorbent)·(1000/$M_{r\ (heparin)}$)/(m^2 OSA/g support).

[b] μmol heparin/$m^2 \cdot 10^{-6} \cdot N_A \cdot 1.15 \cdot 10^{-18} \cdot 100$, where: $N_A = 6.022 \cdot 10^{23}$ (Avogadro's number) and $1.15 \cdot 10^{-18}$=approximate cross-section area of the heparin molecule (m^2).

correlation between heparin content and thrombin binding capacity has been demonstrated through batch adsorption studies with these adsorbents [30].

3.9. Ligand densities on heparin-Fractosil 1000 adsorbents

With a surface area of 20 m^2/g, and an amino-propyl-group content of approximately 20 μmol/g support material, 1 μmol heparin/m^2 would be theoretically expected from a 1:1 molar heparin/NH_2 coupling ratio with the heparin-Fractosil 1000 adsorbent. The experimental coupling yields with this silica material were however found to represent far lower coupling ratios. This result is demonstrated in Table 11, where the measured heparin contents of the heparin-Fractosil 1000 adsorbents have been converted to μmol heparin/m^2. As an example, the

Table 11
Calculated ligand coverage (%) on heparin-Fractosil 1000

Adsorbent	mg Heparin per g adsorbent	μmol Heparin per m^2 [a]	Ligand coverage[b](%)
(a) Average percentile ligand coverage [$M_{r\ (heparin)}$=13 500 g/mol (av. M_r)]			
HFS 1	1.60	0.006	0.4
HFS 4	4.77	0.018	1.2
HFS 2	8.75	0.032	2.2
HFS 3	13.6	0.050	3.5
(b) Possible ranges of ligand coverage ($M_{r\ (heparin)}$=5000–30 000 g/mol)			
HFS 1	1.60	0.003–0.016	0.2–1.1
HFS 4	4.77	0.008–0.048	0.6–3.3
HFS 2	8.75	0.015–0.087	1.0–6.1
HFS 3	13.6	0.023–0.136	1.6–9.4

Theoretical values for the average percentile ligand coverage ant possible ranges of percentile ligand coverage with heparin immobilised on the outer surface area (OSA) of Fractosil 1000 particles (40–63 μm particle size) were made, based on the measured heparin contents on a range of heparin-Fractosil adsorbents, and the specific surface area of 20 m^2/g Fractosil 1000 surface area. Calculations were made based on the average molecular weight 13 500 g/mol for heparin in the 5000–30 000 molecular weight range (a), or based on the whole molecular weight range (b)

[a] (mg heparin/g adsorbent)·(1000/$M_{r\ (heparin)}$)/(20 m^2/g silica).

[b] μmol heparin/m^2·10^{-6}·N_A·1.15·10^{-18}. Where N_A=6.022·10^{23} (Avogadro's number), and 1.15·10^{-18}=approx. cross-section area of the heparin molecule (m^2).

measured heparin content on the HFS 3 adsorbent reflected a ligand coverage of at the most 0.136 μmol heparin/m^2 (see Table 11b, column 3).

The calculated values for percentile ligand coverages revealed major differences between the heparin-Fractosil 1000 and heparin-LiChroprep Si60 adsorbents. Taking HFS 2 as an example, the measured heparin content was 8.75 mg/g adsorbent. Taking into account the M_r range of heparin (see Table 11b), and assuming an even distribution of heparin ligands on the total surface area (20 m^2/g silica) a molar coverage between 0.015 and 0.087 μmol heparin/m^2 is derived, which is equivalent to a coverage between 1.0 and 6.1%. As shown previously, heparin-LiChroprep Si60 adsorbents with similar heparin contents in terms of mg/g adsorbent had much greater ligand densities. As an example, the HLC 22c adsorbent, containing 7.42 mg heparin/g adsorbent, had a percentile ligand coverage of at least 69% (see Table 10b). The binding-site accessibility for proteins on the heparin ligands would thus be greater on the heparin-Fractosil 1000 adsorbents than on corresponding heparin-LiChroprep Si60 adsorbents, and consequently higher capacities would also be anticipated. The proposed difference in ligand density has

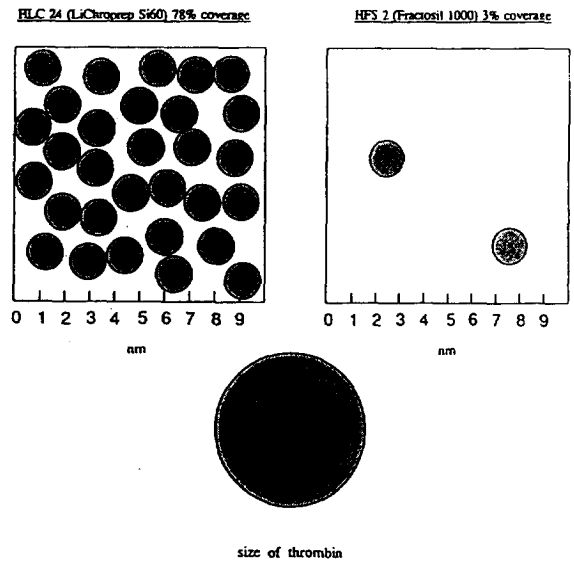

Fig. 4. Schematic representation of the average ligand coverages of heparin immobilised onto LiChroprep Si60 and Fractosil 1000 sorbents (40–63 μm particle size), as shown in Table 10 and Table 11. Circles represent cross-section areas of heparin end-point attached to the adsorbent surface as viewed at a 90° angle relative to the surface.

been represented schematically as spatial ligand distributions in Fig. 4.

4. Conclusions

As documented in this study, it is possible to generate monolayer-coverage heparin-affinity adsorbents by immobilising heparin via its reducing end through reductive amination procedures onto aminopropylated silica-based support materials. Through this approach, the heparin is coupled in a directed manner, thereby promoting the accessibility of binding sites on the heparin molecule to protein solutes in bio-affinity applications. This principle has been explored by other investigators for the synthesis of soft gel heparin adsorbents and rigid polymer-coated heparin adsorbents. In the present investigations, the strategy of immobilising heparin directly onto aminopropylated silica materials was established.

The silica materials were aminopropyl-modified using γ-aminopropyltriethoxy silane, either under aqueous or anhydrous conditions. These two approaches yielded radically different chemically modified silicas with different amino-group contents. Typically, with the Fractosil 1000, the anhydrous procedure yielded aminopropylsilyl coverages between 3.3 and 4.9 μmol NH_2/m^2, whilst the reaction under aqueous conditions only yielded 0.98 to 1.05 μmol NH_2/m^2. The more extensive modification achieved under anhydrous conditions represented an aminopropylsilyl coverage close to values expected for an optimal monomeric silylation reaction. The major proportion of the aminopropylsilane coupled under anhydrous conditions was however found to be unstable in an aqueous environment. When Fractosil 1000 which had been aminopropylated under anhydrous conditions was stored in a buffer mimicking the heparin coupling conditions, the amino-group content was reduced to nearly the same levels as those obtained using the aqueous silylation procedure. Consequently, heparin was coupled onto aminopropyl-derivatised Fractosil 1000, similar heparin contents were obtained regardless which aminopropylation procedure had been employed. These investigations suggested that hydrolysis may have been occurring with the heparin-modified LiChroprep-NH_2 and NH_2-Fractosil 1000 adsorbents. The higher stability of the Fractosil 1000 adsorbent, which was aminopropyl-silylated in an aqueous environment, was probably due to cross-linking of aminopropylsilane occurring under these conditions [18]. The more extensive reduction in amino-group content on the aminopropylated Fractosil 1000 may however also have been due to non-covalently chemisorbed APS silane leaching off the support. Since the loss of aminopropyl groups was also observed on commercial LiChroprep-NH_2, the hydrolysis phenomenon was not unique to supports synthesised in this laboratory. Although leakage from the heparin adsorbents was observed, the ligand loss rate was much slower than the silane leakage with the corresponding aminopropylated support materials. This result indicated that the coupled ligands had a stabilising effect on the intrinsically less stable aminopropylsilyl-modified support material. The blocking of free amino groups was in the present investigations achieved through acetylation with acetic anhydride. Following storage in an aqueous environment, free amino groups were however again detected on the blocked adsorbents. The low observed leakage of the heparin ligands may have been associated with the presence of such free amino groups. The stability of the acetylated heparin adsorbents was improved when storage was carried out at pH 5.0.

With the heparin-LiChroprep Si60 adsorbents, heparin coupling yields between 10 and 20% relative to amount heparin applied were generally achieved. With these yields, heparin contents ranging between 0.6 and 26 mg heparin per g adsorbent were obtained. The measured heparin contents on the heparin-LiChroprep Si60 adsorbents were in the range 0.08–3.4 μmol heparin per m^2, or a ligand coverage between 3 and 100% of the external surface area based on the M_r range of 5000 to 30 000 for heparin. The experimental data with the larger pore size on Fractosil 1000 (1000 Å) suggested that the surface area within the pores participates in the heparin immobilisation to this support material. Coupling yields with the heparin-Fractosil 1000 adsorbents were however lower than with the heparin-LiChroprep Si60 adsorbents. Possible explanations to these low coupling yields could be found

in the lower silane contents compared to LiChroprep-NH_2, and the further loss of the aminopropyl groups during coupling step with the amino-modified Fractosil 1000. The heparin contents, between 1 and 14 mg heparin per g on the heparin-Fractosil 1000 adsorbents, were nevertheless in the same range as those of the heparin-LiChroprep Si60 adsorbents. These values converted to 0.006 to 0.05 μmol heparin per m^2, or a ligand coverage of between 0.4 and 3.5%, based on the specific surface area on Fractosil 1000 of 20 m^2/g support and the M_r range of 5000 to 30 000 for heparin.

Several important features related to the use of immobilised heparin-affinity adsorbents thus arise from these investigations. Firstly, these studies demonstrate that with appropriate attention to the choice of the physical properties of the support material, radically different adsorbents can be prepared. Secondly, the conditions of immobilisation can affect the performance of the derived heparin-affinity adsorbent, both in terms of stability of the ligand and accessibility of the ligand. Thirdly, heparin adsorbents which match or exceed the ligand densities of commercially available adsorbents can be prepared through the end-point-attachment approach documented in these investigations. Fourthly, the use of mechanically rigid particles as the support material offers the use of higher superficial velocities with packed columns and even the opportunity for application at relatively high flow-rates of expanded bed approaches with relatively crude feedstocks. However, the most significant advantage which these adsorbents appear to offer relates to the yields achievable with separations based on the use of end-point-attached heparin vis-a-vis the other types of grafted heparin support materials, both in terms of the relative purification factor differences but also in terms of the subtly different selectivities which are manifested by these new types of immobilised heparin-affinity adsorbents compared to the conventional materials previously used. Documentation of these advantages and further properties of these different heparin-affinity adsorbents in various application studies with crude protein mixtures containing thrombin [30,31], antithrombin III [39] and basic FGF [40], are provided in the associated papers. It can be noted from the results obtained with these latter investigations that higher flow-rates could be routinely employed and higher purification factors achieved with these classes of heparin binding proteins using the new adsorbents, suggesting that in large-scale process applications higher productivity will be feasible with appropriately prepared heparin-silica adsorbents based on the correct selection of particle and pore-size characteristics, drawing on the guidelines presented in this investigation.

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