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# Dual Application of Cryogel as Solid Support in Peptide Synthesis and Subsequent Protein-Capture

**Gry Ravn Jespersen,[1,2] Anders Lærke Nielsen,[2] Finn Matthiesen,[3] Henrik Sune Andersen,[2] Harald Kirsebom[1]**

[1]Department of Biotechnology, Lund University, 221 00 Lund, Sweden
[2]Novo Nordisk A/S, Department of Biopharm Chemistry, Novo Nordisk Park, DK-2760 Måløv, Denmark
[3]Novo Nordisk A/S, Department of Protein Purification Technology, Novo Nordisk Park, DK-2760 Måløv, Denmark
Correspondence to: H. Kirsebom (E-mail: Harald.Kirsebom@biotek.lu.se)

**ABSTRACT:** The use of a cryogel in a combined application as a solid support for automated synthesis of a peptide ligand followed by affinity chromatography of a target protein is evaluated. The advantage, of synthesizing the ligand directly on the cryogel, is the circumvention of the standard process of synthesizing a peptide on a solid support, followed by cleavage, purification, analysis, and finally immobilization on the cryogel. To demonstrate the application, a peptide affinity ligand is synthesized directly on a cryogel with a yield of 28.4 $\mu$mol $g^{-1}$ dry polymer and purity of 45% of crude product. The affinity capture of an antipeptide antibody reveals a specific binding capacity of 0.86 mg $g^{-1}$ dry polymer. To further elucidate the general availability of a peptide ligand to a macromolecular interaction, a trypsin substrate is synthesized on a cryogel. Trypsin cleavage of immobilized substrate is determined to 1.5 $\mu$mol $g^{-1}$ dry polymer. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 4383–4391, 2013

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## INTRODUCTION

As the biopharmaceutical industry expands its portfolio of recombinant protein therapeutics, the need for new and alternative methods within analysis and purification are called for.[1] Chromatography is currently employed as a primary tool in purification and analysis of many biopharmaceutical products, based on the high separation efficiencies achieved through this technique.[2] Of the different chromatographic techniques available, affinity chromatography is highly useful in the capture of a target protein, as it is based on specific interactions between target and ligand. One well characterized affinity-pair is based on an antibody, which specifically binds in a calcium dependent manner to a short peptide termed HPC4, derived from protein C.[3] This affinity tag may be used for isolation of recombinant proteins carrying the HPC4 epitope.[4]

Within affinity chromatography the application of small peptide ligands has increased due to the development of spot synthesis, which facilitates the identification of new peptide affinity ligands.[5] Subsequently, when a suitable ligand candidate has been selected, it is produced in larger scale by solid phase peptide synthesis (SPPS), cleaved off the resin, purified and finally covalently linked onto a chromatography stationary phase.

One particular class of chromatography media are collectively termed monoliths. This term refers to the fact that these stationary phases are cast as one-block, porous materials. Monoliths are characterized by a porous structure constituted by a network of interconnected macropores. These macropores enable mass transport by means of convection and thus resolutions and binding capacities that are unaffected by flow.[6] This property of monoliths makes them ideal as stationary phases in analytical applications, as has been demonstrated in several studies.[7–11] Cryogels comprise a subclass of monoliths that are polymeric gels prepared at temperatures $<0°C$ where the solvent, typically water, freezes and by this acts as porogen in forming of the polymeric network.[12] Cryogels prepared by radical polymerization of water soluble vinyl monomers are endowed with a distinct macroporous morphology and very high mechanical stability, combined with the flexibility and general biocompatibility of a hydrogel.[12,13]

To improve the current procedure for production of peptide-functionalized affinity-chromatography stationary phases it would be a significant advantage, in terms of limiting the steps of preparation and loss of ligand, if the peptide ligand was synthesized directly on the chromatographic support. Until now, only a few studies have investigated the potential of utilizing a monolithic support in peptide synthesis followed by a

chromatographic application and these studies have all been performed on poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monoliths also known as CIM® discs.[14–16]

Cryogels are easily prepared from inexpensive monomeric precursors and, as well as other monoliths, they exhibit flow-independent binding capacities.[17] A distinct feature of cryogels is that, due to their very large macropores, it is possible to apply a crude cell homogenate directly to the column without risk of pores clogging, thus circumventing prior, otherwise mandatory, steps of centrifugation and filtration.[17] These qualities render cryogels as an attractive platform for analytical chromatography applications where speed and cost efficiency are at focus. In addition, due to the potentially low cost of manufacture, cryogels could be considered as disposable materials, as the concept of single-use materials is gaining increasing interest within biotechnology applications.

To the best of our knowledge, no prior work on synthesizing a peptide ligand directly on a cryogel for the purpose of performing protein chromatography has been published. Therefore, in this study we aim at demonstrating the feasibility of the dual application of a cryogel as solid support for the synthesis of a peptide ligand, followed by affinity capture of a target protein. To do this we utilize the HPC4 peptide-tag as the model affinity-ligand synthesized directly on a cryogel, followed by specific capture of an anti-HPC4 antibody. Simultaneously, we aim at validating the applicability of a cryogel to automated laboratory systems and therefore the synthesis of the peptide is performed using a microwave assisted automated peptide synthesizer which constitutes a considerable improvement to lab-bench peptide synthesis in terms of speed and handling. The chromatography is performed using an Äkta Avant liquid chromatography station.

To further demonstrate the application of a cryogel as solid support in SPPS, and to elucidate the general availability of a peptide ligand to macromolecular interactions, we also synthesize a trypsin substrate with varying spacer length and evaluate and compare the yield of synthesis versus the degree of subsequent trypsin cleavage.

## EXPERIMENTAL

### Materials

*N,N′*-dimethylacrylamide, poly(ethylene glycol)diacrylate ($M_n \approx 250$), ammonium persulfate (APS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium chloride, Tween®80, trypsin from bovine pancreas, diisopropylethylamine (DIPEA), ethylenediamine tetraacetic acid (EDTA), and calcium chloride were all purchased from Sigma–Aldrich. *N*-(3-aminopropyl)methacrylamide was purchased from Polysciences Europe, GmbH and tetramethylethylenediamine (TEMED) was purchased from Amresco®. All L-amino acids were purchased from either Protein Technologies, Inc. or Novabiochem®, {2-[2-(fmoc-amino)ethoxy}acetic acid (oligoethylene glycol, OEG) was purchased from Novabiochem®, $N^{\alpha}$-Fmoc-$N^{\beta}$-2,4-dinitrophenyl-L-2,3-diaminopropionic acid (fmoc-Dap-(Dnp)-OH) was purchased from Bachem, 2-(Boc-amino)benzoic acid (Boc-2-Abz-OH) and 4-[(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid (RINK amide linker) were purchased from Sigma–Aldrich. Ethyl cyano(hydroxyimino)acetate (Oxyma Pure), 1-hydroxy-7-azabenzotriazole (HoAt), and 1-hydroxybenzotriazole (HOBt) were purchased from Novabiochem®, piperidine, diisopropylcarbodiimide (DIC), and trifluoroacetic acid (TFA) were purchased from Biosolve Chimie (France), triisopropylsilane (TIPS) was purchased from Fluorochem (UK) and Tentagel® RAM resin was purchased from Rapp Polymere. The 2-propanol, formic acid, *N*-methylpyrrolidone (NMP), dichloromethane (DCM), methanol, acetonitrile, diethyl ether, and acetic anhydride were all purchased from Merck Chemicals, GmbH. Purified antibodies with specificities against interleukin-21 and HPC4, respectively as well as CHO cell harvest containing antibody with alternative specificity was obtained from Novo Nordisk A/S.

### Preparation of Cryogel

Prior to polymerization, inhibitors were removed from dimethylacrylamide and poly(ethylene glycol)diacrylate by passing the respective monomer through a 2 mL column of inhibitor remover purchased from Sigma–Aldrich (product no. 306312). To prepare the cryogels, a 7% (w/w) mixture of *N*-(3-aminopropyl)methacrylmide, dimethylacrylamide, and poly(ethylene glycol)diacrylate (molar equivalents 1 : 9 : 2) in Milli-Q water was prepared. TEMED, constituting 3% (w/w) of monomers, was added and the mixture was degassed with $N_2$ for 15 min and then cooled on ice for 30 min. Then APS, 1% (w/w) of monomers, was added and the mixture was stirred briefly before 0.2 mL of the suspension was added to glass tubes with an inner diameter of 5 mm. The glass tubes were then quickly transferred to a Jalubo, F34-EH refrigerated circulator bath (Jalubo GmbH, Germany) with a fixed temperature of −12°C. Care was taken to ensure that the content of all glass tubes was frozen within a few minutes and they were then allowed to react in the freezing bath for 24 h. The resulting cryogels were thawed at room temperature and washed with 30 column volumes of Milli-Q purified water. The cryogels were then removed from their glass tube and stored in water at 4°C until further use.

### Characterization of Cryogel

To determine the polymerization yield of the prepared cryogel, samples were lyophilized and their dry weight recorded. The polymerization yield was calculated with the use of the following equation:

$$\text{Yield}\,\% = \frac{w_1}{w_0} \times 100$$

where $w_0$ and $w_1$ are the theoretical and actual dry weights, respectively.

The degree of swelling of unmodified cryogels in water and NMP was determined by swelling dry samples in the respective solvent for a minimum of 5 h. The degree of swelling was then calculated with the use of the following equation:

$$\text{Swelling} = \frac{w_2 - w_1}{w_1}$$

where $w_1$ and $w_2$ are the dry and swollen weights of the sample, respectively.

To visualize the pore morphology of the cryogel scanning electron microscopy was used. Samples for microscopy were prepared by cutting cryogels into thin discs which were freeze dried before they were sputter coated with a mixture of gold and palladium (40 : 60). Scanning electron microscopy was performed with a JEOL JSM-80 5600 LV microscope (Tokyo, Japan). The mean pore diameter was determined by measuring the diameter of pores identified as surface pores from representative SEM images, an average pore diameter was determined on the basis of 35 measurements. Analyses of the image were conducted using the ImageJ software, version 1.47 was employed (http//imagej.nih.gov/).

### Primary Amine Loading of the Cryogel

Lyophilized cryogel samples were swollen in NMP and were each added a mixture of (0.030 mmol, 10 equiv) Fmoc-glycine-OH, (0.030 mmol. 10 equiv) HoAt, (0.030 mmol, 10 equiv) DIC and (0.038 mmol, 12 equiv) DIPEA, dissolved in 1 mL NMP. Couplings were conducted for 18 h at room temperature with vigorous mixing, followed by three washes with NMP, one with DCM, one with methanol and finally three washes with DCM. Following the coupling reaction a Kaiser test was performed revealing incomplete coupling.[18] The coupling was repeated, followed by a second Kaiser test which was also positive, thus at this point all primary amines available for coupling had been reacted. Samples were then lyophilized and weighed in order to determine their dry weights. The dry samples were then immersed in 3 mL mixture of NMP with 20% (v/v) piperidine and were allowed to react with this mixture for 1 h at room temperature with rigorous mixing. The supernatants' absorbances at 290 nm were determined by use of a Nanodrop spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo Scientific). The cryogel primary amine loading was calculated based on the concentration of fulvene-piperidine adduct in the mixture by use of Lambert-Beers law and molar absorptivity 5253 $M^{-1}$ $cm^{-1}$.[19]

### Automated Peptide Synthesis on Cryogel

Automated peptide synthesis on cryogels was performed on a Liberty Automated 12-channel Microwave Peptide Synthesizer® (CEM Corporation). For each synthesis 30 mg dry cryogel (equivalent to ~10 $\mu$mol primary amines) was swollen in NMP for minimum 5 h and was then placed in the microwave reactor. The excess of amino acids over cryogel primary amines, used for the couplings, was 100 molar equivalents. Fmoc-deprotection was carried out using 5% piperidine in NMP and the couplings were carried out by applying a mixture of 0.3 M amino acid and 0.3 M Oxyma in NMP and DIC, all steps were carried out applying microwaves. All peptides intended to remain attached on the cryogel support were acetylated using 1 M acetic anhydride in NMP.

When cleavage of the synthesized peptides from the solid support was required, a RINK-amide linker was initially coupled to the cryogel, followed by assembly of the peptide. All couplings were carried out as described above.

For synthesis of HPC4-peptide tag the deprotection mixture was also added 0.75 mM HOBt to minimize the formation of aspartimide by-product.[20]

For peptide synthesis on a conventional resin, amino functionalized TentaGel RAM (Rapp Polymere) (0.21 mmol primary amine/g polymer) was used in a quantity equivalent to 12 $\mu$mol primary amines. For these syntheses the same method as above was applied. For bulk production of trypsin substrate a preset 0.25 mmol method, applying amino acids in fourfold molar excess to resin primary amines, was used.

### Cleavage and Deprotection of Peptides

Resin or cryogel was washed several times with DCM to remove all traces of NMP. Peptides were then cleaved off and deprotected by treatment of the resin or cryogel with a mixture of (v/v) 93% TFA, 4% TIPS and 3% Milli-Q purified water for 2 h at room temperature. The supernatant was then isolated by filtration and was evaporated with $N_2$ to a minimal volume. The peptide was precipitated by the addition of ice cold diethyl ether and the precipitate was isolated by centrifugation.

Peptides that were not to be cleaved off cryogel were deprotected, following the same method as described above, however, after treatment with the TFA mixture the gels were washed with DCM, NMP and Milli-Q purified water before being stored at 4°C in water until further use.

### Analysis of Peptides

Mass spectrometry was performed on a Waters Acquity UPLC with a Waters Acquity UPLC BEH, C-18, 1.7 $\mu$m, 2.1 mm × 50 mm column and a Waters (Micromass) LCT Premier XE detector. Solvent A consisted of milli-Q purified water with (v/v) 0.1% formic acid and the separation was performed by applying a 5–90% linear gradient of solvent B, which consisted of acetonitrile with 0.1% (v/v) formic acid. The gradient run time was 4.0 min and the total run time was 7.0 min applying a flow rate of 0.4mL $min^{-1}$. Column temperature was 40°C.

Peptide purity was assessed by dissolving a small quantity in 10% acetonitrile followed by analysis on a Waters Acquity UPLC system with an Acquity UPLC BEH C18 column (1.7 $\mu$m, 2.1 mm × 150 mm) and a Waters Acquity TUV detector.

Peptide quantitation was carried out using an Antek8060 ChemiLuminescence Nitrogen Detector (CLND coupled to an Agilent 1200 series HPLC-system equipped with a Phenomenex Jupiter 5u C18 300Å (100 × 4.6 $mm^2$) column. The sample nitrogen content was quantified by relative comparison to an internal calibration standard solution of insulin aspart, containing 0.55 mg nitrogen/mL.

### Chromatography

Liquid chromatography experiments were performed on an ÄktaAvant liquid chromatography station (GE Healthcare). The dry cryogel sample was inserted into a Tricorn 5/20 column (GE Healthcare) and then swollen *in situ*. The column was connected to the Äkta system and packed by applying increasing flow until the cross column pressure increased significantly, indicating that the cryogel had reached its pressure limit and the cryogel settled at the bottom of the column. The volume of the packed cryogel was 0.2 mL.

The following method was applied to all experiments: The cryogel was equilibrated with 40 column volumes of equilibration

buffer (20 m*M* HEPES, 1.0 m*M* $CaCl_2$, 100 m*M* NaCl, 0.005% (v/v) Tween80, pH 7.5) at flow rate 2 mL $min^{-1}$. Then it was loaded with 2 mL of one of the following (i) anti-IL21 antibody (0.2 mg $mL^{-1}$) in application buffer (20 m*M* HEPES, 10 m*M* $CaCl_2$, 100 m*M* NaCl, 0.005% (v/v) Tween80, pH 7.5), (ii) anti-HPC4 antibody (0.2 mg $mL^{-1}$) in application buffer, (iii) cell harvest with anti-HPLC4 antibody (0.2 mg $mL^{-1}$) and 10 m*M* $CaCl_2$, (iv) cell harvest with 10 m*M* $CaCl_2$ at flow rate 0.5 mL $min^{-1}$. A wash step was performed with 80 column volumes of wash buffer (20 m*M* HEPES, 1.0 m*M* $CaCl_2$, 1*M* NaCl, 0.005% (v/v) Tween80, pH 7.5) at flow rate 2 mL $min^{-1}$ and the column was eluted by applying 30 column volumes of elution buffer (20 m*M* HEPES, 5 m*M* EDTA, 100 m*M* NaCl, 0.005% (v/v) Tween80, pH 7.5) at flow rate 1 mL $min^{-1}$. As a final step the cryogel was washed with 80 column volumes of equilibration buffer. Flow through and elution was collected as 0.5 mL fractions. All experiments were repeated five times.

### Size Exclusion HPLC-Analysis of Elution Fractions

The relevant eluted fractions were pooled and analyzed for concentration and purity on a Bio Sep-SEC–S3000 column (Phenomenex, Denmark) connected to a Waters Alliance HPLC-system (Waters, Denmark). Running buffer (200 m*M* sodium phosphate, 300 m*M* NaCl, 10% 2-propanol (v/v), pH 6.9), flow rate 1 mL $min^{-1}$, column temperature 30°C. To determine the antibody concentration in the eluates, the peak area was compared to the peak area of an antibody standard of known concentration (2.8 mg $mL^{-1}$) and the concentration was calculated based on this.

### Protein Analysis of Elution Fractions

Qualitative protein analysis of elution fractions was performed by use of Agilent 2100 Bioanalyzer® (Agilent Technologies, Denmark). Prior to analysis, elution fractions were concentrated by centrifugation to a volume of 0.3 mL using Satorius Vivaspin 6 columns (Satorius Stedium, Germany). Protein analysis was performed according to the 2100 Bioanalyzer® Agilent protein 230 Kit protocol with the one exception that for elution fractions 20 $\mu$L was added in the sample preparation instead of 4 $\mu$L (replacing water in final sample).

### Trypsin Cleavage of Substrate on Cryogel

To enable conversion of the fluorescence signal, resulting from a trypsin digest of immobilized substrate, into a measure of fluorophore concentration, a standard curve based on a full trypsin digest of different substrate concentrations, in solution, was prepared. Fluorescence was read at $\lambda_{em} = 414$ nm, $\lambda_{ex} = 325$ nm by use of a SpectraMax M2e multimode microplate reader (Molecular Devices, United States) and the concentrations tested were in the range 0–0.5 m*M*. The fluorescence of fully cleaved substrate was linear with increasing substrate concentration in the range of 0–63 $\mu M$. All trypsin digests were performed in triplicates.

Cryogel samples, functionalized with trypsin substrate, were placed in polypropylene vials (Nunc, Denmark) and were then each added 2.7 mL buffer (50 m*M* HEPES, 0.1*M* NaCl, 0.02% (v/v) tween80) with 0.1 $\mu M$ trypsin. The samples were incubated for 2 h at room temperature with gentle shaking and subsequently the fluorescence was quantified. The same experiments were performed without the addition of trypsin to measure the background fluorescence, which was deducted from the result of the trypsin cleavage. Experiments were performed in triplicates.

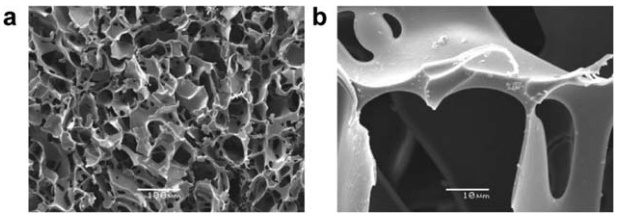


**Figure 1.** Scanning electron microscopy image of the macroporous cryogel morphology at 200 times (a) and 2000 times (b) magnification.

## RESULTS AND DISCUSSION

### Preparation and Characterization of Cryogels

A solid support suitable for peptide synthesis, as well as chromatographic applications must possess the following features (i) a uniform matrix structure, (ii) stability towards applied solvents and reagents, (iii) an appropriate functional group loading, and (iv) high swelling in appropriate solvents. The copolymerization of *N*-(3-aminopropyl)methacrylamide, dimethylacrylamide and poly(ethylene glycol)diacrylate monomers provided cryogels with reaction yields consistently around 80% indicating a straight forward and unhindered radical polymerization reaction at the applied conditions. In support of this, analysis of the pore morphology by SEM revealed a uniform macroporous matrix with pore walls that appeared smooth and with a mean pore diameter of $125 \pm 34$ $\mu$m (Figure 1).

The primary amine loading of the cryogel, prepared from 10% amino functional comonomer, was determined by coupling of Fmoc-protected glycine, followed by quantification of the fulvene-piperidine adduct in the supernatant after cleavage with 20% piperidine in NMP. The reactive amino loading of the cryogels was determined to $0.32 \pm 0.05$ mmol per gram dry polymer. This is in the same range as the amino loading of several commercial synthesis resins, and is generally considered ideal with regard to minimizing steric hindrance during synthesis.[21]

A high material swelling is desirable in SPPS, as it is synonymous with efficient distribution of solvent and reagents throughout the polymer network and thus conveys the delivery of reagents to the sites of reaction. However, monolith chromatography is based on convective flow and thus ligand–target interactions on the surface of the pore walls. Any ligands situated in the pore walls are inaccessible to the protein target and is therefore redundant with respect to chromatographic capture of target. For the specific application of using a cryogel as a solid support for ligand synthesis, followed by protein capture, it could therefore be questioned if swelling is a crucial parameter. In the present study, the swelling of the cryogel in two relevant solvents, namely water and NMP, was investigated and revealed swelling capacities of 24 mL $g^{-1}$ dry cryogel and 19 mL $g^{-1}$ dry cryogel, respectively. In comparison, commercial synthesis resin Tentagel S swells 3.6 mL $g^{-1}$ dry resin and 4.7 mL $g^{-1}$ dry resin in water and dimethylformamide, respectively,

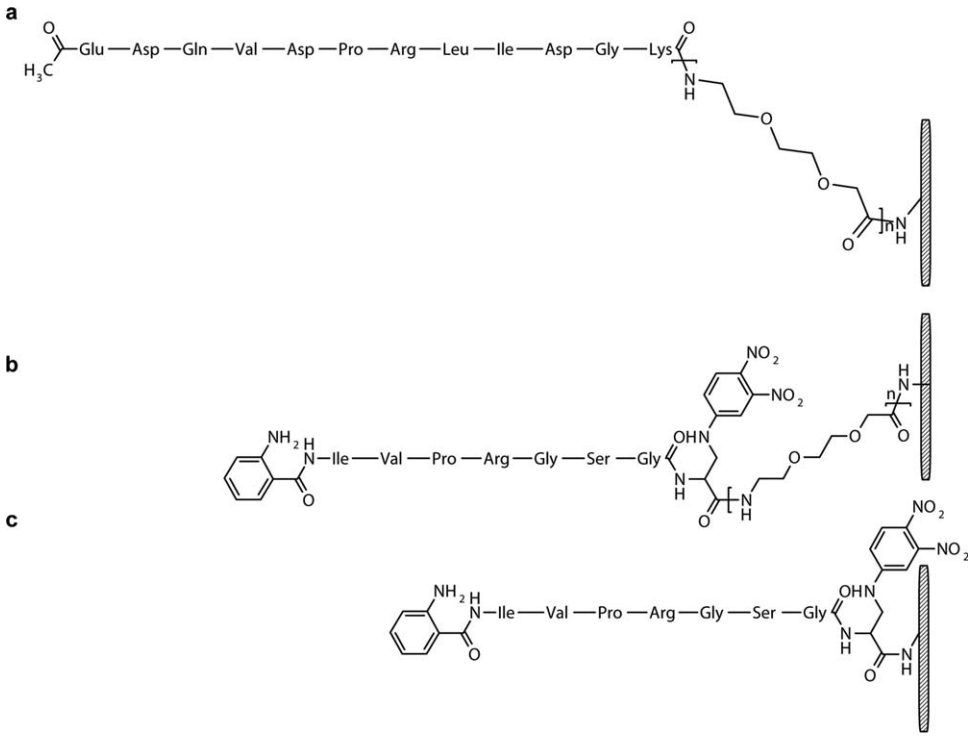


**Figure 2.** Structural presentation of the three peptides synthesized on cryogel. Structure a is HPC4-peptide ligand with a three OEG units spacer, b is trypsin substrate with a six (OEG)-units spacer, and c is trypsin substrate without a spacer. Flanking the trypsin substrate (b and c) is a fluorophore at the N-terminal and a fluorophore quencher at the C-terminal.

as stated by the manufacturer. The much higher swellings of the cryogel compared to Tentagel can be ascribed to the distinct porous morphology of cryogels, which typically represent porosities of >90% and therefore contain a very large fraction of the solvent in the pores.

Cryogels are macroporous, polymeric networks,[22] which in the present work were cast as cylindrically shaped monoliths. An important parameter in liquid chromatography is the mechanical stability of the stationary phase, as it must resist the pressure applied by liquid flow. To test the flow resistance the cryogel column was subjected to increasing linear flow velocities for durations of 2 min and the resulting cross column pressure was recorded. The results of this experiment showed that the cryogel column tolerated linear flow velocities up to 15 mL $min^{-1}$ with negligible cross column pressure, while at flow rates above this, it immediately began to compress. Very high operating flow rates accompanied with low flow resistance has been reported for other cryogel systems[17] as well as for other monoliths[23] and these findings support that the macroporosity of these materials allows for the unhindered flow of water through such a column. To put this result into context, HPLC columns are typically operated in a flow rate range of 0.1–10 mL $min^{-1}$ with concomitant very high pressures,[24] thus in this perspective monoliths could be considered as low pressure alternatives in fast flow analytical applications.

### Peptide Synthesis

In this study, three different peptide constructs were synthesized on the cryogel, by the use of microwave assisted automated peptide synthesizer (Figure 2). The HPC4-peptide tag is a construct of three (OEG)-spacer units and 12 natural amino acids comprising a total of 15 sequential peptide coupling cycles. This peptide contains an Aspartic acid residue that is C-terminally linked to a Glycine residue and is thus prone to aspartimide formation during the Fmoc deprotection step. Aspartimide formation results in the loss of one molecule of water (18 g $mol^{-1}$) caused by a ring-closure between the $\beta$-carboxy sidechain of aspartic acid and the nitrogen of the $\alpha$-carboxamide.[20,25] By addition of HOBt to the cleavage mixture the formation of aspartimide by-product was reduced to ~50% of total product. Analytical UPLC separation of the crude product revealed two large peaks comprising more than 80% of the chromatogram total peak area (Figure 3) and the mass spectrometry confirmed that the crude product consisted primarily of the product and the aspartimide by-product (Figure 4). Based on the information, provided by UPLC- and mass spectrometry analyses, it was not possible to distinguish between the HPC4-peptide tag and its aspartimide derivative. Table I displays the approximate crude product HPC4-peptide tag yield and purity which was determined to 45% and 28.4 $\mu$mol $g^{-1}$ dry polymer, respectively.

The two trypsin substrate constructs contained a fluorophore and a fluorophore quencher, flanking a short peptide with a trypsin cleavage site located on the C-terminal side of the arginine amino residue (Figure 2). The fluorophore quencher was included to prevent the uncleaved substrate from fluorescing when tested in solution. Trypsin substrate I was synthesized directly on the cryogel matrix without insertion of a spacer,

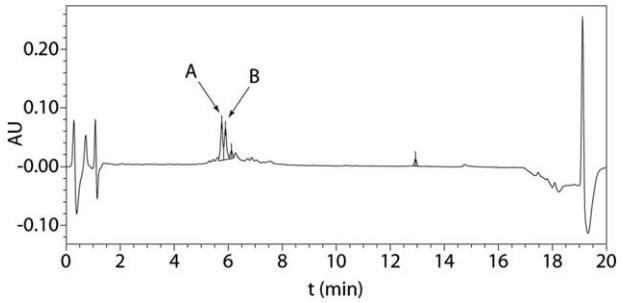


**Figure 3.** UPLC separation of components in the crude product of the HPC4-peptide ligand synthesized on cryogel. The two large peaks at retention times 5.8 min (arrow a) and 5.9 min (arrow b) comprise more than 80% of the total peak area and are identified as the HPC4-product and the aspartimide by-product.

whereas substrate II contained a six unit (OEG)-spacer. The two peptide constructs comprised 10 and 16 peptide couplings including the coupling of the RINK-amide linker, respectively.

UPLC analysis and nitrogen quantification of substrate I and II cleaved from cryogels showed 54 and 57% purity of crude product and 72.2 $\mu$mol and 14.4 $\mu$mol $g^{-1}$ dry polymer, respectively (Table I). To provide a perspective on how the present cryogel performs as synthesis resin, the syntheses of substrates I and II were repeated on a conventional synthesis resin, applying the same equivalents of reagents as for the cryogels.

The synthesis on conventional resin resulted in >80% purity of the crude products and yields of 84.6 and 22.0 $\mu$mol $g^{-1}$ dry resin for substrates I and II, respectively. The much lower purity of crude product, produced on cryogel, may be explained by the macroporous morphology of cryogel. The solute transport in the macropores is mainly driven by convection and is thus dependent on liquid flow, whereas solute transport in micropores is governed by diffusion. In the automated peptide synthesizer mixing is performed by bubbling of air through the reaction mixture and therefore we suspect that the reagents are not as evenly distributed in the cryogel and thus not as effectively delivered to the points of reaction. The TentaGel resin, on the other hand, is comprised of porous beads with a diameter of 90 $\mu$m and thus represents a combination of geometry and surface area which renders a comparably larger number of functional groups more directly available for reaction under the given automated conditions. The yield of substrates I and II produced on cryogels, as determined by nitrogen quantification was a little lower, but in the same range as the yields achieved on synthesis resin. With respect to comparison to other monoliths, Vlakh et al. have investigated the use of a CIM disc monolith as a solid support for peptide synthesis by the synthesis of a four amino acids- and an eight amino acids peptide.[14] The synthesis was performed in a flow-through mode and the purity of both peptides was found to be in the range of 80%. The fact that Vlakh et al. obtained such high product purities on a monolith can probably be attributed to their application of the flow-through mode for the synthesis. In a flow-through set-up, diffusion of reagents into the pore walls is limited and the reaction would be restricted to mainly proceed at the pore wall surface, which represents an easily accessible area with low steric hindrance. However, if it is in fact the case that reaction at the pore wall surface renders a higher purity of product, then this feature is universal to cryogels and would also be the case in the present study set-up using the peptide synthesizer, where reagents are distributed by bubbling of air through the mixture. Thus the same purity of ligand would be available to capture the target regardless of the procedure of manufacture, as ligands situated within the pore walls are inaccessible to the protein. Interestingly Vlakh et al. report to obtain the same ligand loading whether it is produced by direct synthesis or by covalent immobilization of the ligand, which supports the suggestion that application of reagents in a flow-through mode restricts the reaction to proceed solely at the surface of the pore walls.

**Table I.** Yield and Purity of Crude Product Produced by Automated Peptide Synthesis on Cryogel

| Peptide | Yield of product ($\mu$mol $g^{-1}$ dry polymer) | Purity of crude product (%) |
|---|---|---|
| $(OEG)_3$-HPC4 | 28.4 | 45 |
| Trypsin substrate I | 72.2 | 54 |
| $(OEG)_6$-Trypsin substrate II | 14.4 | 57 |

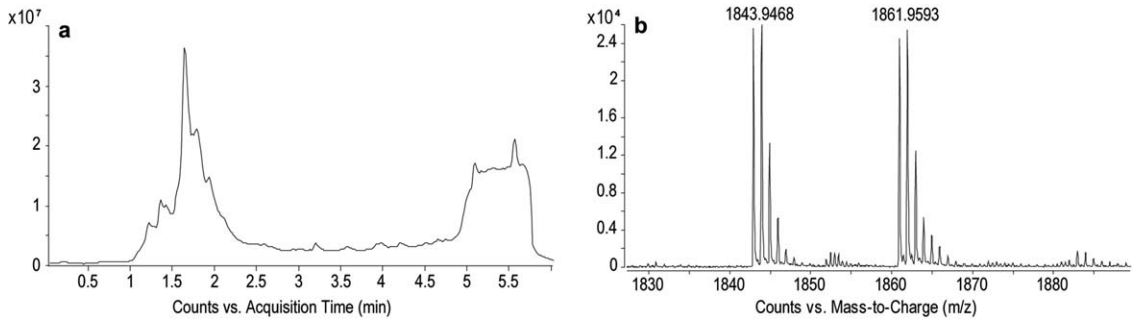


**Figure 4.** LC-MS analysis of crude HPC4-peptide ligand synthesized on cryogel. Image a shows the electrospray ionization TIC signal and image b is the mass spectrum. The analysis clearly shows how both HPC4-peptide (1861 g $mol^{-1}$) and the aspartimide by-product (1843 g $mol^{-1}$), which are both N-terminal acetylated, are present in the crude product.

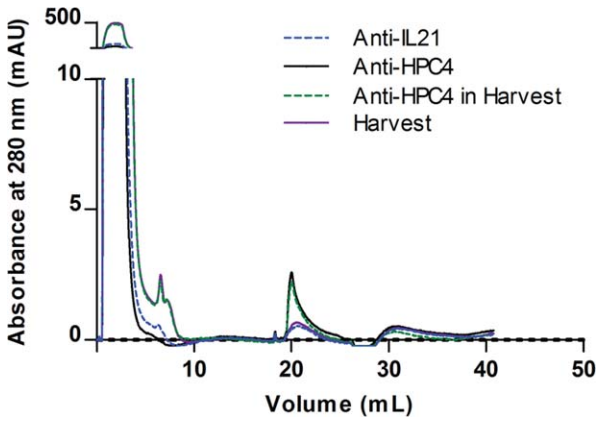


**Figure 5.** Overlay of chromatograms of application of anti-HPC4 antibody (dotted, blue), anti-IL21 antibody (straight, black), anti-HPC4 antibody in cell harvest (dotted, red) and cell harvest (straight, green) on HPC4-cryogel followed by elution with EDTA. Because of the high absorbance of the cell harvest, absorbances above 10 mAU are not displayed here. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### Anti-HPC4 Antibody Binding to HPC4-Cryogel

To evaluate the binding capacity and specificity of the HPC4-peptide tag functionalized cryogels, a series of four experiments were carried out on five individual cryogels. These were mounted in commercially available columns and, connected to an Äkta Avant chromatography station. Once tightly fitted in the column and connected to the chromatography system, each cryogel was loaded with (i) anti-IL21 antibody, (ii) anti-HPC4 antibody, (iii) anti-HPC4 antibody in microfiltered cell culture broth containing antibody with alternative specificity, and (iv) microfiltered cell culture broth containing antibody with alternative specificity alone. After each application, bound protein was eluted with 5 m*M* EDTA. The HPC4-peptide tagged cryogel should have explicit specificity towards the binding of anti-HPC4 antibody and hence the applications of an anti-IL21 antibody and cell harvest served as control experiments. The 280-nm absorbance was recorded to allow comparison of loading and elution profiles of the different applications.

Comparison of the elution profiles of these four applications demonstrates the cryogel's capability of specifically capturing the anti-HPC4 antibody both in purified form and from a cell harvest (Figure 5). At the applied flow rate of 0.5 mL $min^{-1}$ (153 cm $h^{-1}$), the residence time of the respective proteins in the cryogel column is 24 s, which can be regarded as very short. Figure 5 displays an overlay of chromatograms of the four different applications. At 20 mL it is very clear that anti-HPC4 antibody is eluted in a sharp peak from the application containing anti-HPC4 antibody. The sharpness of the elution peak clearly demonstrates the strong $Ca^{2+}$ dependency of the target–ligand interaction of this system, as anti-HPC4 antibody is released from the column immediately upon addition of EDTA. The applications which did not contain anti-HPC4 antibody also show a minor UV signal at 20 mL, which is due the presence of EDTA in the elution buffer. The applications of harvest exhibit a small bump on the hind side of the application peak at ~5 mL. This is probably due to the slight retention of the cryogel column of some of the components in the harvest, which consists of a complex mixture of molecules. This retention can be elicited through either hydrophobic interactions with the cryogel matrix or by sixe exclusion effects.

The elution fractions collected from the four experiments in this study were analyzed by size exclusion HPLC to evaluate their purity and determine the binding capacities. For the application of anti-HPC4 antibody in purified form and as a component in cell harvest, the cryogels had average binding capacities of $0.86 \pm 0.03$ mg $g^{-1}$ and $0.71 \pm 0.10$ mg $g^{-1}$, respectively. The slightly lower binding capacity toward anti-HPC4 antibody in cell harvest can be explained by the concomitant presence of proteins, lipids, and other cell components, which are interacting and interfering with the binding of the antibody to the peptide tag.

As mentioned, monoliths normally have binding capacities that are independent of the flow rate, a feature which most probably also applies to the present cryogel as seen by the fact that target binding is detected at a protein residence time as short as 24 s, which was the case at the conditions under study. Given that the aim of this study was to provide proof of concept that a cryogel can be used as a single solid support for the direct synthesis of ligand followed by protein target capture, no attempt was made to optimize this model affinity cryogel. A natural next step in the further development and optimization of this cryogel would be to characterize it by determining binding kinetics and evaluate how flow rate may affect binding capacity or purity of product.

To obtain an additional qualitative record of the elution fractions, they were analyzed on an Agilent 2100 Bioanalyzer (Figure 6). Prior to the analysis, it was necessary to concentrate the elution fractions, to a level above the technique's detection

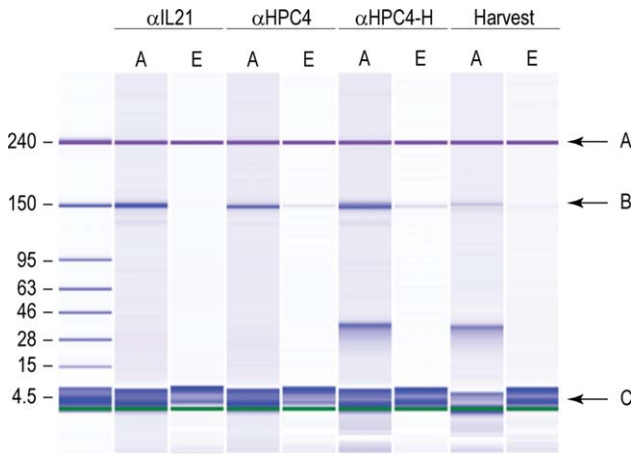


**Figure 6.** Bioanalyzer gel chip with application (A) and elution fractions (E) from application of anti-IL21 antibody (αIL21), anti-HPC4 antibody (αHPC4), anti-HPC4 antibody in cell harvest (αHPC4-H) and cell harvest (harvest) on HPC4-cryogel. The arrows indicate internal upper (1) and lower (3) markers and the band position at 150 kDa (2), which is the molecular weight range of antibodies. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

limit. As the procedure of upconcentration may result in some loss of protein, the Bioanalyzer was not used for quantitation of protein contents. From the Bioanalyzer gel chip it was confirmed that the HPC4-peptide tag cryogel does not bind anti-IL21 antibody. Furthermore it can be seen that the cryogel captures anti-HPC4 antibody both in its purified form and as a component in a cell harvest. Finally it appears that the cryogel captures a faint amount of unspecified antibody present in the cell harvest (Figure 6). This binding could be due to unspecific ionic interaction with charged amino acid residues in the peptide ligand and is so minor that it could not be quantified by SE-HPLC and we therefore consider it as negligible, especially when anti-HPC4 antibody is present in the mixture.

The binding capacity of the HPC4-tag functionalized cryogel, towards the anti-HPC4 antibody, was low, especially when compared to the determined HPC4-peptide tag ligand loading (Table I). It appears that only a very small fraction of the ligands present on the cryogel were available for binding of the antibody. Studies on antibody capture using cryogels generally show markedly higher binding capacities than the ones reported here.[26–30] The binding capacity can be directly related to the surface area of the cryogel that is available for antibody interaction, and in a monolith, such as these cryogels, this is the surface area of the macropores. The size and morphology of cryogel macropores depend on the conditions applied during polymerization and therefore the area of macropores varies between different cryogel systems.[31–33] At present there is no effective method for measuring the specific area of the macropores in a soft monolith, such as a cryogel, and therefore direct comparison of macromolecule binding capacities of different cryogel systems is problematic.

### Trypsin Cleavage of Substrate with Varying OEG-Spacer Synthesized Directly on the Cryogel

As shown above, we were able to manufacture a peptide ligand on a cryogel that selectively binds its target protein, but the binding capacity was unexpectedly low. Therefore, we wanted to elucidate the general availability of peptide ligands synthesized directly on the cryogel matrix and the possible effect of introducing a spacer. To do this, we utilized a trypsin substrate sequence with a C-terminal di-nitrophenyl quencher and an N-terminal 2-aminobenzoic acid fluorophore. This allowed us to monitor substrate cleavage by quantifying the amount of released fluorophore.[34] The amount of cleaved substrate was determined using a standard curve correlating the amount of cleaved substrate and the resulting fluorescence.

The amount of substrate cleaved by trypsin, determined by an end-point measurement, was 1.1 $\mu$mol $g^{-1}$ dry polymer for the substrate with no spacer and 1.5 $\mu$mol $g^{-1}$ dry polymer for substrate with six unit (OEG)-spacer (Table II). Comparing this to the binding capacity of the HPC4-functionalized cryogels toward the anti-HPC4 antibody, which was determined to 5.7 nmol $g^{-1}$ dry polymer, the amount of substrate available for trypsin cleavage is ~200-fold higher. This difference in availability may be explained by two factors, namely the size difference between anti-HPC4 antibody (≈150 kDa) and trypsin (23.8 kDa) and the macromolecules' different mode of interaction

**Table II.** Substrate I and II Loading and Yield of Trypsin Cleavage

| Substrate type | Cryogel substrate loading ($\mu$mol $g^{-1}$ dry polymer) | Substrate cleaved by 0.1 $\mu M$ trypsin ($\mu$mol $g^{-1}$ dry polymer) |
|---|---|---|
| Substrate I, no spacer | 72.3 | 1.1 ± 0.1 |
| Substrate II, $(OEG)_6$-spacer | 14.4 | 1.5 ± 0.2 |

with their targets. First, the hydrodynamic radius of a typical antibody is ~5 nm, whereas the hydrodynamic radius of trypsin is ~2 nm.[35,36] Consequently, the surface area of the cryogel macropores can accommodate more molecules of trypsin compared to the antibody. Secondly, the mode of interaction of the antibody and trypsin with their respective ligands or substrate is different. The interaction between anti-HPC4 antibody and ligand is static in the sense that, when one molecule of antibody is bound it covers a specified area, and possibly other ligands in close proximity, until it is eluted. In comparison, the trypsin-substrate interaction can be described as dynamic in the sense that it is an on/off mode of interaction, thus rendering a larger surface area and amount of ligands available.

Considering the effect of insertion of a spacer on the substrate availability to cleavage, it is seen that 1.5% of the substrate without spacer is cleaved whereas 10% of the substrate with spacer is cleaved. These results indicate that insertion of a spacer enhances the availability of substrate to trypsin cleavage, which seems logical as moving the substrate away from the pore wall surface would minimize possible steric hindrances preventing trypsin from encountering the peptide cleavage site. With regard to optimization of the cryogel for chromatography these results suggest that insertion of a spacer could be an optimization parameter for increasing the ligand availability. In this study the HPC4-ligand was attached to a six-unit (OEG)-spacer, but possibly the binding capacity of the cryogel could be increased by incorporation of an even longer spacer.

The findings in this study show a large discrepancy between the cryogel ligand loading and the macromolecule binding capacity and this indicates that the investigated cryogel encompasses two distinct porous systems; (i) a porous system consisting of micro- and possibly mesopores accessible to small molecule reactions, and (ii) a porous system consisting of highly interconnected supermacropores which is accessible to macromolecules, this can be visualized by SEM (Figure 1). This feature of cryogels has been investigated and characterized in several studies and this collected work is reviewed by Gun'ko et al. 2013.[22] The surface area of the macropores of a cryogel is vanishingly small compared to that of the nano-, micro- and mesopores which comprise more than 95% of the specific surface area of a cryogel.[20] This relationship explains the observed large discrepancy between the present cryogel's ligand or substrate loading and the availability of these for macromolecular interaction.

The low binding capacity of cryogels toward proteins has long been known and recognized, but the rather high capacity

toward small molecule reactions, as observed in this study has not been thoroughly investigated.

## CONCLUSIONS

The feasibility of using a cryogel in dual applications as demonstrated in this study, where a model ligand is synthesized directly on the cryogel followed by capture of the target protein, is an improvement of the existing procedure, by circumvention of time-consuming preparation steps and consequently loss of ligand. Solid phase peptide synthesis, using a cryogel as solid support was successfully performed by the use of a microwave assisted peptide synthesizer, which is a substantial improvement to lab-bench peptide synthesis in terms of reducing time consumption and work load. Furthermore the chromatography was performed by connecting the cryogel to an automated liquid chromatography station which offers the advantages of easy handling and online monitoring of key chromatography parameters such as cross column pressure, UV, pH, and conductivity. The cryogel, which was the subject of the present study, showed binding capacities which were low, but consistently reproducible as tested by five different sample cryogels, proving that the preparation procedure outlined in this study is sound. As mentioned, cryogels offer the possibility of the direct application of crude cell homogenate without prior purification. Combined with the facts that these matrices are easily prepared, inexpensive, customizable and exhibit flow independent binding make cryogels obvious candidates as solid supports in analytical chromatography applications where speed, cost efficiency and material flexibility are desired. Examples of such applications could be within online analysis of fermentation processes or the analysis of radioactive reagents where a disposable material is needed. By following the procedure outlined in this study cryogels carrying peptide ligands for analytical chromatography can easily be prepared, but to improve the purity of ligand and binding capacity of the cryogel further optimization and chromatography characterization studies must be undertaken.

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