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High throughput processing of particulate-containing samples using supermacroporous elastic monoliths in microtiter (multiwell) plate format

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Abstract

Two steps in parallel processing of multiple biosamples, namely, sample clarification and capture of the target protein, were integrated and combined with the direct assay of captured protein using a newly developed microtiter (96-well) plate system based on the monoliths of hydrophilic elastic supermacroporous material, cryogel. Cryogel monoliths have pore size large enough for microbial and mammalian cells to pass through unretained. Moreover, cryogel monoliths are elastic allowing them to be slightly compressed and easily introduced into the wells. When expanded, cryogel monoliths fill the well tightly with no risk of leakage in between the monolith and the walls of the well. The capillary forces keep the liquid inside the pores of the cryogel monolith making the monolith columns drainage protected. The application of a certain volume of liquid on top of a cryogel monolith column results in the displacement of exactly the same volume of liquid from the column. The concept of using supermacroporous gels in 96-well plate format offers new possibilities to the biotechnologist allowing separation of particulate matter, capturing of soluble material from particle containing media, and parallel assay of large number of non-clarified samples.
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Keywords: Supermacroporous monoliths; Parallel processing of particulate samples; Drainage protected columns

1. Introduction

Parallel processing of samples in robotic systems has proven to be the only plausible solution to the challenge of analyzing a rapidly growing number of samples handled in biosciences. A microtiter plate format has become a gold standard in parallel sample processing. Commercially available specialized microtiter plates allow parallel processing of a variety of sample-handling steps like solid phase extraction (SPE), sample filtration, removal of low-molecular-weight impurities or proteins, plasmid DNA binding, besides running immunoassays and cell cultivations (see e.g. www.whatman.com).

The automated SPE in 96-well format has become increasingly popular for the analysis of low-molecular-weight analytes in environmental science, food science, clinical chemistry, pharmaceutical bioanalysis, forensics and analytical biochemistry (for a review see [1]). However, application of SPE for the analysis of proteins has been rather limited so far. The protein complexity and diversity make the task of parallel processing quite challenging. In order to meet the requirements for identification and quantification of proteins from a large number of biological samples, specialized 96-well plates have been developed that clear cell debris via vacuum filtration, and retain chromatography gels allowing binding, washing and elution steps [2]. In most cases, single plate processing cannot meet the high sample-throughput demand [1].

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In the present paper, we are addressing the problem of integrating two steps in biosample processing, namely sample clarification and capture of the target protein, and combining these steps with the direct assay of captured protein. To achieve this goal we have developed a new 96-well SPE system based on the hydrophilic elastic supermacroporous material, cryogel of cross-linked polydimethylacrylamide (polyDMAA). Recently, we have presented polyacrylamide cryogel monoliths as new chromatographic materials for the direct capture of target proteins from unprocessed cell homogenates [3]. A system of large interconnected pores in cryogels allows cell debris and even intact bacterial cells [4] or mammalian cells [5,6] to pass freely through the cryogel column. Moreover, cryogel monoliths have properties that could be highly favorable for using them in 96-well systems. Firstly, elasticity of monoliths allows them to be slightly compressed for them to be easily introduced into the wells and retained therein with no risk of leakage in between the monolith and the walls of the well. Secondly, the capillary forces keep the liquid inside the pores of the gel making the monolith columns drainage protected. On the one hand, the application of a certain volume of liquid on top of a cryogel monolith column results in the displacement of exactly the same volume of liquid from the column, while on the other, it stays filled with the liquid all the time.

2. Materials and methods

2.1. Materials

N,*N*-Dimethylacrylamide (DMAA, 99%), aluminium oxide, ammonium persulfate (APS) and allyl glycidyl ether (AGE, 99%) were bought from Aldrich (Steinheim, Germany). *N*,*N*′-Methylenebis(acrylamide) (MBAAm) was from Acros (Geel, Belgium); iminodiacetic acid (IDA) was from Fluka (Buchs, Switzerland). Lysozyme, Sodium pyruvate, bicinchoninic acid solution, (β-NADH, copper sulphate, EDTA-tetrasodium salt, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and imidazole were purchased from Sigma (St. Louis, USA). High salt LB-Broth, micro agar, sodium ampicillin and Isopropyl-β-D-thiogalactopyranoide (IPTG) were from Duchefa (Haarlem, The Netherlands). The buffer salts used were of analytical quality.

Recombinant strain of *E. coli* TGI cells expressing a thermostable lactate dehydrogenase (from thermophilic *B. stearothermophilus*) carrying a tag of six histidine residues (His_6-LDH) was a gift from Professor Leif Bülow, Department of Pure and Applied Biochemistry, Lund University. Recombinant strain of *E. coli* producing extracellular His_6-tagged single chain Fv-antibody fragments (His_6-scFv) [7] was provided by BioInvent Therapeutics AB (Lund, Sweden) as a kind gift.

Bakers' yeast in the form of pressed blocks was purchased locally.

2.2. Production of cryogel monoliths

DMAA (2.1 ml purified from stabilisator on an aluminium oxide column) and MBAAm (0.75 g) were dissolved in 40 ml of deionized water. Then 0.275 ml AGE was added to the mixture with continuous stirring and the volume was adjusted to 50 ml with deionized water. The mixture was degassed under vacuum for 10 min to eliminate dissolved oxygen. Free radical polymerization was initiated by adding TEMED (35 μl) and APS (30 mg) and the reaction mixture was stirred gently for 0.5 min. Then 0.5 ml of the reaction mixture was added very quickly into each well of a 96-well mold (the mold was cooled at 4 °C for 10 min prior to filling) (Fig. 1a). The solution in the mold was frozen within 20–30 min in the ARCTEST cooling chamber at −10 °C, maintained at that temperature overnight and then thawed at room temperature. The cryogel matrix was washed by passing 10 ml of deionized water through each well. Then the monoliths were transferred from the mold into the plastic 96-well plate (with 96 bottomless round wells, each with a volume of 1.5 ml and a well diameter of 6.9 mm, Fig. 1b and c) developed for chromatography applications.

2.3. Coupling of Cu(II)-IDA ligand to the cryogel monoliths in the 96-well plate

Four milliliters of 0.5 M Na_2CO_3 solution were passed through each well filled with cryogel monolith, after which they were equilibrated with 0.5 M IDA in 1.0 M Na_2CO_3,

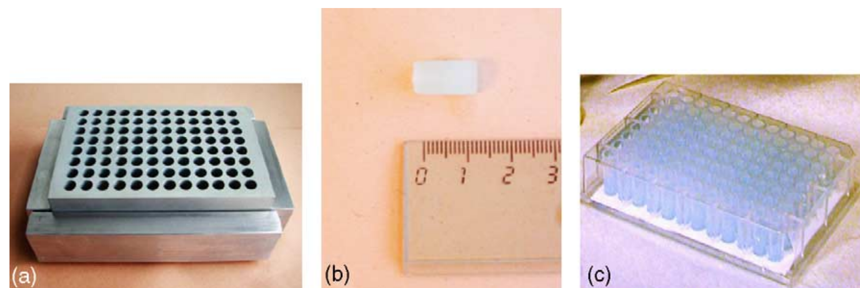

Fig. 1. Photo of the (A) 96-well metal mold used for the production of cryogel monoliths; (B) cryogel monolith removed from the mold and (C) 96-well microtiter plate.

pH 10.0. Finally, the 96-well plate with the cryogel monoliths was placed into a vessel containing 400 ml of 0.5 M IDA in 1.0 M Na_2CO_3, pH 10.0 and was incubated overnight at room temperature with gentle shaking. The modified cryogels in the plate were washed with water until pH became neutral. Cu(II) was bound to the IDA-cryogels by passing 2 ml of 0.5 M $CuSO_4$ (dissolved in distilled water) through each well. Finally, each well was washed with water and the cryogel monoliths were equilibrated with 20 mM HEPES, 0.2 M NaCl pH 7.0. The ligand density was determined as described elsewhere [4].

2.4. Cultivation of E. coli cells producing recombinant proteins

Cultivation of *E. coli* TGI cells producing His_6-LDH, preparation of cell homogenate, and the enzyme and protein assays have been described elsewhere [3].

The recombinant His_6-scFv producing *E. coli* cells were maintained in LB medium (10 g tryptone; 5 g yeast extract and 5 g sodium chloride in one litre distilled water, pH 7.2) containing 0.1 mg/ml ampicillin. The solid medium (with 1.5% w/v agar) in a Petri plate was inoculated with glycerol stock of the culture and incubated overnight at 37 °C. A colony was then inoculated in 7 ml of the LB medium, pH 7.2 supplemented with 1% glucose and ampicillin (0.1 mg/ml), and incubated overnight at 37 °C with shaking (200 rpm). This seed culture was then used to inoculate, at a concentration of 1% (v/v), 0.1 l Erlenmeyer flask containing 25 ml of the TB medium (12 g pancreatic digest of casein; 24 g yeast extract; 9.4 g dipotassium phosphate and 2.2 g monopotassium phosphate in 1 l distilled water, pH 7.2) supplemented with glycerol 4 ml/1 and ampicillin 0.1 mg/ml. The flasks were incubated at 37 °C with shaking (175 rpm). When the optical density at 600 nm reached 0.52 (in about 2.5 h), IPTG was added to a final concentration of 0.1 mM to induce His_6-scFv fragment gene expression and incubation continued overnight.

2.5. Preparation of a cryogel sample for microscopy

Gel discs of about 3–5 mm thickness were cut from the gel column. The gel discs were fixed in 2.5% glutaraldehyde in 0.12 M sodium phosphate buffer, pH 7.2, overnight, and postfixed in 1% osmium tetra-oxide for 1 h. Then the samples were dehydrated in ethanol (0, 50, 75, and 99.5%) and critical point dried. The dried samples were coated with gold/palladium (40/60) and examined using a JEOL JSM-5600LV scanning electron microscope.

2.6. Direct quantification of His-tagged lactate dehydrogenase from crude homogenate using Cu (II)-IDA cryogel monoliths in microtiter plate

Different amounts of *E. coli* cell homogenate containing His_6-LDH were applied to Cu(II)-IDA cryogel monoliths (0.5 ml) equilibrated with 0.2 M Tris–HCl buffer pH 7.3 (buffer A). After 5 min incubation the wells were washed with the same buffer to remove unbound protein and cell debris. Then the reaction mixture (0.5 ml of buffer A containing 0.45 mM NADH and 2.0 mM pyruvate) was added and incubated within the wells for 2 min. After incubation the reaction mixture was displaced from the wells by adding 0.5 ml of buffer A and analyzed by measuring absorbance at 340 nm. In the control experiment buffer A was used instead of cell homogenate. ΔA_{340} was calculated from the difference in A_{340} values of homogenate loaded and control wells. Each homogenate load was analyzed on 10 different randomly chosen wells.

2.7. Binding of His_6-scFv to Cu(II)-IDA cryogel monoliths in microtiter plate

Different volumes of *E. coli* cell culture fluid containing 30 μg/ml His_6-scFv fragments were applied to different randomly selected wells packed with Cu(II)-IDA cryogel monoliths pre-equilibrated with 20 mM HEPES, 0.2 M NaCl, 2 mM imidazole pH 7.0. After 5 min incubation the wells were washed with 1.5 ml of the same buffer to remove cells and non-bound protein. The bound protein was eluted with 1 ml of 20 mM EDTA, 20 mM NaCl pH 7.5. The content of His_6-scFv fragments in the broth and in the eluted fractions was detected by indirect ELISA.

2.8. Quantification of His_6-scFv

The wells of a 96-well microtiter plate were coated with 60 μl anti-6x-His (3 μg/ml) in carbonate–bicarbonate buffer, pH 9.6. After overnight incubation at 4 °C, the solution was poured off and the wells were washed using 300 μl ELISA buffer (10 mM phosphate buffer pH 7.4, 0.15 M NaCl, 0.05% Tween 20). Washing was repeated three times with 5 min incubation during the last washing step. The samples (100 μl) were added in wells in lane 1 of the plate and further double dilutions were made in columns 2–11. The wells in line 12 were used as controls and contained only buffer. The plate was left on a shaker at 100 rpm for 1 h at room temperature. The solution was poured off and the wells were washed as above. In each well 50 μl of secondary antibody, rabbit α scFv(b) (2000× diluted in ELISA buffer) was added and the plate was further incubated for 1 h at room temperature under similar conditions. The antibody solution was poured off and after thorough washing, the wells were treated with 50 μl of enzyme conjugated anti-antibody (porcine anti-rabbit IgG-horse radish peroxidase, 1000× diluted in ELISA buffer) and the plate was incubated for 1 h with shaking. The solution was poured off and the wells were washed as before. The substrate solution (freshly prepared by dissolving one OPD tablet and one urea hydrogen peroxide buffer tablet in 20 ml of deionized water), 200 μl, was added to all wells, and after 10 min incubation at room temperature the reaction was terminated by adding 50 μl of 3 M HC1 and absorbance

was read at 492 nm against reference blank at 650 nm using ELISA reader (ASYS Hitech GmbH, Austria).

2.9. Statistical calculations

Standard deviation, s in series of measurements was calculated as

$$s = [(n-1)^{-1}(x_1^2 + x_2^2 + x_3^2 + \cdots + x_n^2 - nx^2)]^{1/2}$$

where n is the number of measurements, x_i is the individual value in the series and x is the medium value. The data are presented as $x \pm s$. The individual wells used for measurements were selected randomly.

3. Results and discussion

3.1. Production and properties of 96-well microtiter plate with cryogel monoliths

Supermacroporous morphology of a gel formed by a hydrophilic polymer could be achieved when the gel formation takes place in the presence of a template or *porogen* that could be removed later, thus leaving a system of interconnected large pores. An example of such a template is a water immiscible solvent, e.g. cyclohexane, which is emulsified in the solution of gel forming polymer, agarose. After gel formation, removal of cyclohexane by extensive washing with 50% ethanol leaves a system of large (size 20–200 μm) interconnected pores [8]. Supermacroporus matrices from moderately hydrophobic water insoluble polymers like polylactides are formed by suspending salt, e.g. NaCl, that is insoluble in organic solvent, in the polymer solution in 1,4-dioxane followed by solvent removal by freeze drying and salt leaching with water [9].

When the gel matrices are formed in moderately frozen solutions of monomeric or polymeric precursors, the solutes are concentrated in a small non-frozen part of the sample despite that the whole sample looks like a solid block. The polymerization proceeds in the frozen state resulting in the formation of a polymer network around ice crystals, which in this case function as porogen. On melting the ice, a system of large interconnected pores (1–100 μm in size depending on the nature of the polymer and polymerization conditions used) is left in place of the ice crystals [10].

The monoliths for using in 96-well plate were produced by pouring a mixture of monomers, DMAA and allyl glycidyl ether (AGE, a source of reactive groups for further covalent coupling of affinity ligands), cross-linker, *N*,*N*′-methylene-bis(acrylamide) (MBAAm) and polymerization initiators into each well of the 96-well metal mold sealed at the bottom with a removable metal plate (Fig. 1a). The sealed mold was cooled at −10 °C and the polymerization mixture in the wells was frozen within 20 min. After keeping overnight at −10 °C the mold was warmed to room temperature. The cryogel monoliths (rods 1.25 cm × 0.71 cm diameter) (Fig. 1b) were removed from the metal mold, subjected to chemical modification and ligand coupling in a batch mode and finally inserted inside the custom-made plastic 96-well plate (Fig. 1c).

The monoliths produced have pores in the size range of 10–100 μm (Fig. 2) and are of spongy morphology. Around 70–75% of the total liquid in the monoliths is easily pressed

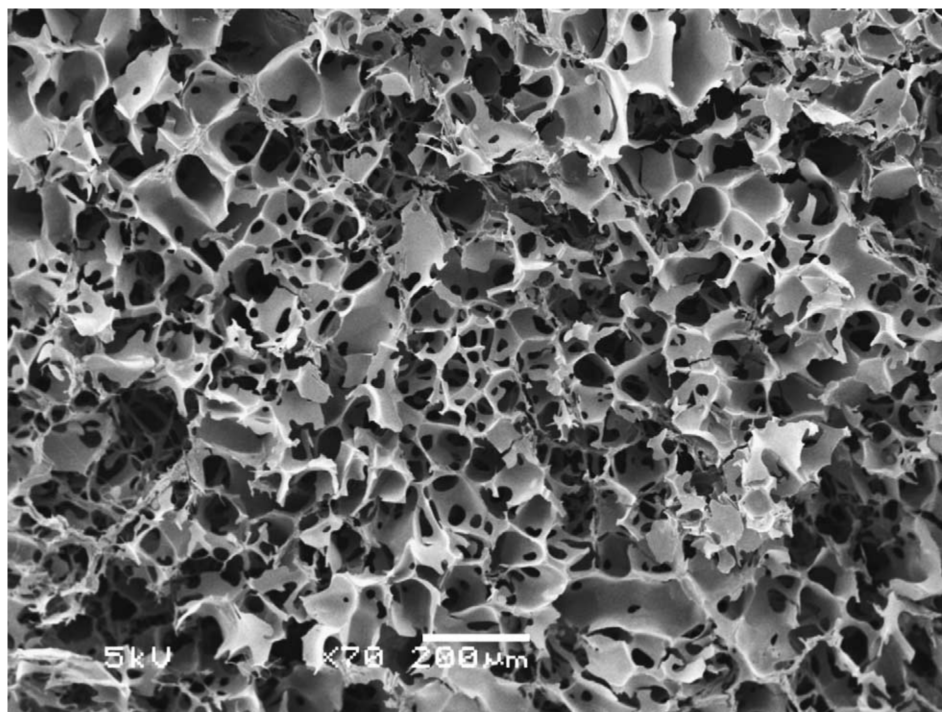

Fig. 2. Scanning electron micrographs of the supermacroporous DMAA cryogel matrix.

out mechanically. Thus, the polymer gel phase (polymer with tightly bound water) occupies less than 1/4 of the total monolith volume. Hence the polymer concentration in the gel phase (which forms the walls of the pores) is rather high, i.e. above 20% (w/v) as the initial total concentration of monomers used was about 5% (w/v). High polymer content in the pore walls makes them strong and lends elasticity to the monoliths. Thus, it was possible to compress monoliths (diameter of 0.71 cm) slightly for inserting them in the wells (diameter of 0.69 cm) of the plastic 96-well plate.

The degree of compression (i.e. the diameter of the well in the plastic plate) was optimized so that the compressed monolith is kept tightly in the well without pronounced shrinking of the pores. It is noteworthy that even uncompressed swelled monoliths have running-dry-protected properties and do not fall out of the wells with the diameter equal to the one of the cryogel itself. The functional pore size of compressed cryogels was evaluated as flow rate of water at the hydrostatic pressure of about 0.01 MPa. The compressed monolith did not retain yeast cells from the applied 1 ml pulse with OD_{600} = 1.31, i.e. 100% of applied cells were found in the flow-through.

Slightly compressed monoliths when inserted into the wells are tightly connected to the walls of the well with no leakage occurring in between the monolith and the walls. Moreover it was interesting to observe that the monoliths in the wells retain the liquid inside them, without any drainage. The application of 0.5 ml liquid into the well with the liquid-containing monolith resulted in displacing of 0.5 ml liquid from the bottom of the monolith. Use of such supermacroporous monoliths leads to a new microtiter format in which the wells are open-ended (without any filters, etc.) allowing free passage of the particles and other contaminants, while the desired protein can be retained and subsequently detected and quantified.

3.2. Direct capture of protein from cell homogenate

Parallel expression and purification of the gene products are often simplified by utilization of fusion tags, among the most common being the histidine tags. Previously we have shown that recombinant His_6-LDH could be captured directly from non-clarified crude homogenate using Cu(II)-loaded IDA-cryogels [3]. The pore size in cryogel was big enough to allow the particulate matter present in the homogenate to pass freely through the monoliths without being retained. The same system has been selected to evaluate the performance of 96-well microtiter plate with cryogel monoliths.

The histidine tag on the recombinant LDH is fairly far away from the active site of the enzyme, hence the enzyme is still catalytically active when bound to Cu(II)-loaded IDA-cryogel, allowing direct quantification of the bound enzyme. Direct capture of His_6-LDH from the crude homogenate on cryogel monolith was followed by washing out non-bound proteins and particulate material and then incubation with

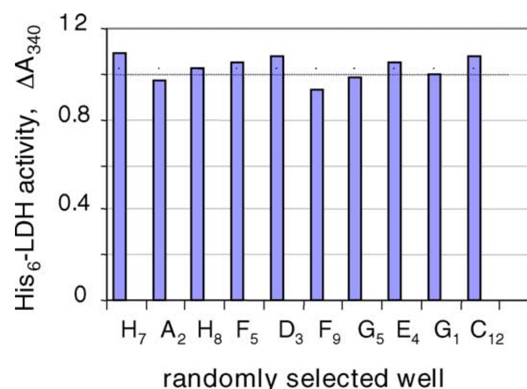

Fig. 3. Binding of His_6-LDH from 100 μl of crude homogenate to Cu(II)-IDA cryogel monoliths (0.5 ml) in 10 randomly selected wells. Non-bound protein and cell debris were washed with 0.2 M Tris–HCl buffer pH 7.3 (buffer A). After 2 min incubation of the substrate and NADH with the cryogel monoliths the reaction mixture was displaced from the wells by adding 0.5 ml of buffer A and analyzed by measuring absorbance at 340 nm. The dashed line represents the average value. The standard deviation for the values in individual wells was 0.08.

the substrate-containing solution. Due to the capillary forces, substrate-containing solution was retained in the monolith with no need in back pressure or valve to stop the flow of liquid through the monolith. The reacted solution was then displaced with a fresh portion of the buffer. In order to evaluate monolith-to-monolith variation the same portion of crude homogenate was applied to 10 randomly selected wells. Fig. 3 presents the data on His_6-LDH binding when applying 100 μl of crude homogenate on 10 different Cu(II)-IDA cryogel monoliths. Standard deviation of the experiment was 0.08.

Fig. 4 presents the data on His_6-LDH binding when applying different amounts of crude homogenate (each homogenate sample was applied to seven randomly selected wells). The amount of cofactor NADH oxidized to NAD, was linearly proportional to the amount of His_6-LDH present in

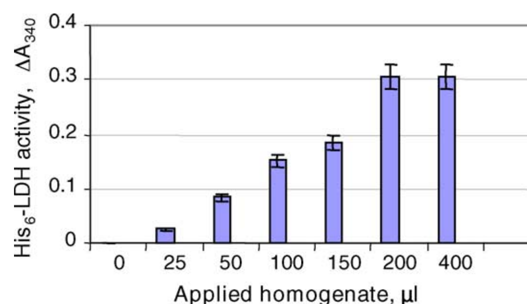

Fig. 4. Binding of His_6-LDH from different amounts of crude *E. coli* homogenate added to seven randomly selected wells containing 0.5 ml Cu(II)-IDA cryogel monoliths. Unbound protein and cell debris were washed with 0.2 M Tris-HCl buffer pH 7.3 (buffer A). After 2 min incubation of the substrate and NADH within the cryogel monoliths the reaction mixture was displaced from the wells by adding 0.5 ml of buffer A and analyzed by measuring absorbance at 340 nm. In the control experiment buffer A was used instead of cell homogenate.

Table 1
Direct assay of His_6-LDH activity in *E. coli* cell homogenate, under varying sonication conditions, using Cu(II)-IDA cryogel monoliths

Sonication conditions							His_6-LDH activity (ΔA_{340})[b]
Cycles per second	Amplitude[a] (%)						
0.5	20						0
0.7	20						0.025
0.5	20	40					0.142
0.7	20	40					0.160
0.5	20	40	60				0.175
0.7	20	40	60				0.183
0.5	20	40	60	60			0.187
0.7	20	40	60	60			0.186
0.5	20	40	60	60	60	60	0.229

[a] Sonication at each amplitude was carried out for 1 min, each treatment followed by 1 min of interruption. His_6-LDH was isolated simultaneously from several samples of cell homogenate (150 μl) obtained under different sonication conditions.
[b] Enzymatic reaction was carried out directly in microtiter plate filled with Cu(II)-IDA cryogel monoliths (0.5 ml).

the sample applied to the well within the range of 0–200 μl of the applied homogenate. No enzymatic activity was observed in the flow-through fractions within this range. Application of 400 μl of homogenate exceeded the binding capacity of the adsorbent which was found to be 67 μg His_6-LDH/ml adsorbent. Integrated direct capture and enzyme assay on the cryogel-containing microtiter plate allows fast quantification of the enzyme in a large number of non-clarified samples. The use of multiwell plate filled with Cu(II)-IDA cryogel monoliths for screening of optimal cell disruption conditions is demonstrated in Table 1.

3.3. Direct capture of protein from cell culture broth

The pore size in the cryogel monoliths is sufficiently large to allow even the whole cells to pass through non-retained. Thus the 96-well plate could be used for the direct capture of extracellularly expressed proteins from cell culture. The feasibility of such an approach was demonstrated using *E. coli* culture broth producing extracellularly recombinant His_6-tagged single chain Fv-antibody fragments (His_6-scFv). About 80–90% of His_6-scFv applied in the crude homogenate (in the range 0.3–3 μg) was recovered from Cu(II)-IDA cryogel monoliths (Fig. 5). The amount of recovered His_6-scFv is linearly proportional to the amount of applied His_6-scFv within the range of 0.3-2.1 μg. The binding capacity of Cu(II)-IDA cryogel for His_6-scFv was 4 μg/ml adsorbent. Low binding capacity can be explained by the fact that some components in the fermentation media surrounding extracellularly expressed protein bound to IMAC-ligands thus reducing the number of available ligands. His_6-scFv in the unknown non-clarified samples can be determined using the calibration curve. The use of 96-well plate allows easy parallel assay of samples, e.g. to monitor the accumulation of the His_6-scFv during the fermentation (Table 2).

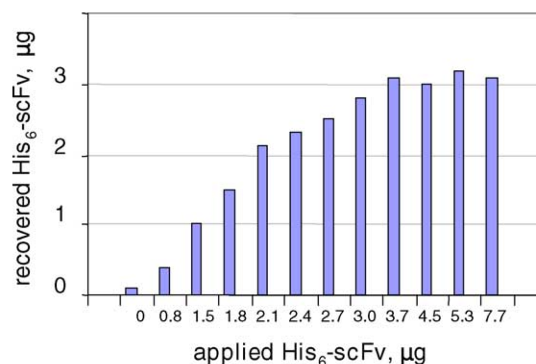

Fig. 5. The dependence of the amount of recovered His_6-scFv on the amount of His_6-scFv applied to randomly selected wells packed with Cu(II)-IDA cryogel monoliths (0.5 ml). Different volumes of *E. coli* cell culture fluid containing 30 μg/ml His_6-scFv were applied on the monoliths equilibrated with 20 mM HEPES, 0.2 M NaCl, 2 mM imidazole pH 7.0 followed by washing with the same buffer to remove cells and non-bound protein. His_6-scFv was eluted with 1 ml of 20 mM EDTA, 20 mM NaCl pH 7.5. His_6-scFv concentration was assayed as described in the text.

Table 2
Assay of extracellularly produced His_6-scFv in the culture broth after induction with isopropyl β-D-thiogalactopyranoside (IPTG) using Cu(II)-IDA cryogel monoliths

Time after induction with IPTG (h)	His_6-scFv (μg/ml broth)
0	0
1	0
2	0
3	1.7
4	3
16	12
19	16
23	18

His_6-scFv was isolated simultaneously from several samples (100 μl sample) using the microtiter plate filled with Cu(II)-IDA cryogel monoliths (0.5 ml) and quantified by indirect ELISA using peroxidase conjugated secondary antibody.

4. Conclusion

A new microtiter plate solid phase extraction system based on the hydrophilic elastic supermacroporous material, cryogel of cross-linked polydimethylacrylamide (DMAA) has been developed allowing the integrated extract clarification and capture of the target protein from large number of samples simultaneously. Moreover, the new 96-well SPE system has some attractive properties for high throughput sample processing. The capillary forces in the pores of the gel keep the liquid inside the monoliths making the monolith columns drainage protected and allowing enzymatic reactions to proceed within the cryogel matrix.

The concept of using supermacroporous gels in 96-well plate format offers many interesting possibilities to the biotechnologist. Separation of particulate matter, capturing of soluble material from particle containing media, parallel assay of large number of non-clarified samples are areas of applications that will become more abundant when the gels

become more easily available. Furthermore, the gels seem suitable for screening of ligands to select optimal chromatography conditions for protein purification.

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