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# Development of wide-pore CLEAR supports for applications involving biological macromolecules

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## Summary

The CLEAR supports were originally developed for solid-phase synthesis. In this study, two wide-pore versions of the resins were prepared by radical suspension polymerization using toluene as the porogen. Trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate was used as the cross-linking agent. Resins of two different porosities were synthesized. The permeability to biological macromolecules was investigated by size-exclusion chromatography, using the resin beads as the stationary phase. One of the resins was able to retard thyroglobulin (669 kDa) while the other, prepared with a lower amount of porogen, showed a lower fractionation range (1-60 kDa).

## Introduction

When Merrifield introduced the concept of synthesizing peptides on a solid support, low cross-linked microporous polystyrene beads were used as the solid phase [1]. This material is still used widely and works well for many applications. A broad range of other materials have been developed and tested since then. Some of these have been shown to work well and have been commercialized, while others have been abandoned. The activities in the area have been spurred by the fact that some peptide sequences are difficult to synthesize on conventional polystyrene supports. The growing interest in solid-phase organic synthesis and combinatorial chemistry has also created a need of novel supports [2].

The Pepsyn [3,4] and the Polyhipe [5] supports were developed based on the idea that the supports should have similar polarity as the peptide backbone. The hydrophobic nature of the polystyrene resins was modified in PEG-PS [6], TentaGel [7], and ArgoGel [8] by the introduction of polyethylene glycol chains. The PEGA support [9] is made from polyethylene glycol dimethacrylamide. The main constituent of the CLEAR support [10] is trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate, a branched cross-linker containing short polyethylene glycol chains. This solid phase was developed to meet the need of biocompatible supports that swell in a broad range of solvents, including water. The support is stable under the conditions used for peptide synthesis and can be applied both in batch-wise and continuous-flow peptide synthesizers. Several „difficult sequences“ have been synthesized successfully on CLEAR.

In solid-phase synthesis, the product is normally cleaved from the support and retrieved at the end of the synthesis. For some applications, it is timesaving to keep the product on the support and use the product-resin conjugate directly for further investigations. To carry out bioassays while the compounds are still attached to the support is attractive for high-throughput screenings of combinatorial libraries. Such on-resin screenings impose requirements on the solid phase regarding solvent compatibility; it must be compatible with both the solvent used during the synthesis and with water. Furthermore, the support should have a sufficient porosity to allow biological macromolecules to enter the material. Another application of resins that work both in organic solvents and in water is for the synthesis of affinity ligands and the direct use of the ligand-resin conjugate as the stationary phase in affinity chromatography or solid-phase extraction. This dual function of the resin obviates the need of first cleaving the ligand from the solid-phase synthesis support and then couple it to a stationary phase.

Several studies have been done to investigate the permeability of commercial supports used for solid-phase synthesis. One study reported that trypsin (23.5 kDa) could penetrate TentaGel [11] while another study showed that chymotrypsin (22 kDa) could not reach and hydrolyze peptide substrates located in the interior of the beads [12]. Low-cross-linked versions of the PEGA resin have been shown to be permeable to antibodies (150 kDa) [13]. In this study, we have prepared wide-pore versions of the CLEAR support and investigated the accessibility of their pores to macromolecules.

## Experimental

### *Materials*

Trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate, methyl acrylate, and lauric acid were obtained from Aldrich (Milwaukee, WI, USA). 2,2'-azobisisobutyronitrile (AIBN) was from Acros (Geel, Belgium). Blue Dextran was purchased from Pharmacia (Uppsala, Sweden). Leu-enkephalin amide and ACP(65-74) amide were prepared as previously described on CLEAR [10]. Cytochrome C (bovine heart), myoglobin (horse heart), soy-bean trypsin inhibitor, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Lactate dehydrogenase (hog muscle) was from Boehringer Mannheim (Germany). Insulin (bovine pancreas) and vitamin $B_{12}$ were from ICN (Aurora, OH, USA). Organic solvents (*p.a.* grade) were from LabScan (Dublin, Ireland).

### *Suspension polymerization*

Spherical beads were prepared as previously described [10] by suspension polymerization using a reactor and an overhead stirrer following the designs described by Arshady and Ledwith [14]. An aqueous phase consisting of deionized water (250 mL; previously purged with a stream of argon for 5 min) and ammonium laureate solution (10 mL of 1% lauric acid in water, adjusted to pH 10.3 with conc. aqueous $NH_3$), and an organic phase (which had been purged with a stream of argon for 5 min) consisting of methyl acrylate (1.72 g, 20 mmol), trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate (18,26 g, 20 mmol), AIBN (1.0 g, 6.0 mmol), and toluene

(70 mL) (*wide-pore CLEAR A*); or trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate (13.7 g, 15 mmol), AIBN (1.0 g, 6.0 mmol), and toluene (100 mL) (*wide-pore CLEAR B*) were stirred (400 rpm) under an argon atmosphere for 1.5 h at 70 °C. The beads were collected on a sintered glass filter funnel, washed with water (~ 10 L) and methanol (~ 500 mL) and sieved in water using 125- and 150-µm sieves. Wide-pore CLEAR A beads (125-150 µm) were collected on a sintered glass filter funnel, dried with methanol, and finally dried *in vacuo* overnight (yield: 16.1 g). Wide-pore CLEAR B beads (> 150 µm) could not be dried on a glass filter funnel since they had a hydrogel-like character and were therefore packed directly into the column.

*Scanning electron microscopy (SEM)*

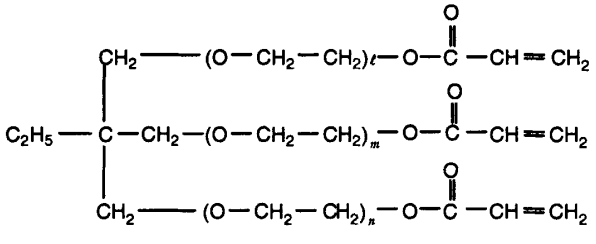
The textures of CLEAR materials were studied by scanning electron microscopy (SEM) using an ISI-100A scanning electron microscope. Polymer samples were mounted on SEM stubs and were coated with platinum. Images were recorded at accelerating voltages of 15 kV.

*Size-exclusion chromatography*

The polymer beads (4.6 g of wide-pore CLEAR A and 2.2 g of wide-pore CLEAR B) were suspended in buffer and packed at 0.2 mL/min into Bio-Rad Econo-column chromatography columns (1.5 x 20 cm) using a 2232 Microperpex S peristaltic pump (LKB, Bromma, Sweden). The final bed height of wide-pore CLEAR A was 148 mm and of wide-pore CLEAR B 145 mm. The eluent during both the packing and the chromatography was 10 mM sodium phosphate buffer pH 7.0. For wide-pore CLEAR A, the molecular weight markers included ACP (65-74) amide, insulin, soybean trypsin inhibitor, cytochrome C, myoglobin, and BSA. For wide-pore CLEAR B, vitamin B12, insulin, soybean trypsin inhibitor, cytochrome C, myoglobin, BSA, lactate dehydrogenase, and thyroglobulin were used as molecular weight markers. Each molecular weight marker (1.5-2 mg) were dissolved in buffer and applied to the column bed manually using a pipette. Elution was carried out at 0.06 mL/min. Detection was done at 278 nm or 206 nm (the latter wavelength for insulin, Leu-enkephalin amide, and ACP (65-74) amide) using a 2138 Uvicord S detector (LKB, Bromma, Sweden). The void volume ($V_o$) was determined with Blue Dextran. The distribution coefficient ($K_{av}$) was calculated as $K_{av} = (V_e-V_o)/(V_t-V_o)$ where $V_e$ is the elution volume of the sample and $V_t$ is the total bed volume.

## Results and discussion

Two wide-pore CLEAR supports (A and B) were prepared by radical suspension polymerization using toluene as the porogen. The main component of the polymers was the branched cross-linker trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate (**1**).



**1** $\ell + m + n \sim 14$

We anticipated that both the amount of the porogen used during the polymerization and the degree of cross-linking could affect the porosity and the swelling properties of the resulting beads. Higher amounts of the porogen were used in this study compared to the amounts normally used for the synthesis of the CLEAR supports [10]. The volume ratio of porogen to cross-linker was 2.5 times higher in wide-pore CLEAR A than in regular CLEAR and 5 times higher in wide-pore CLEAR B. This resulted, as expected, in a more porous nature of both A and B. Regular CLEAR supports are prepared by co-polymerization of the cross-linker with an amino-functionalized monomer [10]. In wide-pore CLEAR A, methyl acrylate was substituted for the amino-functionalized monomer to maintain the same degree of cross-linking as in regular CLEAR (amino-functionalized monomers were not used since the free amino groups could interact with the peptides and proteins used as molecular weight markers in the size-exclusion chromatography). In resin B, the co-monomer was omitted and this resin therefore had a higher degree of cross-linking. Despite this, B was more porous than A and showed a hydrogel-like character. The higher porosity was due to the larger amount of porogen used during the polymerization. The swollen beads of both A and B were spherical as seen by light microscopy. The beads of B appeared to be softer than those of A as they were easily deformed and either collapsed or broke upon drying in vacuum. Figures 1 and 2 show the shape and the texture of dried beads as viewed by scanning electron microscopy.

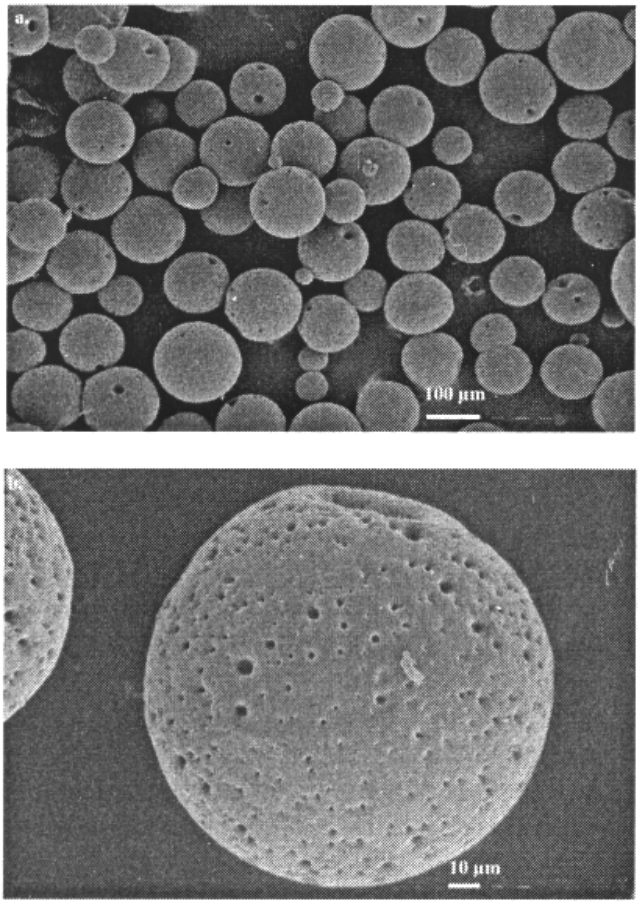



**Fig. 1** Scanning electron micrographs showing the shape and texture of dried (solvent-free and non-swelled) wide-pore CLEAR A. Accelerating voltage: 15 kV. Working magnification: (a) 100 × and (b) 600 ×.

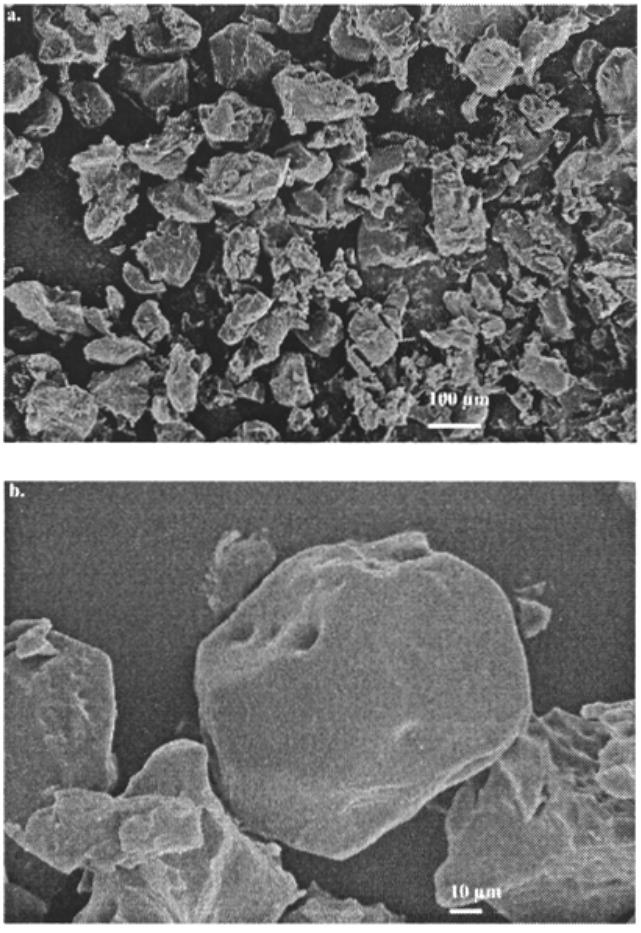



**Fig. 2** Scanning electron micrographs showing the shape and texture of dried (solvent-free and non-swelled) wide-pore CLEAR B. Accelerating voltage: 15 kV. Working magnification: (a) 50 × and (b) 600 ×.

Wide-pore CLEAR A was easier to handle than wide-pore CLEAR B and could be filtered and dried using a glass filter funnel or a syringe reaction vessel (the latter is used commonly in solid-phase syntheses and solid-phase extractions). Wide-pore CLEAR B could not be dried in this way due to the hydrogel-like character. When it was dried under vacuum, the beads broke or collapsed and were thereby destroyed irreversibly as can be seen in Figure 2.

The purpose of this investigation was to prepare biocompatible porous resins that can be used as the solid phase in solid phase synthesis. Resin A is expected to be suitable for this, since it can be handled in similar ways as the regular CLEAR supports. Further investigations need to be carried out to assess whether resin B can be used for such applications.

Both of the two wide-pore CLEAR supports prepared showed good compatibility with water. The porosity was evaluated by size-exclusion chromatography. The polymer beads were packed into columns and protein samples were eluted with phosphate buffer pH 7. As can be seen in the selectivity curves (Figure 3) and in Table 1, the fractionation range was broader for CLEAR B than for CLEAR A. This was due to the more porous character of CLEAR B. A macromolecule as large as thyroglobulin (669 kDa) was retarded on the column packed with resin B and hence entered the pores of the beads.

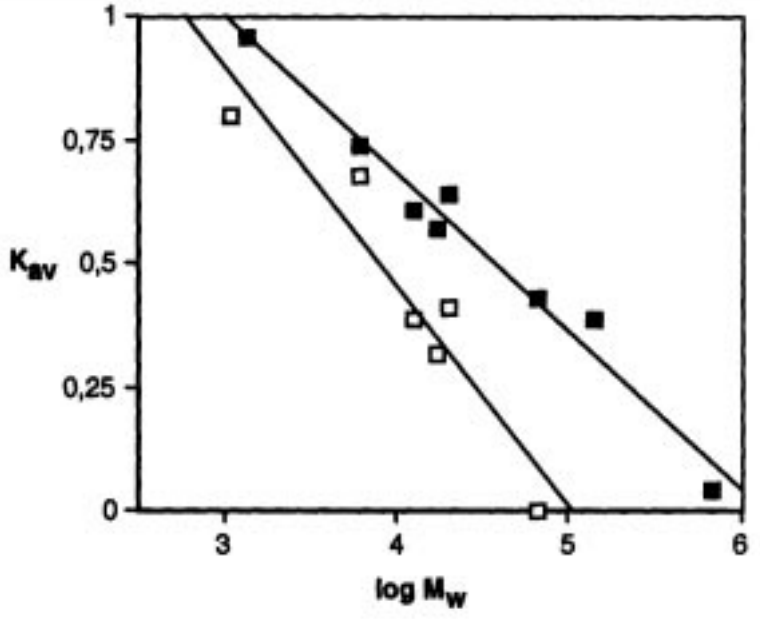



**Fig. 3** Selectivity curves of wide-pore CLEAR A (□) and B (■). Molecular markers applied to the column packed with wide-pore CLEAR A included ACP(65-74)amide (1.1 kDa), insulin (6 kDa), soybean trypsin inhibitor (20 kDa), cytochrome C (12.3 kDa), myoglobin (16.9 kDa), and BSA (66 kDa). Molecular markers applied to the column packed with wide-pore CLEAR B included vitamin B12 (1.4 kDa), insulin (6 kDa), soybean trypsin inhibitor (20.1 kDa), cytochrome C (12.3 kDa), myoglobin (16.9 kDa), BSA (66 kDa), lactate dehydrogenase (140 kDa), and thyroglobulin (669 kDa). Each molecular weight marker was applied to the column and eluted separately. The distribution coefficient ($K_{av}$) was calculated as described in the experimental section.

The swelling of the polymers were estimated from the bed volume of the packed columns (Table 1). The swelling of wide-pore CLEAR B was approximately twice that of wide-pore CLEAR A. As expected, there seems to be a correlation between both the swelling and the porosity of the beads and the amount of the porogen used during the polymerization.

**Table 1** Characteristics of wide-pore CLEAR supports.

| | Volume ratio cross-linker : porogen | Swelling in water (mL/g) | Fractionation range[a] (kDa) |
|---|---|---|---|
| Wide-pore CLEAR A | 1 : 4 | 6 | 1 – 60 |
| Wide-pore CLEAR B | 1 : 8 | 12 | 2 – 660 |
| Regular CLEAR[b] | 1 : 1.6 | 4 | n.d.[c] |

[a] *The indicated fractionation ranges correspond to $K_{av}$ between 0.1 and 0.9 in Figure 3.*
[b] *The data on regular CLEAR was obtained from ref. 10.*
[c] *The fractionation range of regular CLEAR was not determined (n.d.) since it contains polar groups that could interact with eluted proteins used as molecular weight markers.*

## Conclusions

These results show that the CLEAR supports can be made porous enough to allow macromolecules as large as thyroglobulin (669 kDa) to permeate the material. Further investigations will be on the evaluation of wide-pore CLEAR supports for solid-phase synthesis of peptide ligands and the subsequent utilization of the peptide-resin as the stationary phase in affinity chromatography.

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