

Review

# Large Volume Monolithic Stationary Phases: Preparation, Properties, and Applications

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*Dedicated to the memory of the late Prof. Dr. Valentin Koloini*

## Abstract

Monoliths represent the 4<sup>th</sup> generation of chromatographic supports. They consist of a single piece of highly porous material with interconnected flow through pores. Because of that transport is based on convection what results in a flow unaffected separation and dynamic binding capacity. This is especially important when large molecular weight molecules such as proteins, DNA or viruses have to be purified. For this purpose large volume monolithic columns are needed. In this article preparation of such columns is described together with their main applications. The article is dedicated to Prof. Tine Koloini who substantially contributed to this topic.

**Keywords:** Chromatography, monoliths, preparative, macromolecules

## 1. Introduction

Chromatographic monoliths are a particular type of chromatographic stationary phases consisting of a single piece of porous material. Because of the highly interconnected open and large pores – channels the mobile phase is flowing through them without severe flow restrictions. Therefore, the main transport mechanism of the molecules to be separated is convection. Because of that, a mass exchange between the mobile and stationary phase is very fast, similar to the membrane supports, which are however by definition very thin. On the other hand, monoliths can be prepared in various shapes and dimensions. They also exhibit several other advantageous features:

- flow unaffected separation resolution and dynamic binding capacity<sup>1,2</sup>
- high porosity reaching values up to 90%<sup>3</sup> and significantly exceeding the porosity of the particulate beds
- high dynamic binding capacity for very large molecules<sup>4</sup> due to unhindered surface accessibility

Because of the described properties, monoliths have been implemented in several areas of separation science.

Due to an easy covalent bonding to various surfaces they are an almost ideal matrix for flow-through microchips or capillaries and plethora of examples are reported in the literature.<sup>5,6,7</sup>

Despite significant success of the monoliths on a micro and analytical scale, few reports in the literature describe applications of large volume monoliths.<sup>8,9,10</sup> This is rather surprising taking into account several important features, especially low pressure drop combined with flow unaffected dynamic binding capacity, resulting in a very high productivity of purification process. Lack of the large monolithic units must therefore be attributed to the difficulties related to their preparation. In this article, we briefly describe the preparation of cylindrical shape large volume monoliths and their application, the field where Prof. Koloini contributed significantly.

### 1. 1. Design of the Large Volume Radial Methacrylate Monolithic Columns

Currently, there is still ongoing discussion about the proper ways for scaling-up the chromatographic columns, especially in the field of biochromatography, where the

large scale monoliths are a support of choice. To preserve constant quality of the product obtained from purification process, several criteria were proposed, among them constant column height or length, constant residence time, constant  $L/d_p$  ratio, etc.<sup>11</sup> For some of them, like for  $L/d_p$  criterion for which the particle diameter should be determined, implementation on the monoliths is not obvious since it is difficult to define a particle diameter. This difficulty has been overcome by Tallarek et al. proposing a phenomenological approach introducing an equivalent of the particle diameter based on the estimation of friction factor.<sup>12</sup> For methacrylate monoliths  $d_p$  was estimated from the measurement of peak broadening and pressure drop.<sup>13</sup> Besides considering the abovementioned approaches, methacrylate based monoliths were scaled-up following two main ideas:

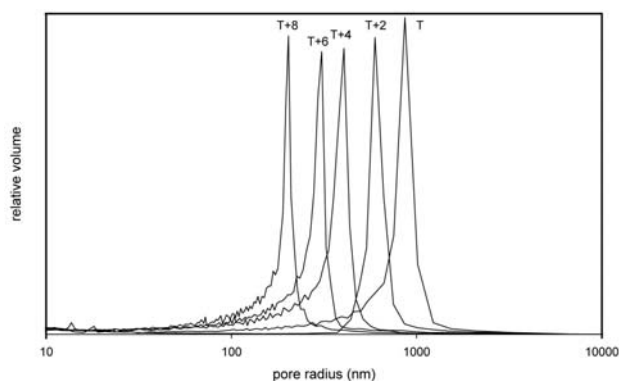
- main field of usage is the purification of large molecules like large proteins, DNA or even viruses
- microscopic structure reflected in pore size distribution should be constant regardless the monolith volume

Purification of macromolecules has several peculiarities, which significantly influence the design of chromatographic columns. Macromolecules interact with the surface via several binding sites.<sup>14,15</sup> Consequently, their adsorption isotherm is extremely steep, almost rectangular, reflecting in a very narrow mobile phase window within which they are eluted under isocratic conditions. Because of that, mobile phase gradients are implemented for their purification. For this type of elution, the effect of column length on the resolution is significantly reduced in comparison to isocratic elution. In fact, there is an optimal column length for a given system and a gradient slope, which is found to be very short for large molecules.<sup>16,17</sup> Because of that, even very short columns exhibit high separation efficiency when elution via linear or stepwise gradient elution is used.<sup>18</sup> Very recently it was demonstrated that at extremely high number of interacting sites between the molecules and the matrix encountered at DNA purification, significant self-sharpening effect for convective media like monoliths is present. This results in a further increase of column efficiency.<sup>19</sup>

Preservation of monolithic microstructure is not related directly to any physical phenomenon but provides simplicity to chromatographic methods transfer. Keeping the constant structure, the resin efficiency, normally expressed in terms of HETP, is also kept constant.

Structure of the methacrylate monoliths depends on several factors: on the composition of the monomer mixture comprising of monomer, crosslinker, porogens and initiator as well as on the polymerization temperature. Especially temperature is a very powerful tool since it enables preparation of different structures of the monolith without changing its chemical composition. This phenomenon arises from the very nature of initiator. During initiator degradation free radicals are released. The higher the

temperature, more radicals are formed per time unit initiating formation of a higher number of nuclei, which continuously grow. Because the total amount of the monomers is limited, higher number of the nuclei results in reduction of their size and consequently in formation of smaller pores. This process is extremely sensitive since even a variation of the polymerization temperature for only 8 °C shifts pore size for almost an order of magnitude.<sup>20</sup> (Figure 1) Being an excellent tool for tailoring the monolith structure, temperature also represents one of the main challenges for the preparation of large volume methacrylate monolithic columns.



**Figure 1:** Effect of the polymerisation temperature on the pore size distribution. At the highest temperature ( $T + 8$ ) the pore radius is 200 nm while at the lowest  $T$  the pores are much larger with the pore radius of 850 nm (Reprinted with permission from 20).

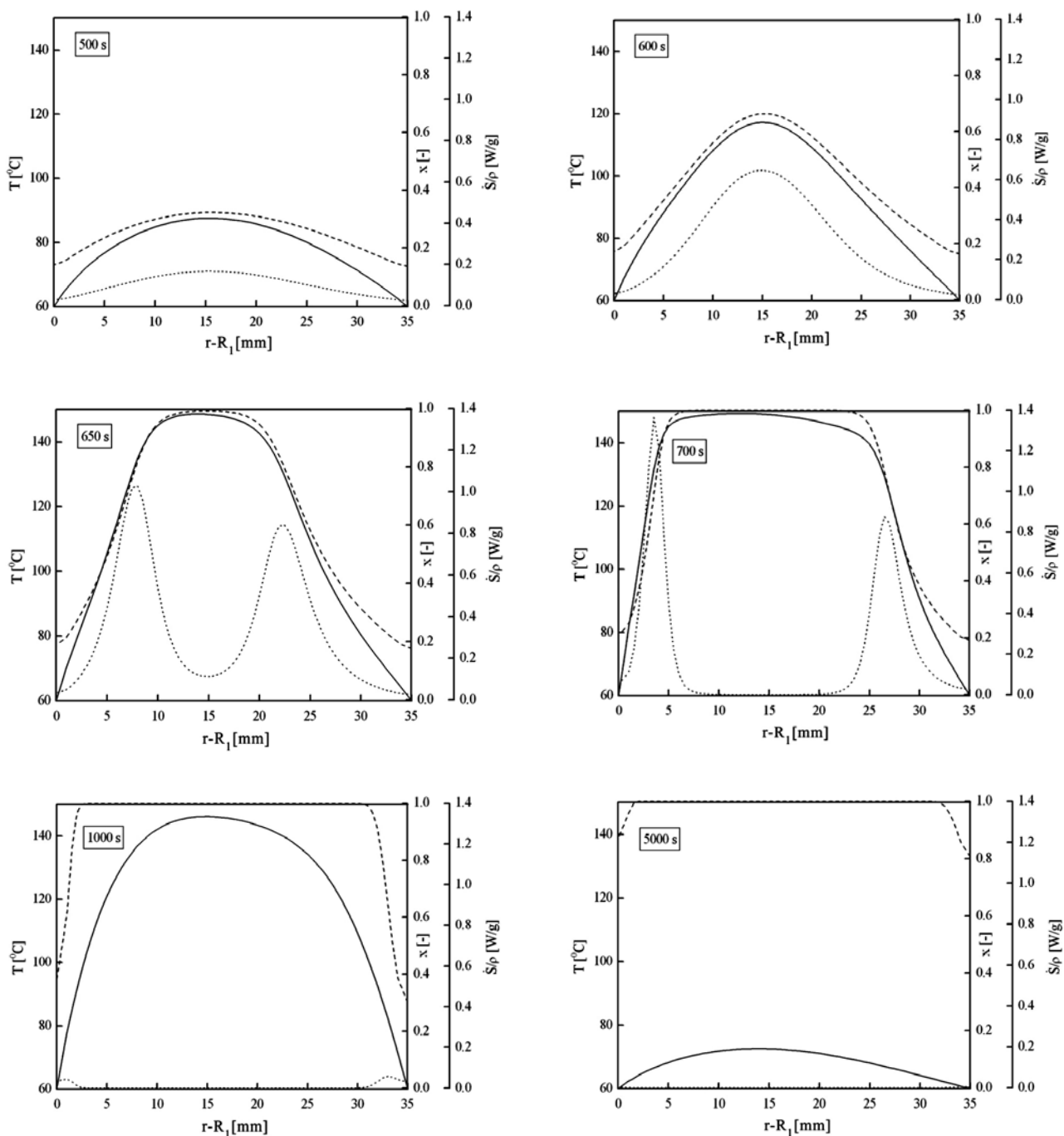
Methacrylate polymerization is a very exothermic reaction releasing during polymerization of methacrylate monoliths around 190 J/g of heat.<sup>21</sup> To obtain single piece monolithic structure bulk polymerization has to be implemented and any mixing has to be avoided. Because of that, generated heat can not be effectively dissipated, therefore an increase of the temperature inside the polymerization mixture during the polymerization occurs. At the maximal polymerization rate the increase can be as high as 80 °C.<sup>20</sup> Taking into account that already one tenth of this value dramatically changes the pore size it can be anticipated that such increase results in extremely non-homogeneous pore distribution, indeed experimentally demonstrated.<sup>10</sup>

To obtain a uniform monolithic structure, the temperature increase inside the monomer mixture during the polymerization should be small. Due to a limited flexibility in heat dissipation the monolith thickness should be carefully controlled.

The appropriate maximal monolith thickness was determined by mathematical modeling. Heat is released through a chemical bond formation during polymerization. Therefore, the amount of heat released should be proportional to the reaction kinetics. By measuring the heat release we were able to determine the reaction order

as well as to determine heat release per volume unit of polymerization mixture. Details about the determination of reaction kinetics and mathematical modeling can be found elsewhere.<sup>21,22</sup> The outcome of this analysis is the maximal allowed monolith thickness providing uniform monolith structure, together with a time required to complete polymerization over the entire monolith volume (Figure 2).

As already stated, Convective Interaction Media monoliths were developed to be used for purification of large molecules, typically proteins, DNA or viruses. Therefore, columns with rather short bed can be used. Since we are not any longer bound to chromatographic column of substantial length, different scale-up designs are possible. Especially three of them seems to be very suitable: rod format, disk format and tube format. As described in



**Figure 2.** Time sequences of the temperature, conversion and generated heat flow distributions in an annular shaped mould. Dimensions of the mould:  $R_1 = 5$  mm,  $R_2 = 40$  mm. Time scale is given in the Figure. Solid line represents temperature distribution; dashed line represents conversion profile and dotted line represents generated heat flow (Reprinted with permission from 22).

details elsewhere,<sup>23</sup> the tube format was found to be the most advantageous since it gives a high degree of flexibility (tube diameter as well as tube height can vary) combined with the low pressure drop. That is the main reason for the construction of tubular shaped large volume methacrylate monolithic columns. Monoliths of different volumes up to 8000 ml were constructed (Figure 3). Details about the chromatographic columns comprising such monoliths are described elsewhere.<sup>24,25</sup>



Figure 3: Cylinder shape monoliths of different volumes.

Despite the limitation of the monolith thickness related to the preparation problems, cylindrical shape monolith of desired thickness can easily be constructed, polymerizing several cylinders of appropriate dimensions and inserting one into another, so called “tube-in-tube” approach. Furthermore, each of the cylinders can bear different chemistry, therefore multidimensional chromatographic column, named CLC (Conjoint Liquid Chromatography) for the methacrylate monoliths, can be prepared. In this way, extra column broadening, which occurs when connecting several columns consecutively, is significantly reduced.<sup>26,27</sup>

## 1. 2. Properties of Radial Large Volume Methacrylate Monolithic Columns and Their Applications

To exploit all the benefits of a tubular approach, we have to change the commonly applied axial flow of the mobile phase into a radial one. Entering into the chromatographic column, mobile phase is directed into the chan-

nel extending over the entire outer surface of the monolith. In this way, the mobile phase is uniformly spread over the monolith. Since the monolith is sealed between two plates, the only possible direction for the mobile phase is to penetrate into the monolith pores. After passing the monolith, the mobile phase is collected in the middle

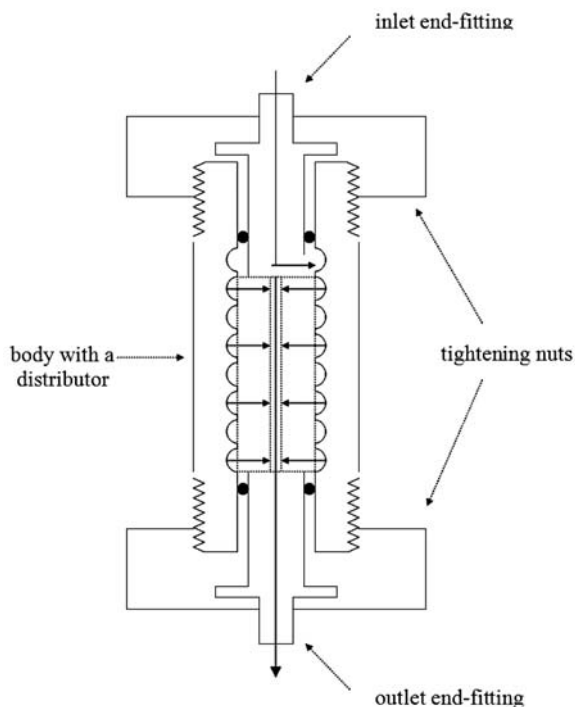


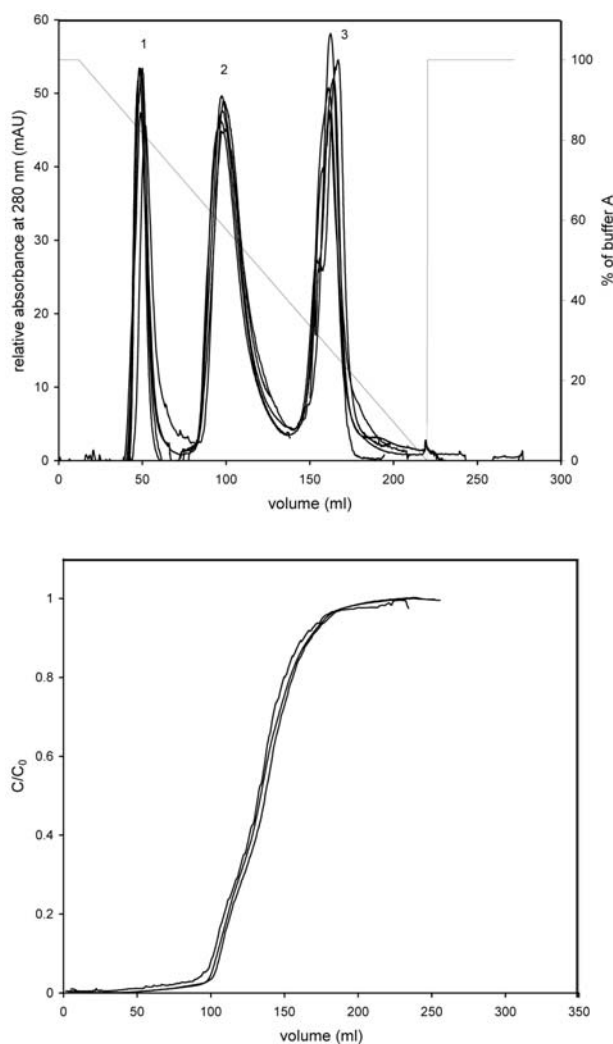
Figure 4: Schematic presentation of the chromatographic housing comprising a large volume annular monolith. Solid arrows indicate the direction of the mobile phase (Reprinted with permission from 24).

hole and directed into capillary connected with the column exit. The construction is shown in Figure 4. In this way, the entire monolithic bed is uniformly used. This can be concluded from the capacity per monolith unit which is the same as for the monolithic disks of the volume 0.34 ml, operating in an axial mode.

As already mentioned, tubular shaped monolith can be prepared in various thicknesses using a “tube-in-tube” approach. Since radial chromatographic mode is applied, the linear velocity of the mobile phase increases when passing through the monolith.<sup>24</sup> In the case of particle shaped supports, change of linear velocity results in a change of column efficiency according to the Van Deemter equation. For the monoliths, due to an extremely fast exchange between the mobile and stationary phase, diffusion limitations can be neglected resulting in flow independent resolution and dynamic binding capacity. Jungbauer and Hahn<sup>11</sup> investigated the bed usage for the 8 ml tubular shaped monolithic column assuming a certain linear velocity (critical velocity) beyond which diffusion restrictions become significant. They showed that for a critical velocity

of 1500 cm/h over 99% of bed is used. These theoretical findings are also experimentally confirmed. In Figure 5 it is demonstrated that the change of the linear velocity in the practical range of flow rates does not change neither resolution neither dynamic binding capacity. The same group also demonstrated that dispersion for a radial and axial column is comparable<sup>28</sup>. Therefore, although a radial flow is implemented, one can not discriminate by performance a radial monolithic column from an axial operating column.

So far, radial monolithic columns have been successfully implemented for purification of different types of macromolecules. In the field of proteins, anion exchan-



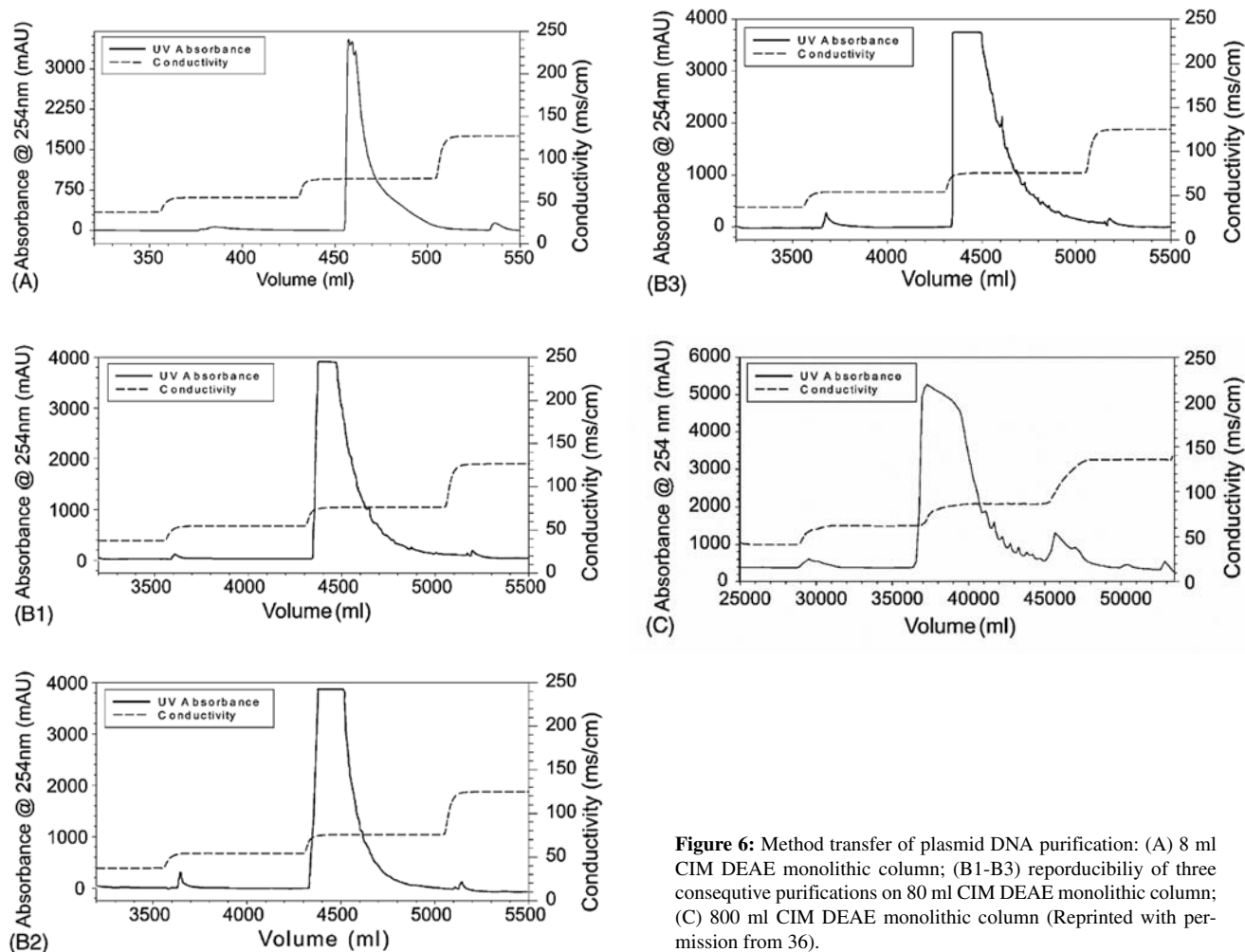
**Figure 5:** a) Effect of the flow rate on the separation efficiency. Separation of a protein mixture at six different flow rates (40, 80, 120, 160, 200 and 240 ml/min) normalised to the elution volume. Stationary phase: 80 ml CIM QA monolithic column. Sample: myoglobin (peak 1), conalbumin (peak 2) and soybean trypsin inhibitor (peak 3). b) Effect of the flow rate on the dynamic binding capacity. Flow rate: 50, 100 and 150 ml/min. Stationary phase: 80 ml CIM QA monolithic column. Sample: Bovine serum albumin (BSA) (Reprinted with permission from 24)

ge tubular monolithic columns were used for purification of lignin peroxidase isoforms,<sup>29,30</sup> having very similar molecular mass and isoelectric points. Using a combination of pH and salt gradient purification of manganese peroxidases was possible, too.<sup>30</sup> There are several reports implementing radial monolithic columns for purification of clotting factors from human plasma. Quaternary amine (QA) 8 ml unit was used for purification of factor VIII,<sup>31</sup> while up to 500 ml DEAE unit was used for purification of clotting factor IX.<sup>32,33</sup> All purifications were completed in shorter time as purification performed with conventional supports.

An interesting application was shown for preparative purification of clinical grade IgMs.<sup>34,35</sup> A three step bind-elute chromatographic process, based on hydroxyapatite as a capturing step, followed by a two step polishing performed on anion-exchange and cation-exchange short monolithic columns was developed. Since IgM displays considerable structural and biochemical heterogeneity, the sequence of chromatographic steps can be exchanged and it is dependent on the individual IgM to be purified. Due to a high binding capacity of the short monolithic columns for IgM (exceeding 30 mg/ml) which is flow-unaffected the purification productivity for 8 L monolithic column was shown to be 3 kg of IgM per day.

Another important molecule for treatment of humans is plasmid DNA (pDNA). Application of pDNA for gene therapy requires removal of impurities like RNA, g-DNA, proteins and plasmids and also open circular and linear pDNA forms. The potential of monoliths for pDNA purification was first explored by Štrancar and co-workers.<sup>20</sup> They demonstrated that short methacrylate monolithic columns can be efficiently used for intermediate purification step and exhibit the highest capacity of all tested resins.<sup>20</sup> This initial work was followed by an extensive study of Urthaler and co-workers.<sup>36</sup> Among different anion exchangers DEAE chemistry was selected due to the highest resolution, purity and recovery approaching 100%. Optimized process was scaled from 8 ml to 800 ml monolithic column (Figure 6)<sup>36</sup> and recently to 8 L monolithic column.<sup>37</sup> It was calculated that the use of monolithic columns as compared to conventional packed bed columns increased productivity 15 fold.

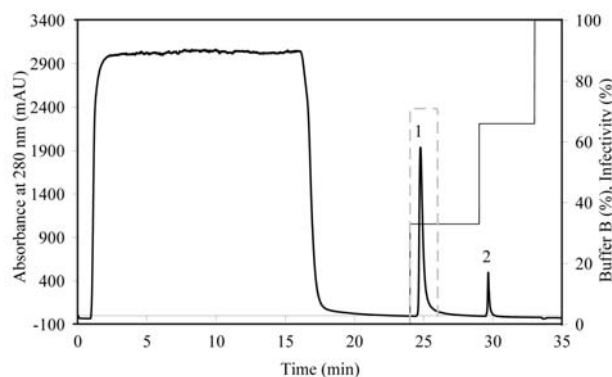
Recently, several reports about implementation of these columns for purification and concentration of viruses were published. Maurer and co-workers reported purification of H1N1 and H5N1 influenza A viruses using strong IEX monolithic chromatographic support.<sup>38</sup> By using strong anion exchange monolithic column for intermediate step 95% removal of DNA and protein was obtained combined with virus concentration. Because of assay variability of infectivity test, recovery of virus was estimated as 50–100% in this step. Monolithic columns were also implemented for purification of bacteriophages (phages).<sup>39</sup> Fast purification was performed in a few minutes giving the reproducible reco-



**Figure 6:** Method transfer of plasmid DNA purification: (A) 8 ml CIM DEAE monolithic column; (B1-B3) reproducibility of three consecutive purifications on 80 ml CIM DEAE monolithic column; (C) 800 ml CIM DEAE monolithic column (Reprinted with permission from 36).

very (measured by virus infectivity) in the range of 70% (Figure 7).

Besides for purification, large volume monolithic columns were implemented also for virus concentration from environmental waters.<sup>40</sup> In spite of being present in extremely low concentration, they could lead to pande-



**Figure 7:** Gradient elution of phage T4 on the CIM QA monolithic column. Peak 1 represents phage fraction, peak 2 is DNA (Reprinted with permission from 39).

mics or being misused as a bioterrorism weapon. With concentration of rotavirus, they showed significant decrease in limit of detection using PCR down to 100 rotavirus particles in ml of water.<sup>40</sup> Similar work was performed by Kovač and co-workers<sup>41</sup> who developed a method with monolithic columns for concentration of *Feline calicivirus* and *hepatitis A virus* in water samples, which enables detection with RT-PCR.

## 2. Conclusions

Monolithic columns combine several features, such as high porosity, flow independent resolution and dynamic binding capacity. Because of that they are very suitable for purification of large molecules. Although challenges related to the preparation of large volume monoliths with uniform structure slowed down their implementation in purification processes, using a mathematical modeling and appropriate column design this problem was successfully solved. Columns of the volumes up to 8L operating at low pressure drop were recently successfully imple-

mented in an industrial processes of pDNA and virus purification. Because of their advantageous properties it is expected that they will become a method of choice in many down stream processes of biologic macromolecules.

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## Povzetek

Monoliti predstavljajo četrto generacijo kromatografskih nosilcev. Monolit je narejen iz enega kosa poroznega materiala, ki vsebuje povezane pretočne pore. Zaradi tega transport molekul temelji na konvekciji, kar se odraža v tokovno neodvisni ločbi in dinamični vezni kapaciteti. Te lastnosti so še posebej pomembne pri čiščenju molekul z visoko molekularno maso kot so proteini, DNA in virusi. V ta namen potrebujemo monolitne kolone velikega volumna. V tem prispevku opisujemo pripravo takih kolon kot tudi njihovo uporabo na različnih primerih. Članek je posvečen prof. dr. Tinetu Koloiniju, ki je znatno prispeval k razvoju tega področja.