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New developments and possibilities of wide-pore superficially porous particle technology applied for the liquid chromatographic analysis of therapeutic proteins

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Abstract

This review paper discusses about the success of columns packed with superficially porous particles (SPP) in liquid chromatography for the analysis of peptides and proteins. First, it summarizes the history of SPP, including the development of different SPP generations from particles of 50 µm to sub-2 µm. It also critically discusses the improved kinetic performance of SPP particles in comparison to fully porous particles. The current trends and applications of columns packed with SPPs for the analysis of peptides and proteins (including mAbs and ADC at the intact and sub-unit levels) are shown, as well. Finally, some of the potential perspectives for this technology are also described, including the radially oriented mesopores or the applicability of the technology for chiral separations.

Keywords:

Superficially porous particles, core-shell, wide-pore, protein, monoclonal antibody, antibody-drug-conjugate

1. History of columns packed with SPP particles

In the recent development of particle technology targeted for liquid chromatography (LC), the use of superficially porous (SPP or often called as shell, core-shell, fused-core or partially-porous) particles has received considerable attention [1,2]. SPPs manifest the advantages of porous and nonporous particles. Knox was the first to recommend the use of thin films of the stationary liquid phase in liquid–liquid chromatography [3]. The concept of superficial stationary phases in LC, was first introduced Horváth and co-workers in the late 1960s [4,5]. Horváth applied 50 μm glass bead particles covered with styrene-divinylbenzene based ion exchange resin, known as pellicular packing material for the separation of nucleotides. Later, Kirkland showed, that 30–40 μm diameter SPPs provide much faster separations, compared to the large porous particles used earlier in LC [6]. The motivation behind the development of such materials was that columns packed with partially porous particles would have a higher efficiency than those packed with fully porous particles, because diffusion through the thin porous layer surrounding the particles would be faster than diffusion through the whole particles [2]. This acceleration of diffusion would reduce the time required for solute equilibration between the porous layer and the mobile phase or, more exactly, would effectively reduce the resistance to mass transfer through the stationary phase [2]. This feature should be especially beneficial for the separation of large molecules possessing low diffusivity. This idea made sense at a time when the average particle sizes were ca. 80 μm. Therefore 30-50 μm particles with very thin porous shell have been commercialized in the 1970s under different brand names such as Zipax, Corasil and Pellicosil [6,7,8].

In the 1990s, non-porous particles have also been considered as a valuable option for protein separations. Issaeva *et al.* showed an extremely high speed separation of proteins and peptides using 1.5 μm non-porous particles (Micra) [9]. Barder *et al.* also demonstrated that the efficiency of columns packed with non-porous silica particles was considerably higher than that of columns packed with porous particles, especially at high flow-rates [10]. Non-porous particles can indeed provide lower mass transfer resistance and higher efficiency than porous particles, but they are suffering from a lower specific volume and sample loading capacity. Seifar *et al.* estimated a 50-fold higher sample capacity for porous particles compared to non-porous particles of the same size [11]. In another work, the loading capacity for the 1.7 μm fully porous Acquity C18 particles was found to be 16 times larger than for non-porous 1.5 μm particles [12]. Another issue related to the use of non-porous particles is its very low retention capacity compared to fully porous ones. It was shown that the average carbon load for 1.5 μm non-porous particles was about 56 times lower than for 1.7 μm Acquity C18 porous particles [12]. The lower carbon load provides a lower phase ratio for non-porous particles, which leads to significantly lower retention. Due to the above mentioned limitations, non-porous materials never had too much success.

SPP materials had had a regain interest in the year 2000 and the second generation of SPPs then appeared [13]. At this time, the commercial 5 µm particles, having an average pore size of 300 Å and 0.25 µm shell thickness (it was called Poroshell), showed excellent efficiency for macromolecule separations. Few years later a new generation of SPPs has been developed and particles having standard-pores (90 and 100 Å) were successfully applied for small molecules separations. These were the so-called sub-3 µm SPPs and their structure was very close to the optimum morphology, offering a good compromise between column efficiency and loadability. They were commercialized under the brand names of Halo, Ascentis Express and Kinetex [14,15,16]. A sub-3 µm particle with the pore size of 160 Å packing was introduced in 2010 by Advanced Material Technology (AMT) and Supelco under the brand names of Halo Peptide ES-C18 and Ascentis Express Peptide ES-C18, respectively [17,18]. An average pore size of 160 Å allowed the unrestricted access of molecules up to approximately 15 kDa, depending on the molecular conformation [19]. Kirkland *et al.* compared the efficiency of the 160 Å and 90 Å SPPs for mixtures of peptides and small proteins [18]. Small proteins (i.e., ribonuclease, insulin, cytochrome C and lysozyme) exhibited broadened peaks with the 90 Å SPP, indicating restricted diffusion, but they eluted in narrow peaks from the 160 Å SPP column.

In 2012, a larger (3.6 µm) SPP wide-pore material (0.2 µm shell thickness) was launched under the name Aeris Widepore, and seemed to be particularly promising for large protein separations including monoclonal antibody (mAb) fragments [20,21]. Its relatively large particle diameter afforded low column pressures, which could help to minimize potential on-column degradation of pressure sensitive proteins, by avoiding high shear forces, and to minimize pressure induced increases in hydrophobic retention that can contribute to peak broadening [22,23]. To analyse intact large proteins and their sub-units, the particle size and shell thickness were further optimized [24]. Both theory and previous experimental studies indicated that a thin shell should be used to compensate for the low diffusion coefficients of large molecules. To find the optimum particle morphology, three different batches of 3.4 µm particles with 400 Å pores and thick shells of 0.15, 0.20 or 0.25 µm were compared in an experimental study [24]. It was found that a 0.20 µm shell thickness (400 Å) provided the highest chromatographic performance for proteins. This material is now commercially available under the brand name HALO Protein. It was found that the larger pore size actually had more impact on the kinetic performance achieved with mAbs, than the particle size and shell thickness. The SPPs with larger particle size (3.5 µm) and pore size (450 Å) showed the highest resolution for mAbs [25]. This results led to the optimal particle design with a particle size of 3.5 µm, a thin shell of 0.25 µm and pore size of 450 Å. This material is now commercialized as AdvanceBio RP-mAb. Later, SPPs with 1000 Å pores designed specifically for separating large biomolecules and industrial polymers have been described and showed benefits compared to

300-400 Å SPPs [26]. Very recently, another wide-pore silica-based SPP with a high coverage phenyl bonding has been released and successfully applied for the analysis of mAbs and antibody-drug conjugates (ADCs) [27]. This new material (BioResolve RP mAb polyphenyl) is based on 2.7 µm particles having a shell thickness of 0.40 µm and average pore size of approximately 450 Å.

As shown, there is still a continuous development in SPP technology and more and more efficient stationary phases are regularly released. Various particle morphologies (i.e. particle size, shell thickness, pore size) are now available for protein separations, and figure 1 illustrates the history of SPP development. The aim of this paper is to review the latest developments and applications of wide-pore SPPs applied for large molecule separations and provide some guidelines for method development. Some future perspectives are also presented.

2. Advantages of SPP technology

The peak dispersion in chromatography is generally characterized by the theoretical plate height (*H*) and the number of theoretical plates (*N*). The treatment of mass transfer processes and the distribution equilibrium between the mobile and stationary phase in a column lead to equations which link the theoretical plate height to the properties of the chromatographic systems, such as the linear velocity. First, Van Deemter proposed an equation, which described the column performance as a function of the linear velocity [28]. Since then, several plate height and rate models were derived for LC, by numerous researchers. Knox suggested a useful empirical three term equation to describe the dependency of the theoretical plate height of a column as a function of linear velocity [29]. In this well-known equation, the three parameters *A*, *B*, and *C* are determined by the magnitude of band broadening due to eddy dispersion, longitudinal diffusion, and mass transfer resistance, respectively. The *A*-term depends on the quality of the column packing including: (1) the homogeneity of the packed bed structure, (2) the arrangement of the particles in the wall and the central regions of the column and probably (3) on the particle size distribution. The *B*- and *C*-terms of the plate height equation depend on analyte retention. The *B*-term increases with analyte retention, as more time is available for diffusion to take place in the stationary phase (surface diffusion). The *C*-term expresses the resistance to mass transfer and can be divided into trans-particle (or intra-particle) and external- (or film-) mass transfer resistance contributions. It is expected by the theory that the eddy dispersion contributions to the efficiency of columns packed with SPPs would correspond to the external diameter of the particle, but the internal mass-transfer resistances and longitudinal diffusion would correspond to the thickness and pore diameter of the porous layer. The initial idea of preparing SPPs was to increase the column efficiency by reducing the mass transfer resistance across the particles. However, it seems that trans-

particle mass transfer resistance is far from being the dominant contribution to band broadening in HPLC [1,2]. Indeed, the columns packed with the new generation of SPPS are successful, but for other reasons [2].

According to the theory, the intra-particle diffusivity depends on the ratio of the solid core diameter to that of the particle diameter in a SPP. This ratio is often called as *rho* (ρ) and used as a measure of particle morphology of SPPs. As this ratio increases, the mass transfer kinetics becomes faster through the particles. Similarly, the external mass transfer also depends on the structure of the particles. According to some recent experimental measurements, the mass transfer kinetic is mostly accounted for the external film mass transfer resistance across the thin layer of the mobile phase surrounding the external surface area of the particles [2]. This suggests that, the initial idea of preparing SPPs with the purpose to increase column efficiency by reducing the mass transfer resistance across the particles might provide only modest practical gains for the separation of low or medium molecular weight compounds [2]. To conclude on the mass transfer resistance, approximately 2 times lower *C*-term is expected with current SPP than with the same size fully porous particles. The gain in the *C*-term is more important for large biomolecules and less for small molecules possessing high diffusivity.

On the other hand, the presence of a solid core inside the particles has a direct consequence on the longitudinal diffusion term (*B*-term), since it decreases this contribution to the plate height by about 20 % when the ratio of the core to the particle diameter is $\rho \sim 0.6$ (which is very common for normal pore SPPs) [1,2]. However, the reduced internal porosity of the SPPs brings a limited improvement in their efficiency. Experimentally, it was implied that the solid core reduced the *B*-term by not more than 30 % in comparison to fully porous particles [30]. As a conclusion, it can be stated that recent SPPs manifest a gain of approximately 20-30 % in the longitudinal diffusion. This causes only a gain of a ~10 % increase in the total column efficiency compared to that of columns packed with fully porous particles. However, we have to keep in mind that for large molecules (possessing low diffusivity), the *B*-tem contribution is practically negligible. This benefit is only important for small molecules and for separations performed at low flow rates (long separations).

Finally, according to several experimental results, the eddy dispersion term (*A*-term) of the columns packed with SPPs is significantly smaller (~30-40 %) than that of the columns packed with fully porous particles [1,2]. It is surprising, since particle morphology should not affect the zig-zag multipath dispersion of solutes occurring in the interstitial column volume. It is still unclear whether this significant improvement in efficiency is due to the narrow particle size distribution (PSD) of SPPs [31]. Some recent studies have indeed suggested that particles displaying a very narrow PSD can lead to unprecedented low minimal plate heights [1,2]. It is however uncertain whether this finding can be purely related, because there are also other

factors that might influence the packing quality. Superficially porous particles have indeed a higher density and some of them are rougher than fully porous particles [32]. This might also have had an influence on the achieved packing quality, apart from the PSD.

To conclude on the efficiency of SPPs, the success of these materials in the separation of small molecules is not primarily a result of the decrease of the *C*-term, as it is often claimed in commercial brochures. Most importantly, the exceptional performance of columns packed with SPPs is probably caused by the important reduction of the eddy dispersion term. For large molecules, however the decrease of the *C*-term is the most important benefit of SPPs. Theoretically, as the ρ-value increases (the thinner the porous shell is), significantly lower *C* term contribution is expected. This is probably the reason why commercial wide-pore SPP materials possess higher ρ-values ($0.70 < \rho < 0.94$) than standard pore SPPs ($0.62 < \rho < 0.76$) [27,33]. On the other hand, wide-pore SPPs possess larger particle diameter ($2.7\ \mu m \leq d_p \leq 5\ \mu m$) compared to standard pore ones ($1.3\ \mu m \leq d_p \leq 5\ \mu m$), to avoid very high pressures which can drastically affect protein separations (retention, degradation, conformation).

3. Commercially available wide-pore SPPs

Currently available SPPs for proteins and peptides applications are predominantly RPLC phases, but some SPPs are also available for HILIC peptide applications [34]. RPLC offers the highest separation efficiency for mAb and related protein separations [35]. Its robustness, ease of use and possibility to hyphenate with mass spectrometry (MS) made RPLC well suited both for routine applications and R&D environment. Current RPLC SPP stationary phases applied for the separation of proteins and peptides are silica based, hydrophobically modified particles. For the analysis of moderate size proteins (15 - 20 kDa) and peptides, 1.3 - 3.6 µm (ρ = 0.60 - 0.76), 100 - 200 Å pores size SPPs can be used with mechanical and thermal stability of 600 - 1000 bar and 45 - 90°C, respectively. For the separation of larger proteins up to ~400 - 500 kDa, 2.7 - 5.0 µm (ρ = 0.63 - 0.90), 200 - 1000 Å pores size SPPs are available and can be operated at 400 - 1000 bar and 60 – 90 °C. All of these materials are well suited for use in acidic and neutral conditions, and some of them can also withstand slightly basic mobile phases as well. Where no stability data is available, columns are suggested to be operated in conditions commonly used for similar particles (e.g. max. ~600 bar for 2.6 - 3.5 µm particles, pH 2 - 7 and the lowest possible temperature at which adsorption can be avoided). Most of these widepore SPPs are modified with C18, C8 or C4 chains, some are C3, or phenyl modified using various bonding technologies. An exhaustive list of the RPLC SPPs commercially available for peptides and proteins applications are shown in Table 1.

4. Current applications of wide-pore SPPs

4.1. SPPs for the analysis of intact proteins and subunits

When separating proteins and peptides, several additional features have to be considered, compared to what is typically encountered with small molecules. One of the most important differences is the need for larger pores, to have full access to the surface and unrestricted intra-pore diffusion for large solutes. Thus, peptide separations generally require 100 - 200 Å pores, while 300 - 1000 Å pores are suitable for larger protein fragments and intact proteins.

The second generation of widepore core-shell particles (Poroshell 300) showed excellent performance in bio-macromolecule separations [13]. Roth *et al.* packed these particles into 75 µm x 150 mm capillaries and applied state-of-the-art MS analysis enabling the determination of intact proteins from complex matrices at sub-femtomole levels with excellent peak shapes and loading capacity [36]. The same material has been used for the separation of human IgG2 disulfide isomers. The method has been qualified and allowed a fast separation of the isoforms, substantially improving the throughput [37]. Li *et al.* used a Poroshell 300 column for IgG biosimilarity studies [38]. Staub *et al.* compared the possibilities of 2.6 - 2.7 µm SSPs and 1.7 µm fully porous materials using model peptides, tryptic digest and moderate size model proteins. In their work, 120 Å Poroshell and 100 Å Kinetex particles showed very similar performance to the 1.7 µm fully porous phase when separating ~12 - 64 kDa model proteins and peptides [39].

Variants of recombinant human Interferon alpha-2a have been analyzed using an Aeris Widepore C18 column. The method has been qualified and enabled the separation of the N-methionylated and oxidized variants in slightly degraded samples, more efficiently compared to the European Pharmacopeia RPLC method [40]. Separation performance of the same Aeris material was systematically evaluated in other studies and found to outperform various reference stationary phases when separating G-CSF oxidized and reduced variants [20], mAbs and mAb fragments [35,41]. Aeris Widepore columns have also been used for the separation of ADCs [27,42] and interferon related proteins [21]. It was demonstrated that depending on the gradient conditions (i.e. column length, gradient span and temperature), Aeris Widepore SPPs are suitable for both ultra-fast and ultra-high resolution separations of therapeutic proteins [41,43]. Loading capacity and kinetic efficiency of the latter was comparable to fully porous 1.7 µm particles. Interestingly, retention mechanism seemed to consist of a mixture of weak hydrophobic and pronounced strong polar interactions [21].

Morphology of wide pore SPP materials has been systematically optimized for intact protein and IgG subunit separations. It was found that 400 Å, 3.4 µm particles with 0.2 µm shell thickness and 1000 Å, 2.7 µm particles with 0.35 µm porous shell provided superior chromatographic performance for the separation of proteins up to ~ 400 kDa [24]. SPP particles of 1000 Å and 2.7 µm with 0.35 µm porous shell have been developed for the analysis of large proteins up to ~ 500 kDa and its efficiency has been compared to existing SPP and fully porous materials using a model mAb and various other model proteins [26]. Halo Protein

columns (3.4 µm particles with 0.20 µm shell thickness and 400 Å) have been used for the analysis of IgGs and their fragments [27,44] and for the analysis of antibody drug conjugates [27,42].

Possibilities of new 3.5 µm particles possessing 0.25 µm shell and 450 Å pores (AdvanceBio RP-mAb) have been demonstrated by analyzing various model proteins, intact mAbs, their fragments and peptides [25,27,45], as well as ADCs [46,47].

Recently, a high coverage phenyl bonded SPP has been introduced under the name BioResolve RP-mAb. The material has been characterized and compared to reference widepore RPLC phases using intact and fragmented IgGs and ADC. It was found that this column advantageously marries the kinetic properties of modern SPPs with desirable chemical properties of some polymeric material (such as the Agilent PRLP-1 column) [27,48]. Figure 2 shows some optimized separations of mAb and ADC subunits on a few selected columns packed with SPPs [27].

4.2. SPPs for the analysis of peptides

SPPs with 160 Å pores (Halo Peptide) have been successfully applied for the analysis of peptides and moderate size proteins up to 15 kDa [17-19,49]. Staub *et al.* reported similar results for 2.6 µm Kinetex and 2.7 µm Poroshell 120 particles when separating model and tryptic peptides and comparing the separation power of these shell particles to 1.7 µm fully porous ones [39]. Li *et al.* applied AdvanceBio (Poroshell 120) column for peptide mapping in biosimilarity studies [38].

Columns containing 2.6 µm Kinetex particles were coupled into series to explore the possibilities of high resolution peptide mapping in 1D LC. The best kinetic performance was observed when coupling six columns of 150 mm, and operating the system at 1200 bar, yielding a flow rate close to the optimum of the van Deemter curve and resulting in peak capacity of 1360 for a 480 min gradient [50]. Kinetex 2.6 µm column was used for the analysis of polypeptide antibiotics from food matrices [51]. Very fine prototype core-shell particles of 1.0 - 1.4 µm were also produced and tested using various analytes including tryptic digest. The finally commercialized 1.3 µm SPPs showed 20 – 40 % higher peak capacities compared to the reference 1.7 µm fully porous particles [52]. On the other hand, UHPLC systems with very low extra-column variance (< 10 μl^2) and high operating pressures (e.g. 1200 - 1500 bar) must be used with such columns packed with 1.3 µm SPP to attain their full benefits [53]. Tryptic mAb peptides were successfully separated on columns containing small core-shell particles of 1.3, 1.6 and 1.7 µm. The 1.3 µm particles provided the best separation, but suffered from low permeability. Alternatively, 1.6 or 1.7 µm shell particles can be applied since they showed comparable peak capacity at only two third of the operating pressure of 1.3 µm particles [54]. Indeed, 1.7 µm SPPs showed ~50% improvement in plate heights compared to 1.7 µm fully

porous materials when separating peptides and moderate size proteins, offering the possibility to achieve faster separations [16]. Figure 3 shows the separation of panitumumab tryptic peptides mAbs obtained on 1.3, 1.6 and 1.7 µm SPPs using the same chromatographic conditions [54]. Interestingly, due to differences in diffusion properties, 1.3 µm particles seemed to be more favourable for the separation of peptides than for small molecules when using longer columns and longer gradient times [55]. Finally, the 1.6 µm SPPs have been used for the determination of PEGprotein from biological matrix following tryptic digestion [56].

For interested readers, numerous further applications can be found in brochures of manufacturers reflecting that widepore SPP technology has gained more and more interest in modern analytical laboratories. Stability of wide pore SPPs at high temperatures and acidic conditions, generally applied in peptides and proteins separations are also demonstrated in many of those documents.

5. Current trends and possible developments in SPP technology

The thickness of the porous shell of SPPs influences both the separation power and retention. Decreasing the thickness of the porous layer potentially results in lower values of the *C*-term and minimum plate heights [57]. Poppe plots calculated for various shell thickness predict that the analysis time to attain a given peak capacity decreases significantly with decreasing thickness of the porous layer, but this gain is more important for large molecules [57]. However the eluent strength has to be reduced to compensate for the decrease in retention caused by the reduction of the stationary phase surface area (decrease of volume fraction). Figure 4 shows the impact of shell thickness on the plate height (*h*) as a function of mobile phase velocity (*v*) for peptides and proteins.

An interesting idea was suggested by Guiochon and co-workers to increase efficiency with SPPs for large molecules [58]. Mass transfer kinetics of proteins was found to be the fastest for columns packed with SPP particles having either a large core-to-particle ratio or having a second, external shell (bi-shell particle) made of a thin porous layer with large mesopores (200 – 300 Å) and high porosity (50 – 70 %). The structure of this external shell seems to speed up the penetration of proteins into the particles.

All the above mentioned fundamental studies suggest that thinner shells and larger pore diameters can further improve the efficiency of current SPPs for large molecule separations. As today, mAbs and related compounds (MW ≥ 150 kDa) seem to be the most promising candidates of protein therapeutics, the common wide-pore SPPs (possessing 200 – 300 Å pore diameter) need to be revised. To further optimize the efficiency of commercially available wide-pore SPPs, recently 400, 450 and 1000 Å SPPs were commercialized to analyse large proteins in reversed phase (RP) chromatography [24,25,26,27].

Today, SPPs are mostly used for RP of proteins however obviously, SPP advantages exist in most modes of liquid chromatography (sorptive modes) and therefore new SPPs are expected in hydrophobic interaction chromatography (HIC), hydrophilic interaction liquid chromatography (HILIC) and ion-exchange (IEX), as these modes are routinely used for protein characterization. Recently a HILIC 2.6 µm SPP was introduced for released and labelled glycan analysis of proteins [59]. This column contains a unique titanium based biocompatible hardware and frits.

Surprisingly, it seems that SPPs can provide high efficiency in size exclusion chromatography (SEC) as well. The thermodynamic retention factor and therefore the useful elution window is indeed limited by the ratio of pore porosity (internal) and external porosity in SEC. Therefore, large pore volume is thermodynamically advantageous in SEC, and this is why porous particles provide wider elution window and more chance to separate compounds (improved selectivity). Despite this expected behaviour, a few recent publications showed that the loss of pore volume of SPPs can be compensated by the shortening of the solute diffusion length inside the pores [26,60,61]. Then, even if the elution window is narrower, similar efficiency can be achieved due to the narrower peaks. Moreover, the analysis time can be shortened in proportion to the internal porosity.

5.1. Radially oriented mesopores (ROM-SPP)

Very recently, the so-called radially oriented mesoporous SPPs were proposed to decrease longitudinal diffusion and hence to improve the overall separation efficiency. A new process was developed (called as pseudomorphic transformation (PMT)), which is a form of micelle templating. This PMT technology produces straight, unconnected, and radially-oriented mesopores (ROMs) (Figure 5.). SPP particles with ROMs possess many advantages such as a narrower particle size distribution, thinner porous layer with high surface area and - most importantly - highly ordered, non-tortuous pore channels oriented perpendicular to the particle surface [62]. The improved efficiency of such ROMs mostly related to a smaller *B*-term probably due to the thinner porous layer and/or the unique, ordered porous straight channels. In addition, Poppe plot calculations showed superior performance of these PMT-SPPs across the entire analysis time range compared to conventional SPPs and fully porous particles. A theoretical study also confirmed the gain in longitudinal diffusion, but this gain is only interesting for molecules possessing high diffusivity (small molecules) [63].

Gritti assumed various pore shapes for ROMs in a fundamental study [64]. It was found that the eddy dispersion does not depend on neither the pore structure nor the shape. However, when assuming conical mesopore shape a decrease in *C*-term is expected. Therefore, this conical shape ROM material suits well for protein separations.

In agreement with the theory, some ROM SPPs have already been recently applied for large molecule separations. The pore size of silica SPPs with fibrous shell structure could be tuned by using organic solvents of different polarities [65]. By using benzene as oil phase, SPPs with pore size of up to 370 Å and surface area of 61 m^2/g were prepared and successfully applied for the rapid separation of small solutes, peptides, and proteins. The wide pores of the porous spheres were especially useful for separating large proteins with excellent efficiency without restricted diffusion [65].

5.2. Molecularly imprinted polymers

Molecularly imprinted polymers (MIPs) have been utilized as recognition elements for a wide range of analytes due to their high stability and remarkable mechanical properties. However, the traditional MIPs suffered from some limitations for practical applications. To broaden the application scope, multifunctional SPP MIPs have attracted increasing attention in separation. A current review discusses the recent developments of MIPs with a nonimprinted core (Core@MIP particles) and MIPs with a non-imprinted shell (MIP@Shell particles) [66]. In addition, other novel miscellaneous SPP MIPs with a hollow-core, a semi-shell, or an empty-shell are summarized.

5.3. Magnetic materials

The separation and enrichment of proteins and peptides from complex mixtures is of great importance to provide a successful identification. Core–shell structured magnetic microspheres have been widely used for the enrichment and isolation of proteins and peptides, thanks to their unique properties such as strong magnetic responsiveness, outstanding binding capacity, excellent biocompatibility, robust mechanical strength and admirable recovery [67]. The advances in the application of core–shell structured magnetic materials for proteomic analysis, including the separation and enrichment of low-concentration proteins and peptides, the selective enrichment of phosphoproteins and the selective enrichment of glycoproteins were recently reviewed by Deng *et al.* [67]. Although much progress has been made in the application of core–shell structured magnetic microspheres to the enrichment of low-abundance peptides, the design of novel functionalized magnetic nanocomposites with well-defined nanostructures and surface properties for the application in proteomics remains an area of intense research interest.

5.4. Chiral separations

In the field of enantioseparations by LC, we can expect a real revolution thanks to the use of chiral SPPs of latest generation [68]. Over the years, this field has fallen behind compared to achiral RP separations as regards ultrafast and highly efficient separations. However, new

developments in chiral particle technology may change this trend. In particular, extraordinary results and very fast enantioseparations are expected by the employment of latest generation chiral particles in supercritical fluid chromatography (SFC) [69]. Moreover, chiral stationary phases made on SPPs could be suitable, thanks to their high efficiency, in the case of challenging enantiomeric separations (e.g., chiral impurity profiling), where an extremely low concentration of one enantiomer has to be detected [70].

6. Conclusion

Columns packed with superficially porous particles are widely used in LC and gain more and more interest over the years thanks to their excellent kinetic performance and moderate operating pressure. Standard pore materials (90 – 120 Å) became very successful in the last decade for small molecule separations. Thanks to new developments, widepore SPPs are now also available and offer new possibilities for large molecule separations.

As the pore diameter is larger and shells are thinner, the mass transfer kinetics becomes faster for large solutes (possessing low diffusivity). For such reasons, very efficient separations are routinely achieved on these materials. Current particle structures and pore sizes applied for peptides (100 – 200 Å) and proteins (200 – 1000 Å) separations with very thin porous layer seem to be close to the optimal morphology. Various chemistries are being developed to avoid undesired secondary interactions which often lead to significant band broadening and poor recovery.

Second and third generation SPPs are now regularly used for the analysis of therapeutic proteins, mAbs, ADCs and related proteins at intact, sub-unit and peptide levels. Very high peak capacity can be attained on 50 – 150 mm long columns, typically within 5 – 15 minutes. When combining the exceptional kinetic performance of wide pore SPPs with the reliability of computer assisted retention modeling, significant resources can be saved during method development. From initial experiments to robustness testing and documentation of the optimized method, the whole process usually takes only few working days.

Future trends in SPP developments are focused on further morphology optimizations and finding new fields of application. Radially oriented mesopores with various shapes show significant advantages in diffusion properties which can be beneficial for the separation of large molecules.

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9. Figure/Table captions

Table 1. List and physico-chemical properties of currently available SPPs applied for peptides and proteins separations.

Figure 1. History of the development of SPP phases. The source of pictures/drawings (papers) used for this figure are referred during the text of this review. The particles (spheres) in colour are taken from available (free) brochures from different column providers.

Figure 2. Optimized separations of mAb (A) and ADC (B) subunits on selected columns packed with SPP. Illustration is based on a figure published in ref [27].

Figure 3. Chromatograms of a tryptic digest of Panitumumab on Cortecs 1.6 µm (A), Kinetex 1.7 µm (B) and Kinetex 1.3 µm (C). Mobile phase A: 0.1% TFA in water, B: 0.1% TFA in ACN. Flow rate: 0.5 mL/min, gradient: 10–30% B in 10 min. Column temperature 30 ºC, detection: UV at 210 nm (with permission from ref [54]).

Figure 4. Structure of a SPP (A) and calculated plate height curves for model peptide (B) and model protein (C) assuming various SPP structure with ρ = 0, 0.5, 0.7, 0.85 and 1 (with permission from ref [57]).

Figure 5. Schematic view of radially oriented SPP (A) and conventional SPP (B) (cross section view). Black arrows indicate the solute diffusion.

column name			particle size (µm)	core (µm)	rho	pore size (Å)	max Temperature (°C)	pH range	max pressure (bar)	total porosity	permeability (cm^2*10^{-11})
Aeris (Phenomenex)	Widepore	XB-C18	3.6	3.2	0.89	~ 200	90	1.5-9	600	0.52	21 [25]
		XB-C8$					60				
		C4$									
	Peptide/Kinetex	XB-C18	1.7$	1.3	0.76	100	90		1000	0.56	3.1 [40]
			2.6$	1.9	0.73					0.52	5.8 [39]
			3.6	2.6	0.72				600	n.a.	n.a.
Halo (Advanced Materials Technology)	Protein	ES-C18	3.4#	3	0.88	400	90	1-8	600	0.58	26 [25]
			2.7	2	0.74	1000				0.35	6.4 [39]
		C4	3.4#	3	0.88	400		2-9		0.58	26 [25]
			2.7#	2	0.74	1000				0.35	6.4 [39]
	Peptide	ES-C18	2.0#	1.2	0.6	160	90	1-8	1000	n.a.	n.a.
			2.7*#	1.7	0.63				600	0.35	6.4 [39]
			4.6#	3.3	0.72					n.a.	n.a.
		ES-CN	2.7#	1.7	0.63					0.35	6.4 [39]
			4.6#	3.3	0.72					n.a.	n.a.
		Pheny-Hexyl	2.7	1.7	0.63			2-9		0.35	6.4 [39]
AdvanceBio (Agilent)	RP-mAb	C4	3.5	3	0.86	450	90	1-8	600	0.56	18 [25]
		SB-C8									
		Diphenyl									
	Peptide Mapping	EC-C18	2.7	1.7	0.63	120	60	2-8	600	0.37	4.6 [39]
	Peptide Plus	charged C18									
Accucore (Thermo)	150	C18	2.6	1.6	0.62	150	60	1-11	1000	n.a.	n.a.
	150	C4						2-9			
Poroshell (Agilent)	300	SB-C18	5	4.5	0.9	300	90	1-8	400	0.62	73 [41]
		SB-C8									
		SB-C3									
		Extend-C18									
BioResolve (Waters)	RP mAb	Polyphenyl	2.7	1.9	0.7	450	90	2-7	689	0.65	14 [25]
Cortecs (Waters)		C18	1.6	1.1	0.7	90	45	2-8	1000	0.56	3.5 [40]
			2.7	1.9	0.7					n.a.	n.a.
Meteoric Core (YMC)	BIO	C18	2.7	1.7	0.7	160	70	1.5-10	600	n.a.	n.a.
SpeedCore Bio (Fortis)	Peptide	C18	2.6	2	0.77	160	80	1-8	600	n.a.	n.a.
	Protein	C18	3.5	3.1	0.89	300				n.a.	n.a.
		C8								n.a.	n.a.
		C4								n.a.	n.a.
Capcell (Shiseido) Core	MP	C18	2.7	1.7	0.63	160	n.a.	2-10	600	n.a.	n.a.
	WP					300			480	n.a.	n.a.
SunShell (Chromanik Technologies)	WP	C18	2.6	1.6	0.62	160	n.a.	1.5-10	600	n.a.	n.a.
Aurashell (Horizon)	PEP	C18	2.7	1.7	0.63	200	n.a.	1-9	n.a.	n.a.	n.a.
	PRO	C18	3.5	3	0.86	300	n.a.		n.a.	n.a.	n.a.

$these phases are available packed into biocompatible titanium infused flow path column hardware under the name of bioZen

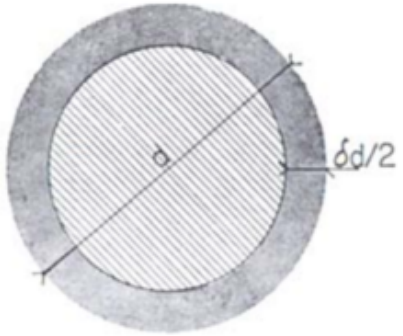
*this phase is available under the brand names of Ascentis (Supelco) Express Petide ES-C18 and Brownlee (Perkin Elmer) SPP Peptide ES-C18

#these phases are available under the brand names of Bioshell (Supelco) A160 Peptide, A400 Protein and IgG 1000Å

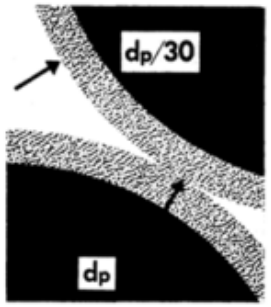
Table 1

Knox:
Idea of thin films for stationary phase

Horvath and co-workers:
50 µm pellicular particles



Kirkland:
30-40 µm controlled surface porosity supports

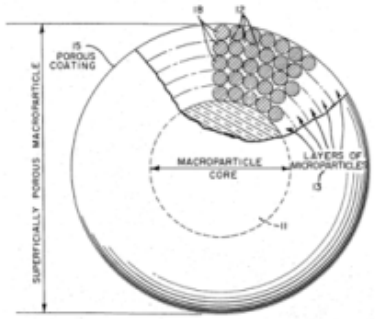


Commercially available 30-50 µm, 1st generation SPPs:

Zipax® (DuPont)

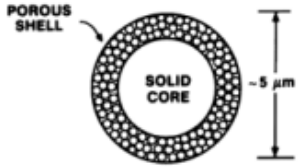
Corasil® (Waters Associates)

Pellicosil® (Macherey-Nagel)

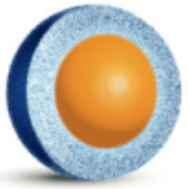


5 µm and sub-3 µm 2nd generation SPPs:

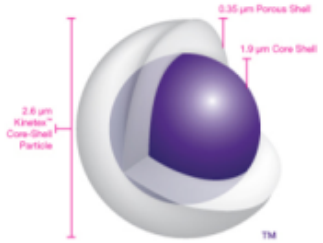
Poroshell® (Agilent)



Halo® (AMT)



Kinetex® (Phenomenex)



Widepore SPPs:

Halo® Protein and Peptide (AMT)

Aeris® (Phenomenex)

AdvanceBio® (Agilent)

Accucore® (Thermo)

BioResolve® (Waters)

Sub-2 µm 3rd generation SPPs:

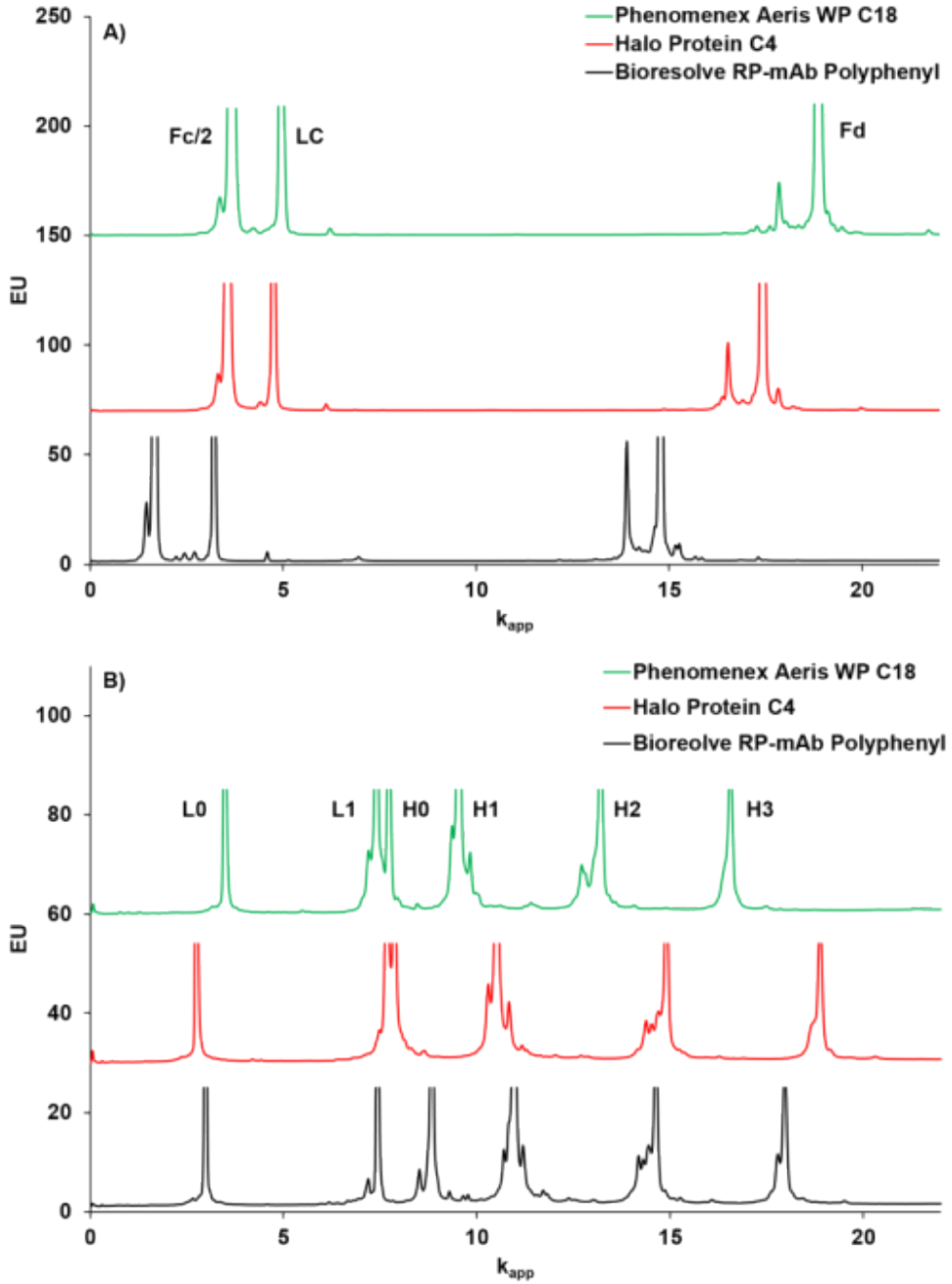
Kinetex® (Phenomenex)

Cortecs® (Waters)

Market diversification for sub-3 µm SPPs

1960s **1970-1990s** **2000s** **2010s**

Figure 1


A)
Phenomenex Aeris WP C18
Halo Protein C4
Bioresolve RP-mAb Polyphenyl
Fc/2
LC
Fd
EU
250
200
150
100
50
0
0
5
10
15
20
k_app
B)
Phenomenex Aeris WP C18
Halo Protein C4
Bioreolve RP-mAb Polyphenyl
L0
L1
H0
H1
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100
80
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20
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Figure 2

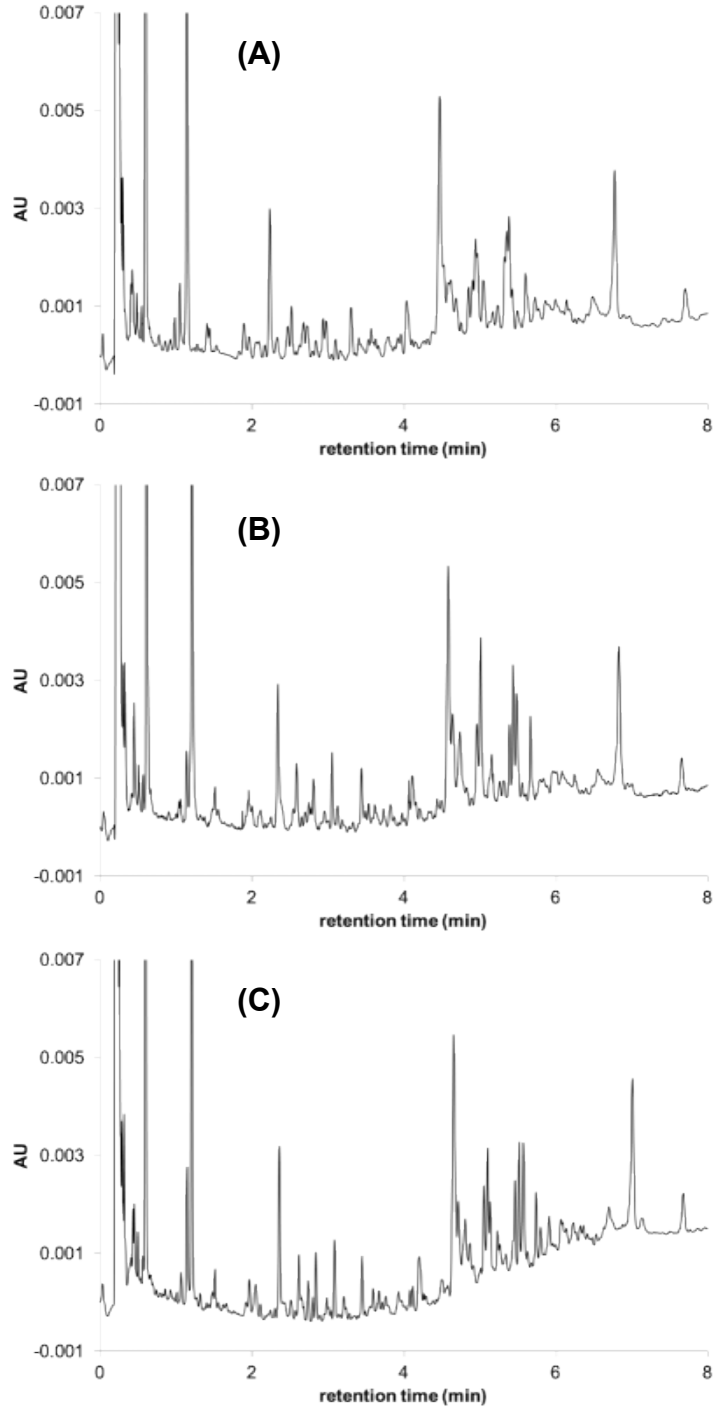

Figure 3

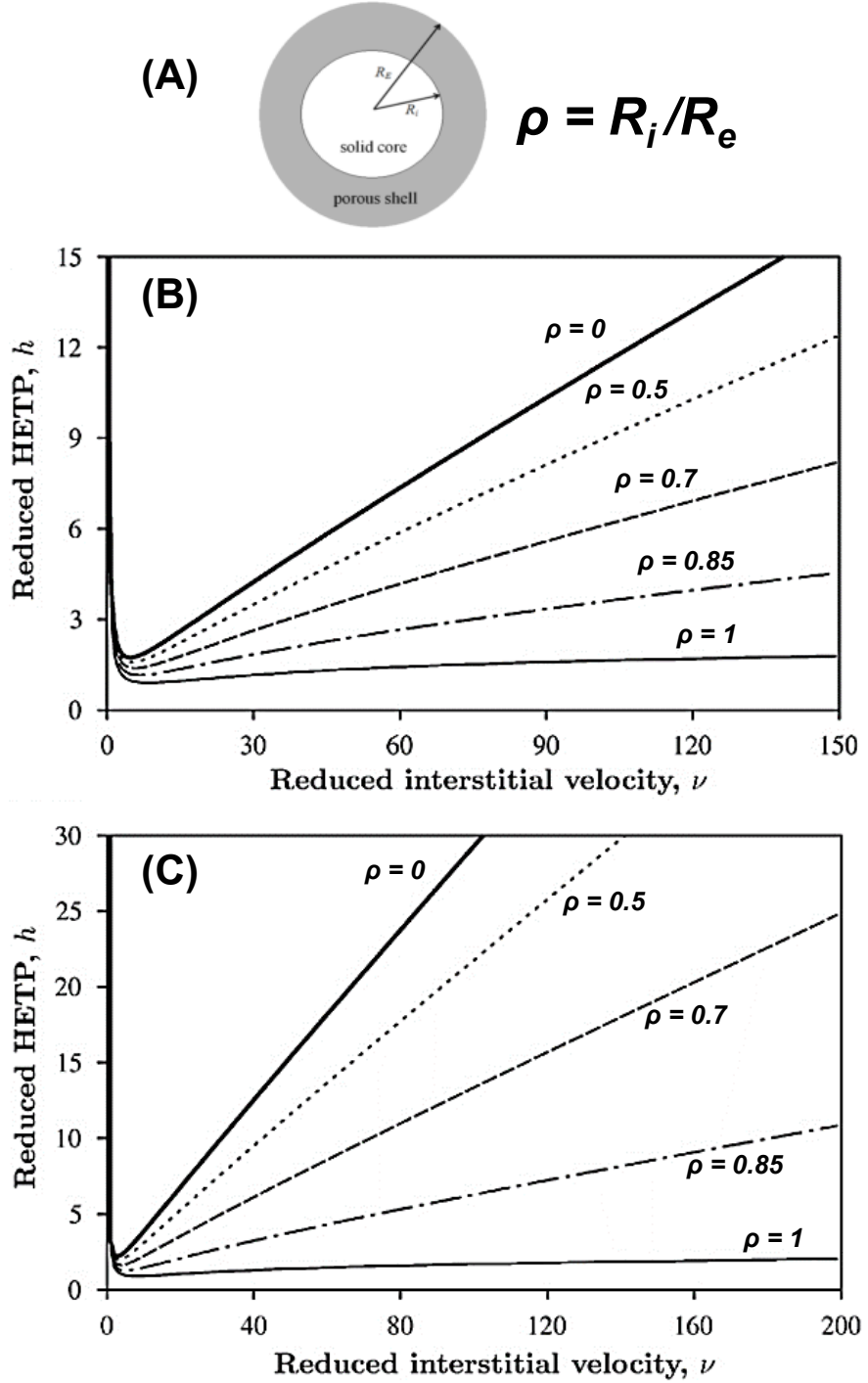
(A)
R_e
R_i
solid core
porous shell
$\rho = R_i/R_e$
(B)
Reduced HETP, h
Reduced interstitial velocity, ν
$\rho = 0$
$\rho = 0.5$
$\rho = 0.7$
$\rho = 0.85$
$\rho = 1$
0
3
6
9
12
15
0
30
60
90
120
150
(C)
Reduced HETP, h
Reduced interstitial velocity, ν
$\rho = 0$
$\rho = 0.5$
$\rho = 0.7$
$\rho = 0.85$
$\rho = 1$
0
5
10
15
20
25
30
0
40
80
120
160
200

Figure 4

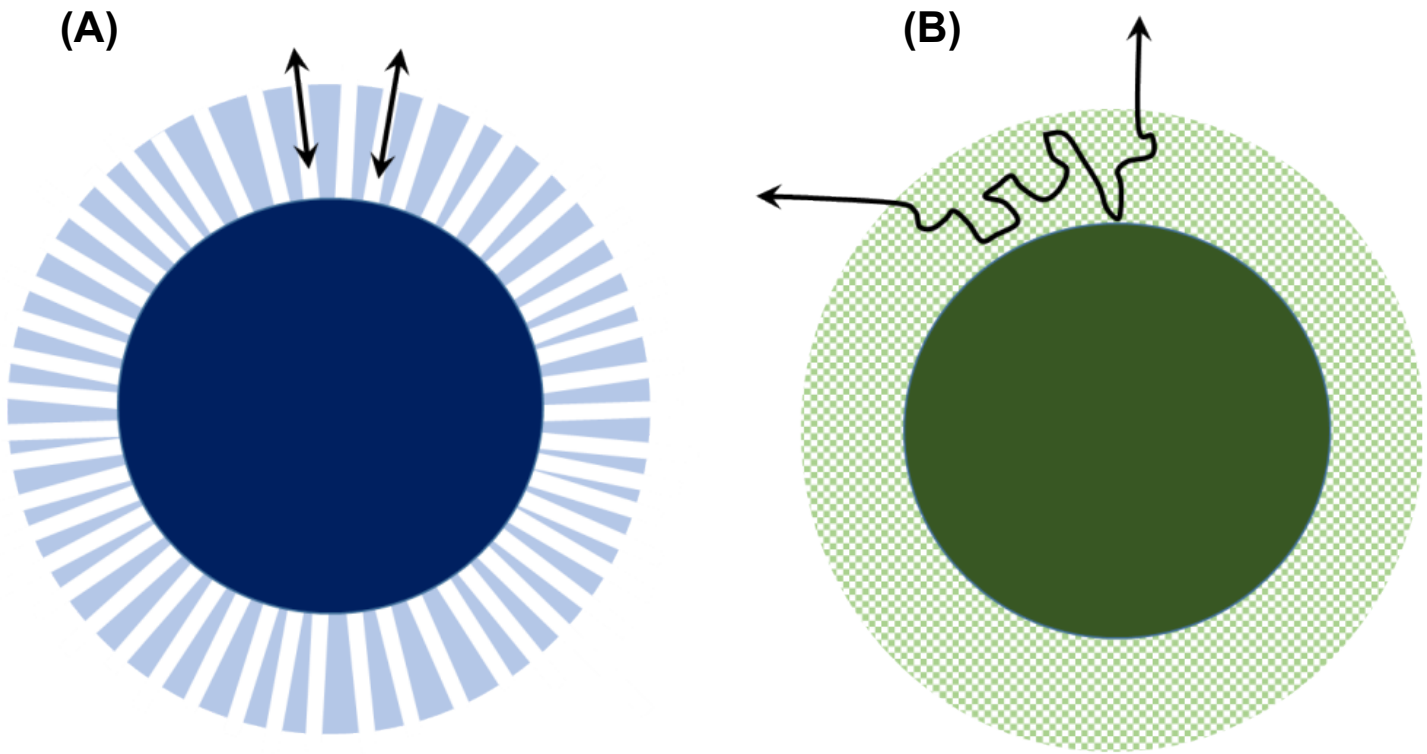

Figure 5