Recent Trends and Advances in Superficially Porous Particle Technology: Application to Large Molecule Separations

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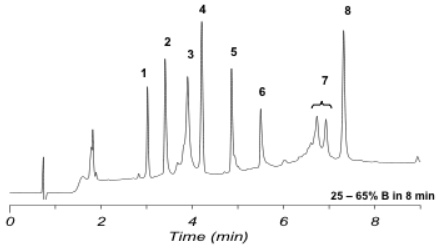
Superficially porous particles have become a major platform for the development of high performance liquid chromatography columns. Since their modern introduction in 2006, most major column manufacturers have adopted the technology. A number of particle sizes and a variety of chemical modifications are now available. In more recent years, the technology has been adopted for larger molecule separations. This contribution aims to provide some highlights of these recent trends.

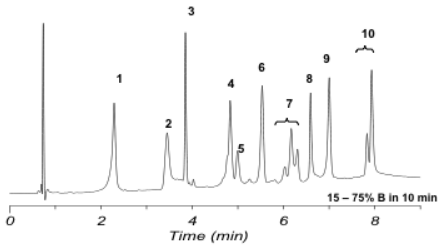
Introduction:

Over the past decade there has been a concerted effort to increase separation efficiency in high performance liquid chromatography (HPLC). The benefits of increasing efficiency include faster analysis times and enhanced resolution. The trend began with the development of smaller particles (generally termed sub-2µm) and the instrumentation advances required to handle the resultant high-pressure requirements, dawning the age of ultra-high performance liquid chromatography (UHPLC). UHPLC using sub-2µm particles has gained widespread use in many industries; however, the financial burden of purchasing new instrumentation has hindered adoption by many potential users. The modern introduction of superficially porous particles (SPP) in 2006 (Halo, Advanced Materials Technology) provided a means of attaining high separation efficiencies with less backpressure burden. The lower backpressure afforded by the SPP architecture has allowed users of both traditional HPLC systems and UHPLC alike to realise high efficiency separations.

There have been many reviews published regarding SPP properties, theoretical treatments and uses over the past several years [1-3]. This contribution is intended to provide a review of some recent trends in the development of SPP based columns, namely the adaptation of SPP technology towards large molecule separations.



Protein	kDa
1. Lysozyme	14.3
2. Haptoglobulin, phenotype 1-1	96
3. Conalbumin	76
4. Trypsin Inhibitor, soybean	20.1
5. Glutamate dehydrogenase	332
6. ß-Galactosidase	465
7. Serum albumin, chicken	44.3
8. Lactate dehydrogenase	140



Protein	kDa
1. Superoxide Dismutase	32.6
2. Serum albumin, bovine	66
3. α-lactablumin	14.2
4. ß-lactoglobulin A	18.4
5. Catalase	250
6. Carbonic Anhydrase	29
7. ß-Amylase	200
8. Glucose-6-phosphate dehydrogenase	103
9. Thioredoxin	11.7
10. Phosphorylase b	97.2

Figure 1: Protein Mixtures on BIOshell A400 Protein C4

Advantages of Superficially Porous Particles

Superficially porous particles or solid core, core-shell and Fused-Core®, as they are often termed, are characterised by having a solid core (typically nonporous silica) surrounded by several layers of porous silica. The first modern SPP phase was introduced by Advanced Materials Technology (HALO) in 2006. The particle design provides a number of advantages over fully porous materials. Peak broadening is generally modelled using the van Deemter equation ($H = A + B/\mu + C\mu$) where A represents eddy diffusion, B; axial diffusion and C; mass transfer effects. The shorter diffusion path within the 'working' porous shell yields improved mass transfer kinetics over fully porous materials (lowering the C term) allowing higher flow rates to be utilised without significantly deteriorating peak efficiency. This effect is most notable for large molecules that exhibit slower diffusion constants. Secondly, the inherent small particle size distribution resulting from the construction process has been attributed to increasing the quality of column packing homogeneity [4]. This may lead to lessening peak broadening due to eddy diffusion. Lastly, the greater permeability of SPP over similar sized fully porous particles allows for higher flow rates and thus effectively reduces axial diffusion that contributes to band-broadening. Although there is still some debate regarding the entire fundamental reasons SPP provide increased efficiency over fully porous particles, it is clear that greater efficiency with respect to

Table 1: Listing of major brands and their offerings

Brand	Manufacturer	Pore Size (Å)	Available Chemistries	Particle Size (mm)	Outer Shell Thickness (mm)
Accucore	Thermo Scientific	80	C18, C8, RP-MS, aQ, PhenylHexyl, Phenyl X, PFP, Polar Premium (RPAmide), HILIC and HILIC Urea.	2.6	0.5
Accucore Vanquish	Thermo Scientific	80	C18	1.5	
Accurcore XL	Thermo Scientific	80	C18, C8	4.0	0.6
Ascentis Express	Supelco	90	C18, C8, PhenylHexyl, RPAmide, F5, ES-CN, HILIC, OH5	5.0	0.6
Ascentis Express	Supelco	90	C18, C8, PhenylHexyl, RPAmide, F5, ES-CN, HILIC, OH5	2.7	0.5
Ascentis Express	Supelco	90	C18, F5, HILIC, OH5	2.0	0.4
BlueShell	Knauer	80	C18, C8, PhenylHexyl, PFP	2.6	0.5
Brownlee SPP	Perkin Elmer	90	C18, C8, PhenylHexyl, PFP, RPAmide, HILIC		
Capcell Core	Shiseido	90	C18	2.7	0.5
Coresep	SIELC	90	Mixed Mode: RP/ SCX; RP/WCX; RP/SAX	2.7	0.5
Cortecs	Waters	90	C18, HILIC	2.7	*
Cortecs	Waters	90	C18, HILIC	1.6	*
Cosmocore	Nacalai	90	C18	2.7	0.5
Flare	Diamond Analytics	120	C18/IEX, C18, HILIC (carbon based particle)	3.6	*
Halo	Advanced Materials Technology	90	C18, C8, PhenylHexyl, PFP, ES-CN, HILIC, Penta-HILIC	5.0	0.6
Halo	Advanced Materials Technology	90	C18, C8, PhenylHexyl, RPAmide, PFP, ES-CN, HILIC, Penta-HILIC	2.7	0.5
Halo	Advanced Materials Technology	90	C18, PFP	2	0.4
Kinetex	Phenomenex	100	C18, XB-C18, EVO-C18, C8, PhenylHexyl, Biphenyl, PFP, HILIC	5.0	*
Kinetex	Phenomenex	100	C18, XB-C18, EVO-C18, C8, PhenylHexyl, Biphenyl, PFP, HILIC	2.6	0.35
Kinetex	Phenomenex	100	C18, XB-C18, EVO-C18, C8, PhenylHexyl, Biphenyl, PFP, HILIC	1.7	0.23
Kinetex	Phenomenex	100	C18	1.3	*
Meteoric Core	YMC	80	C18, C8	2.7	*
Nucleoshell	Macherey Nagel	90	RP 18 & 18 Plus, PhenylHexyl, PFP, HILIC AmmSulphonic acid	2.7	0.5
Nucleoshell	Macherey Nagel	90	RP 18 & 18 Plus	5.0	0.6
Poroshell	Agilent Technologies	120	EC-C18, C8 & CN; PhenylHexyl, SP-C18, C8 & Aq; Bonus-RP, HILIC; HPH-C18 & C8	2.7	0.5
Raptor	Restek	90	C18, Biphenyl	2.7	*
Raptor	Restek	90	C18, Biphenyl	5.0	*
Ultracore	Advanced Chromatography Technologies	95	C18, PhenylHexyl	2.5	*
Ultracore	Advanced Chromatography Technologies	95	C18, PhenylHexyl	5.0	*

** Not stated in the literature*

Table 2: Commerically Available Superficially Porous Particle Columns with Larger Pore Size Structures

Manufacturer	Brand	Pore Size	Available Chemis-tries	Particle Size	Outer Shell Thickness
AMT	Halo	160	C18, CN	2.7	0.5
AMT	Halo	160	C18, CN	4.6	0.6
AMT	Halo	400	C4, C18	3.4	0.2
Supelco	BIOshell	160	C18, CN	2.7	0.5
Supelco	BIOshell	160	C18, CN	4.6	0.6
Supelco	BIOshell	400	C4, C18	3.4	0.2
Phenomenex	AERISWidepore	200	C4,C8, C18	3.6	0.2
Agilent	AdvanceBio	450	C4, C8, diphenyl	3.5	na
Agilent	Poroshell 300	300	C3, C8, C18, C18Extend	5	0.25
Thermo	Accucore 150	150	C4, C18, Amide HILIC	2.6	0.5
Perkin Elmer Brownlee	SPP	160	C18	2.7	na
YMC	MeteoricCore	160	C18	2.7	na
ChromaNik	SunShell	160	C18, RP-Aqua	2.6	0.5
ChromaNik	SunShell	300	C4, C8, C18	2.6	0.5

similarly sized fully porous particles is realised.

Since the release of HALO columns to the market, most of the major column manufacturers have adopted some form of SPP technology. A listing of major brands and their offerings are provided in Table 1. Since the onset of SPP adoption, the manufacturers have added many of the classical HPLC surface chemistry modifications such as C8, cyano and aromatic phases.

Most manufactures have also added different overall particle sizes and, in some cases, pore sizes to accommodate the increased breadth of applications. The initial columns released were primarily packed with 2.6 and 2.7 µm SPP particles with pore sizes around 100 Å. Larger pore sizes (150 – 200 Å) that allowed separations of polypeptides were furthermore introduced [5]. Sub 2-µm versions of SPP based columns have been developed that combine both the efficiency advantages of the smaller particle with the architecture of the core-shell. Additionally, 5 µm SPP sizes have been developed by several manufacturers. These larger particles allow more direct transfer of classical 5 µm applications, such as USP methods, to the newer technology. Most recently, the focus in the industry has been moving toward the adaptation of SPP technology for large molecule separations.

Large Molecule Separations

Reversed-phase (RP) separations of proteins and peptides present some different aspects than what is typically encountered with small molecule RP separations. Probably the most clear is the need for larger pores, in order for the biomolecules to have full access to the surface area afforded by the porous particle. Therefore, typically for peptides, pore sizes of 120 – 200 Å are ideal, while for proteins, 300 Å or larger is generally found to be optimal. Of course, if the pore size gets too large, the physical strength of the particle may be compromised. Mechanistically, probably the most distinctive feature of protein retention by RP is the fact that partitioning between the stationary phase and mobile phase takes place over a much narrow range of solvent strength, than that of small molecules. To an approximation, the larger the protein, the narrower the range of solvent strength, that partitioning takes place. As such, this is often referred to as an on-off phenomenon. The operational implication for this is that for all practical purposes, any RP separation of proteins or peptides requires gradient elution. Perhaps the other more distinctive feature of protein and peptide RP separations with silica particle columns is the operation at low pH with an ion-pairing reagent. Prior to the popularity of MS detection, this was typically done with trifluoroacetic acid (TFA), or other fluorinated organic acids. Proteins and peptides are typically polyionic, and of particular concern, in the case of silica particle columns, are the basic moieties of polypeptides. These can readily undergo cation exchange interactions with the silica surface. Therefore, to mask the positive charges, a strong acid, such as TFA can ion-pair with the basic side chains, and N-terminus, to minimise the ion-exchange behaviour. Indeed, inclusion of an ion-pair reagent is typically necessary to achieve acceptable chromatography of polypeptides. An additional advantage of the strong acid is to minimise the ion-exchange activity of the silica particle, simply by virtue if pH control; additionally the low pH can neutralise carboxylate moieties on the polypeptide so as to enhance retention. Thus, a strong acidic ion-pair reagent, such as TFA, has multiple advantages for RP separations of proteins and peptides. In the case of MS detection, formic acid has become the de facto ion-pair acid of choice, because at optimal concentrations of TFA typical of protein or peptide separations, TFA can still mask charge in the gas phase, thus impeding sensitivity of the MS detector. Lastly, high pH stable columns (unlike pure silica particles) can provide options for RP polypeptide separations that would preclude the need for an ion-pair reagent, and have largely been unexplored with modern high-performance, small particle technologies.

Table 2 lists manufacturers and selected stationary phase characteristics of larger pore SPP columns. According to a recent review by Fekete et. al., large molecule separations suffer from a potential issues due to adsorption, secondary interactions, low diffusion coefficients and poor kinetics [6].

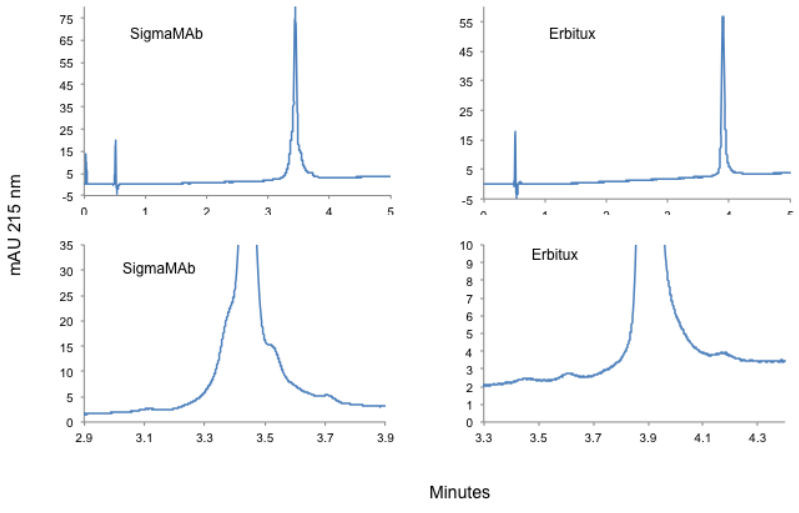


Figure 2: Fast Chromatography of intact monoclonal antibodies on BIOshell A400 Protein C4

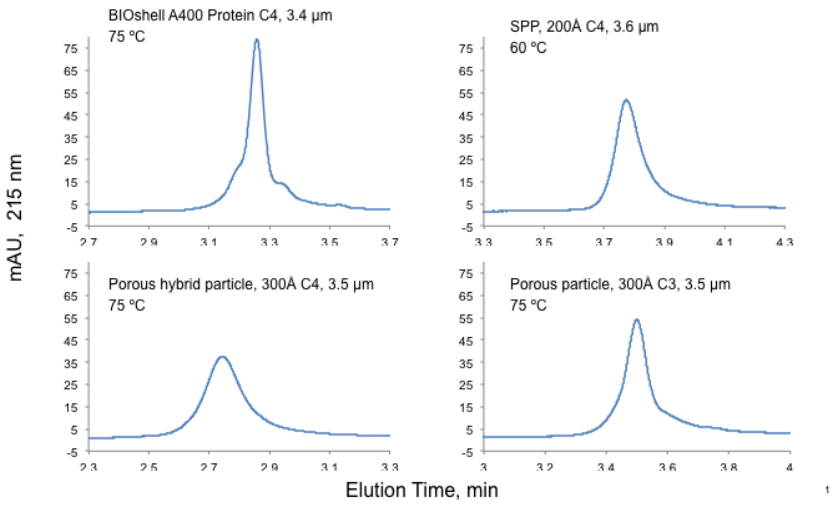


Figure 3: Column Performance comparison with SigmaMAb at optimal temperature

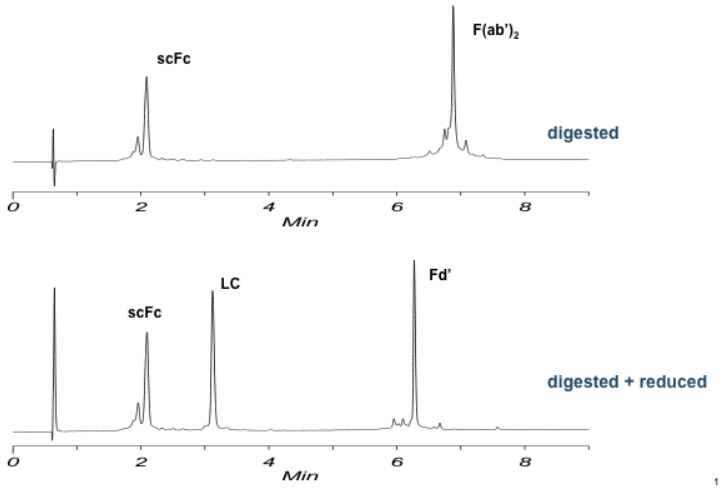


Figure 4: Resolution of IdeS fragments of SigmaMAb (p/n MSQC4) on BIOshell A400 Protein C4, before and after reduction

The characteristics of SPP phases that have proven effective in small molecule separations appear to be suitable for large molecule separations as well; however, in order to allow unrestricted access into the pores for these large molecules, wider pore structures are required. According to Schuster et. al, 90 Å pore structures allow unrestricted access to the internal volume for compounds up to about 5 kDa. Particles constructed with 160 Å pores provide access to compounds up to about 15 kDa and 400 Å pores provide access for structures up to around 500 kDa [7]. Particle with pore sizes around 160 Å are therefore recommended for peptides and small proteins, however for larger proteins larger average pore sizes are required.

As shown in Table 2, the shell thicknesses for particles intended for large molecule separations are, in general, thinner than SPP first introduced for small molecule separations. Due to the relatively slow diffusion of large molecules, thin shells are preferential in terms of efficiency. Schuster et. al compared several shell thickness layers for efficiency in protein separations [7]. It was demonstrated that the thinnest shell thickness resulted in the highest separation efficiency, albeit at the cost of retention and loading capacity. The authors, along with others, have reported that the optimal shell thickness for particles around 3.5 µm is a compromise between efficiency and loading capacity (surface area) [8].

In order for analytes to have adequate retention, they need to have access to the total surface area of the particle. For this reason, large biomolecules require a larger pore in order to provide unhindered access into the pore. This is demonstrated for a variety of proteins, in the example of BIOshell A400 Protein C4 (Figure 1). In this case, proteins varying in size from less than 12 kDa up to 465 kDa are well-retained. The resolution afforded by this SPP column can reveal those components of the sample that are highly pure (single, sharp peak), to those that are inherently more heterogeneous, that may well reflect various glycoforms.

Performance evaluations of some of the more popular wide-pore SPP phases have been published. Fekete et. al., compared the performance of Aeris WIDEPORE SPP to a number of fully porous and smaller pore SPP phases [9]. The combination of the larger pore structure and the SPP design resulted in improved separation power for large proteins over smaller pore SPP columns as well as wide-pore fully-porous columns. Similar efficiencies for small proteins were observed between both narrow and wide pore SPP phases. They also demonstrated that the larger SPP phase, in a similar fashion to the attributes for small molecules, exhibited increased peak capacity per time (and pressure) relative to sub-2 µm wide pore, fully porous platforms.

Temperature is an important variable for protein and other large molecule separations. Increased temperature improves peak shapes, reduces adsorption and improves kinetic performance [6]. Schuster et. al., have demonstrated high stability of C4 and C8 stationary phases at temperatures up to 90°C under acidic mobile phase conditions.

An application that is getting more attention recently is the RP chromatography of monoclonal antibodies (mAb) and/or fragments or subunits thereof, as development of biotherapeutics continues to grow. An example of this is shown in Figure 2, for two intact mAbs. One example is a mAb standard from Sigma-Aldrich (SigmaMAb) that is available as an unlabelled protein, as shown, or is also available uniformly labelled with ^{13}C,^{15}N-arginine and lysine, designed as a universal IgG standard for quantitative assays. The other example shown in Figure 2 is ERBITUX® (cetuximab). In both cases, even at the moderately fast flow, the SPP column can resolve various contaminants or isoforms in the samples. The elevated temperature is necessary for good chromatographic performance of the mAb. Moderate temperatures result in poor recoveries, as evidenced by broad peaks, and low peak areas (data not shown). Similar results have been reported elsewhere [7].

Not all modern, large-pore RP columns exhibit the same performance. Take the example in Figure 3. This compares two SPP columns and two conventional porous particle columns, although one of the porous particle columns is not pure silica (as are the other three), but is a carbon-silica hybrid; all particle sizes are approximately 3.5 µm, and of similar bonding chemistry. None of the other three columns provided the efficiency and resolution of the 400 Å SPP column. The results provide further

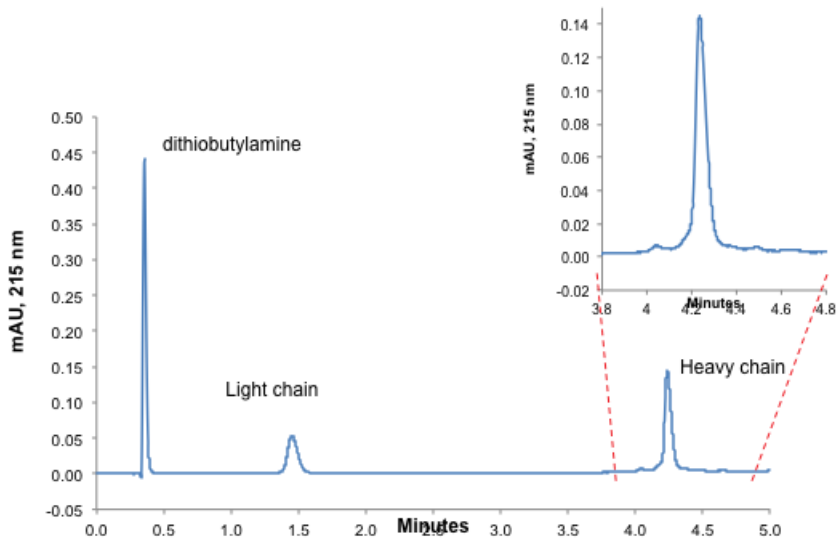


Figure 5: Resolution of SigmaMAb subunits on BIOshell A400 Protein C4w

evidence that it is the combination of the SPP architecture and pore size that yields the best results [7, 8]. That said, it must be noted that according to the manufacturer, the 200 Å SPP column should not be operated at as high as a temperature as shown for the other columns, and so its realised performance for this application may be limited by this constraint.

A particular strategy to mAb characterisation that is increasingly popular is a so-called middle-down approach. That is, instead of doing a top-down approach that requires the best MS instrumentation for protein characterisation, or instead of taking the bottom-up approach on the intact mAb, an intermediate strategy is taken, in which the mAb is cleaved into a few fragments, and each of the fragments characterised further. One such protease that is often used for this application is an internal protease from Streptococcus pyogenes [10]. This enzyme cleaves at the hinge region of human IgG with a high degree of specificity, just seven amino acids downstream from the two disulphide bonds. Thus, the fragments $F(ab')_2$ and the individual Fc domains are generated. Subsequent disulphide reduction dissociates the $F(ab')_2$ fragment into the light chain (LC) and the Fd' fragments. The results of this process are demonstrated in Figure 4. The 400 Å SPP column provides a good solution in this example with the SigmaMAb mAb. For this particular sample, the high temperature is critical to achieve the excellent chromatographic performance as exemplified with this particular column. The high -resolution separation of the individual fragments makes possible the subsequent middle-down characterisation of each polypeptide.

Another possibility for a middle-down approach is to simply dissociate the intact mAb into its component subunits. This is shown, again, with SigmaMAb as an example, in Figure 5. In a neutral pH buffer, the mAb is denatured (so as to render the disulphides readily accessible to the reductant) and reduced. In this case the reductant dithiobutylamine was used because it is much more effective at neutral pH than dithiothreitol. [11]. The sample is then injected onto the column, following a simple dilution with water. The two subunits are readily resolved on the 400 Å SPP column, through application of a shallow acetonitrile gradient. Various likely isoforms of the heavy chain are evident in the expanded view of the chromatogram.

Surface chemistry modifications consisting of C4, C8 and C18 on wide pore SPP phases are available. Schuster et al., compared a densely bonded C4 and sterically protected C8 and C18 bonding chemistries for a set of proteins ranging from 12.4 kDA to 250 kDa [7]. The C4 provided slightly improved separations over the longer alkyl bonded phases, however the overall quality of the separations was similar. It has also been suggested that shorter C4 and C8 ligands may be preferential over C18 phases for protein analysis as mass transfer may be limited for the longer chain bonded phases [12]. It is anticipated that additional bonded phases will emerge to enhance alternative selectivity for peptides in the near future.

Conclusion

Columns developed using superficially porous particle technologies have become a mainstay in the modern analytical laboratory. Like the preceding fully porous particle platforms, SPP continues to expand into a wealth of application domains. Alternative bonded phases and particle sizes have increased utility considerably over the past decade. More recently, SPP with wider pore structures have been made available to enable separations of large molecules such as proteins and large peptides. Pore sizes as large as 400 Å have been shown to provide unrestricted access to proteins as large as 500 kDA. Multiple manufacturers have developed stationary phases that have demonstrated stability at the high temperatures and low pH conditions most suitable for efficient protein separations. Even further developments in both particle architecture and surface chemistry modifications are anticipated that will further extend the utility of the SPP platform.

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