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The utility of porous graphitic carbon as a stationary phase in proteomics workflows: Two-dimensional chromatography of complex peptide samples

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ABSTRACT

We present the first investigation into the utility of porous graphitic carbon (PGC) as a stationary phase in proteomic workflows involving complex samples. PGC offers chemical and physical robustness and is capable of withstanding extremes of pH and higher temperatures than traditional stationary phases, without the likelihood of catastrophic failure. In addition, unlike separations driven by ion exchange mechanisms, there is no requirement for high levels of non-volatile salts such as potassium chloride in the elution buffers, which must be removed prior to LC–MS analysis. Here we present data which demonstrate that PGC affords excellent peptide separation in a complex whole cell lysate digest sample, with good orthogonality to a typical low pH reversed-phase system. As strong cation exchange (SCX) is currently the most popular first dimension for 2D peptide separations, we chose to compare the performance of a PGC and SCX separation as the first dimension in a comprehensive 2D-LC–MS/MS workflow. A significant increase, in the region of 40%, in peptide identifications is reported with off-line PGC fractionation compared to SCX. Around 14,000 unique peptides were identified at an estimated false discovery rate of 1% ($n = 3$ replicates) from starting material constituting only 100 μg of protein extract.

1. Introduction

The comprehensive analysis of complex peptide mixtures, such as those derived from a whole cell lysate (WCL), by LC–MS/MS represents a significant challenge due to acquisition rate limitations and consequent under sampling by current generation mass spectrometers. Since the mass spectrometer only has a finite time (<30 s elution time) in which to detect (MS/MS) co-eluting peptides, many peptides which co-elute result in some of them remaining undetected within that time window. Pre-fractionation prior to RPLC–MS/MS such as in a MudPit approach is often used as a way of increasing the number of peptide (and associated protein) identifications [1]. Strong cation exchange (SCX) chromatography is currently the most widely utilized first dimension for such analyses [2–4]. However, SCX requires the use of high salt concentrations which need extensive removal prior to electrospray ionization. It has also been shown that peptides tend to group in relatively few SCX fractions due to the low resolving power of the intrinsic mechanism, which separates according to their solution-phase charge [5].

As an alternative to SCX, separation on a reversed-phase column at elevated pH (in the region of pH 10.5) has been used [6–8]. This mode of separation offers higher resolution than SCX, and has proved very effective at enabling deeper proteome penetrance compared to those previously observed [9]. Since most reversed-phase columns are based upon a silica support, the challenge to manufacturers is to produce columns capable of withstanding alkali conditions (pH 10.5) which would normally dissolve silica. In addition, the chromatographic system itself must be capable of withstanding these higher pH conditions. The column and system compatibility issues at elevated pH have limited the utility of this approach in the proteomics community.

Recently, McNulty and Annan described the use of an alternative separation mode for the first stage of global enrichment of phosphopeptides, termed hydrophilic interaction chromatography (HILIC) [10] first described by Alpert [11]. Gilar et al. reported the potential of HILIC as a suitable chromatography separation mode with good orthogonality to reversed-phase [5]. However, since solutes are required to be dissolved in high organic solvents (70% acetonitrile) the solubility of certain peptides in such systems may be problematic. SCX is therefore still the dominant first dimension of choice in these 2D peptide separations despite its limitations.

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PGC has been reported to show a mixed mode of separation combining both RP-like hydrophobic interactions as well as electronic, ion-exchange type behavior [12–14]. The material is also very stable, both mechanically and chemically, and is resistant to extremes of pH unlike most silica-based columns. We postulated that these characteristics would make PGC an ideal candidate for proteomic workflows, such as 2D-LC–MS/MS.

Firstly, we assessed the performance of PGC (in this case Hypercarb™) as a first dimension stationary phase, in terms of its resolution and orthogonality to a traditional low pH reversed-phase second dimension. Next, we conducted a like-for-like comparison of peptide identification rates to that of strong cation exchange chromatography, which is currently utilized most often as the first dimension fractionation method for complex peptide separation [15].

2. Experimental

2.1. Materials

The following reagents were purchased from the specified companies: trifluoroacetic acid (TFA), glufibrinopeptide, ammonium bicarbonate, formic acid and trypsin (proteomics grade) were purchased from Sigma–Aldrich (Gillingham, UK). Potassium chloride (AnalaR) was obtained from BDH chemicals (Poole, UK). HPLC grade acetonitrile was purchased from Sigma–Aldrich (Gillingham, UK) and HPLC grade water was obtained from Rathburn (Walkerburn, Scotland).

All off-line first dimension fractionation was performed on an Ultimate 3000 (Thermo Scientific, formerly Dionex, The Netherlands) liquid chromatography system which consisted of a ICS-3000 SP pump, Ultimate 3000 column compartment, Ultimate 3000 autosampler and Ultimate 3000 variable wavelength detector (UV).

The on-line second dimension separations were carried out either on a nanoAcquity UPLC (Waters, Milford, USA) system with column oven, sample manager and binary solvent manager modules (PGC performance assessment), or an Ultimate 3000 RSLCnano system (Thermo Scientific, formerly Dionex, The Netherlands) comprising a 3000 pump, heated column compartment and autosampler (SCX/PGC comparison). In both cases the LC was coupled via a nanospray interface (Proxeon, Denmark) directly to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, USA).

Details of chromatographic conditions and column dimensions are given in the relevant sections below.

2.2. Initial assessment of PGC performance

A sample of *Schizosaccharomyces pombe* prepared using a filter-aided sample preparation (FASP) protocol [16,17] was used to assess the performance of a PGC column. Approximately 400 μg of digested (trypsin) whole cell lysate was loaded onto a PGC column (2.1 mm × 100 mm × 5 μm Hypercarb™, Thermo Scientific) in water containing 0.1% trifluoroacetic acid (TFA). Peptides were separated over a linear gradient of 2% PGC Buffer B (acetonitrile + 0.1% TFA) to 96% PGC Buffer B over 40 min at a flow rate of 250 μL min^{-1}. The column was maintained at a temperature of 30 °C by means of a column oven (Ultimate 3000 column compartment, Dionex). PGC Buffer A consisted of HPLC-grade water containing 0.1% TFA. Fractions were collected at 30 s intervals which corresponded to typical peak width at base based upon glufibrinopeptide (Glufib) (data not shown). Next, fractions were dried on a SpeedVac (Eppendorf, Germany) to remove the acetonitrile, and resuspended in 50 μL PGC Buffer A. A 2 μL aliquot of each of the apparent peptide-rich fractions (from fraction number 10 to number 80) was then injected onto a trapping column (Symmetry™ C18 180 μm × 20 mm × 5 μm) for 5 min at a flow rate of 5 μL min^{-1} then separated on a C18 analytical column (75 μm × 250 mm × 1.7 μm BEH130, Waters) online to an LTQ OrbitrapXL mass spectrometer (Thermo Scientific). This corresponded to a total loading of approximately 16 μg on column. The column was maintained at a temperature of 55 °C by means of a column oven (Waters, MA). The mass spectrometer was set to automatically acquire in parallel acquisition up to six MS/MS spectra in the ion trap segment of the instrument and one high resolution FTMS spectrum per scan cycle. The peptides were separated over a reversed-phase gradient from 3% acetonitrile + 0.1% formic acid to 25% acetonitrile + 0.1% formic acid over 30 min at a flow rate of 400 nL min^{-1}. An overall schematic representation of the experimental set-up is shown in Fig. 1.

Upon closer examination of the MS total ion current (TIC) data, it was clear that the fractions containing the greatest number of peptides were not being sufficiently separated on the relatively short RP gradient and the MS therefore suffers from under sampling i.e. the speed of acquisition was insufficient to allow comprehensive tandem mass spectrometry data to be collected over the elution period. Fraction numbers 30–46 were therefore reanalyzed over a more appropriate separation space from 3% acetonitrile + 0.1% formic acid to 25% acetonitrile + 0.1% formic acid over 60 min in and attempt to alleviate, to some extent, this issue.

2.3. Comparison of PGC to SCX fractionation

A sample of *human* SD1 (pre-B acute lymphoblastic leukaemia cell line) was used to compare the performance of a PGC column against that of an equivalent SCX column in terms of its suitability as an off-line fractionation technique. Approximately 600 μg of digested (trypsin) whole cell lysate was split equally into six aliquots. This provided sufficient material to perform triplicate analyses on both PGC and SCX columns using a total sample loading of 100 μg for each experiment. A 100 μg aliquot of digested material was loaded onto a PGC column (2.1 mm × 50 mm × 5 μm Hypercarb™, Thermo Scientific) in water containing 0.1% TFA. Peptides were separated over a linear gradient of 2% PGC Buffer B (acetonitrile + 0.1% TFA) to 96% PGC Buffer B over 40 min at a flow rate of 400 μL min^{-1}. The column was maintained at a temperature of 50 °C by means of a column oven (Ultimate 3000 column compartment, Dionex). PGC Buffer A consisted of HPLC-grade water containing 0.1% TFA. Fractions were once again collected at 30 s intervals which corresponds to peak width at base for Glufib (Fig. 2a). Next, fractions were dried on a SpeedVac (Eppendorf) to remove the acetonitrile, and resuspended in 50 μL PGC Buffer A. A 5 μL aliquot of each of the apparent peptide-rich fractions (from fraction number 11 to number 55) was then injected onto a trapping column (100 μm × 20 mm × 5 μm Acclaim PepMap 100 C18) for 5 min at a flow rate of 5 μL min^{-1} before being separated on a C18 (75 μm × 500 mm × 3 μm, Acclaim PepMap 100, Dionex) column online to an LTQ OrbitrapXL mass spectrometer (Thermo Scientific) at a flow rate of 300 nL min^{-1}. This corresponded to a total loading of approximately 10 μg. The LC–MS/MS instrumentation was configured the same as in previous experiments detailed above. The entire experiment was performed in triplicate.

As a direct comparison to PGC, equivalent 100 μg samples (triplicate) of SD1 digests were analyzed using a strong cation exchange fractionation approach. A 100 μg aliquot of digested material was loaded onto a typical SCX compatible column (2.1 mm × 50 mm × 5 μm PolySULFOETHYL A ™, (PolyLC inc, Columbia, MD)) in SCX Buffer A (20% acetonitrile: 80% HPLC-grade water containing 0.1% formic acid). Peptides were separated over a linear gradient of 0% SCX Buffer B (20% acetonitrile: 80% 1 M potassium chloride + 0.1% formic acid) to 30% SCX Buffer B over

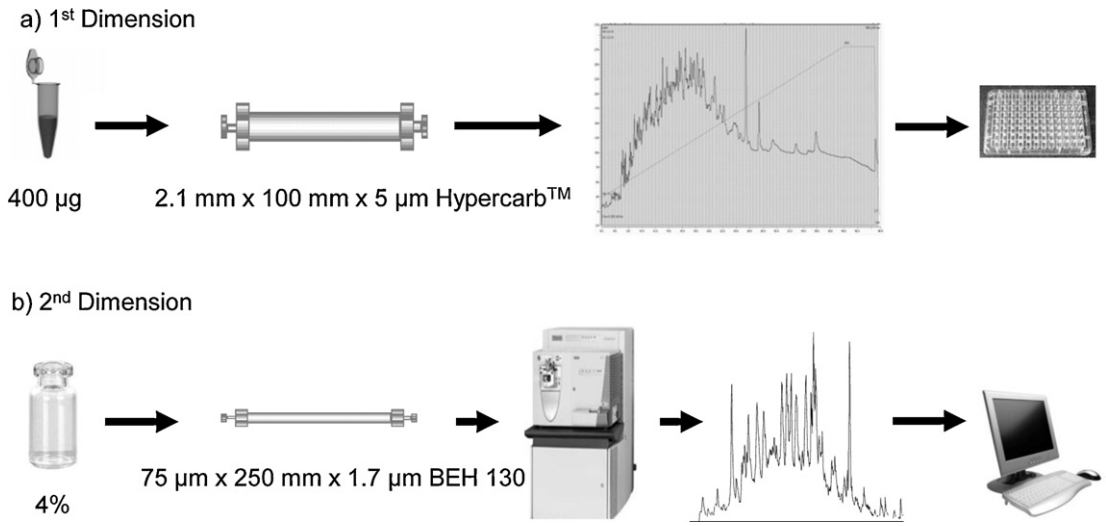

Fig. 1. Schematic diagram showing the experimental procedure for 2-D LC–MS/MS of a complex peptide mixture. (a) First dimension fractionation using PGC column for separation with automated fraction collection, (b) second dimension with reversed-phase separation on-line to a mass spectrometer followed by informatics.

30 min then to 90% Buffer B over a further 7 min, at a flow rate of 400 μL min^{-1}. Due to the amount of non-volatile KCl present in most of the SCX fractions, a longer wash time was required on the trapping column (trapping at a flow rate of 10 μL min^{-1} for 20 min) prior to separation on the analytical column. The column was not heated above ambient and fractions were once again collected at 30 s intervals which corresponded to typical peak width at base based upon glufibrinopeptide (Glufib) (Fig. 2b).

2.4. Data analysis

To assess the effectiveness of PGC/C18 two-dimensional chromatography it was necessary to calculate the number of unique peptides identified using this approach. Data was searched using the Mascot search engine [18] which applies a decoy database approach and peptides reported at a 2% estimated false discovery rate (FDR) [19,20]. For the purposes of this study, a unique peptide is defined as a peptide with a unique amino acid sequence, with or without modifications. The same peptide which occurs in multiple charge state is regarded as the same entity, whereas peptides containing modifications on different residues are regarded as unique.

Data analysis for the comparison of the performance of PGC with SCX was performed using the ProteinPilot™ (v4) software which utilizes the Paragon™ Algorithm (AB SCIEX, Foster City, CA) [21]. Total number of peptides identified with an estimated false discovery rate of 1% were reported for $n = 3$ using both fractionation strategies.

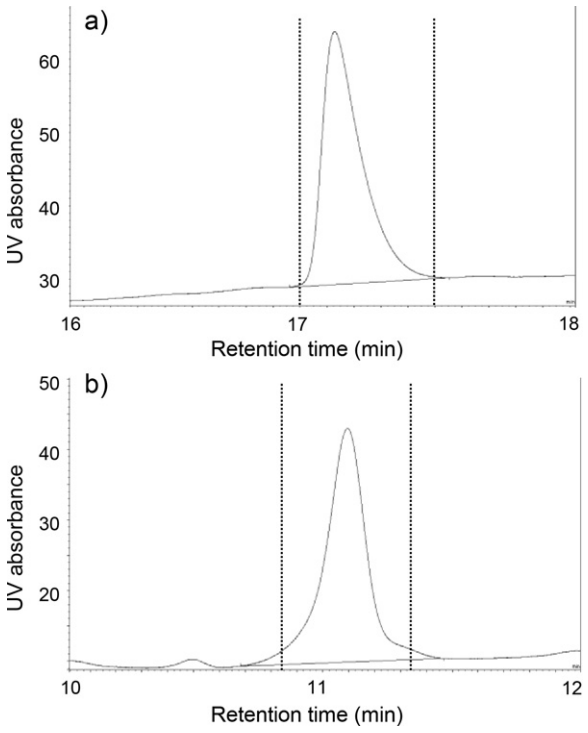

Fig. 2. Region of chromatogram showing the peak shape for 5 μg loading of glufibrinopeptide on (a) PGC, and (b) SCX columns. Peak width at base for both is shown to be around 30 s.

3. Results

3.1. Results of PGC performance assessment

The metrics chosen to assess the performance of PGC as the first dimension for a 2D proteomic analysis were peak shape, orthogonality to C18 and level of peptide identification. The typical peak shape for a standard of glufibrinopeptide (5 μg loading) is shown in Fig. 2a for PGC and Fig. 2b for SCX. The vertical lines correspond to a time window of 30 s. As can be seen, both peaks fall predominantly within this 30 s time window, and are reasonably symmetrical. We can therefore conclude that, at least for the test compound of Glufib, the efficiency of PGC is similar to that of SCX.

Fig. 3 shows a typical UV signal obtained for the PGC fractionation of 400 μg of trypsin-digested whole cell lysate. As can be seen from the UV (214 nm) signal, there appears to be a reasonable spread of peptides throughout the gradient development. Replicate fractionations were performed on three separate FASP vessels with similar UV profiles (Data not shown). Fig. 4 demonstrates the reversed-phase peptide spread in the second dimension versus the PGC fraction number, where each data point represents the presence of a unique peptide in the fraction. As can clearly be seen from this plot there is a significant peptide distribution throughout each on-line separation. The figure demonstrates that PGC and C18 are complimentary in terms of their selectivity and suggests that the combination of the two phases should facilitate the identification

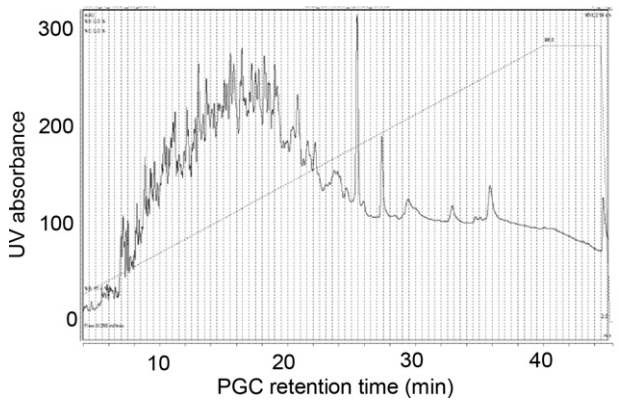

Fig. 3. UV (214 nm) signal obtained from 400 μg of WCL on a Hypercarb™ (100 mm × 2.1 mm × 5 μm) column with 30 s fractionation.

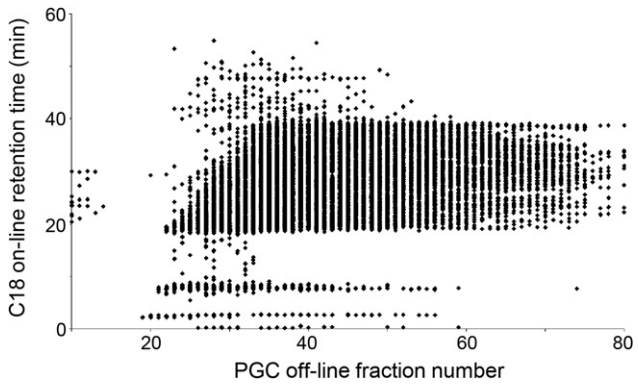

Fig. 4. Plot of retention time spread on the second dimension C18 column versus the first dimension, PGC, fraction number demonstrating excellent orthogonality between the two phases (each point represents an identified unique peptide in the fraction).

of a significantly greater number of peptides than by either phase alone.

The distribution of observed peptides throughout the fractions is shown in Fig. 5 (solid bars). Globally, this represents a total of approximately 14,000 unique peptides, 74% of which were only observed in a single fraction. When a selection of the most peptide-rich fractions was reanalyzed by LC–MS/MS using a longer gradient

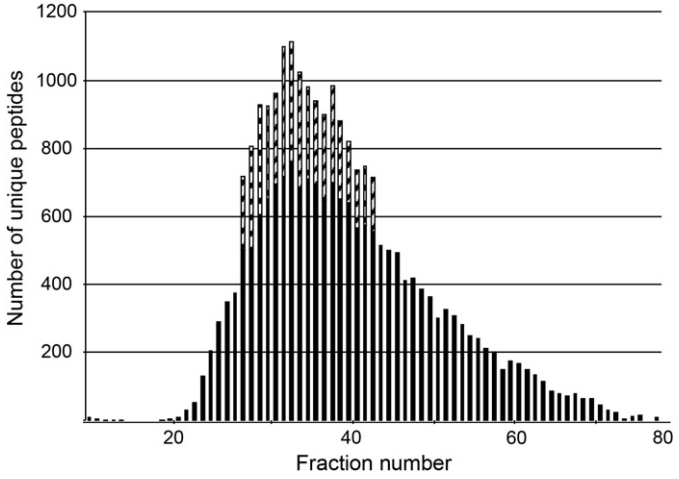

Fig. 5. Number of unique peptides in each PGC fraction from 4% of original material (equivalent to 16 μg total loading). Solid bars = shorter gradient, hatched bars = longer gradient for a selected number of fractions.

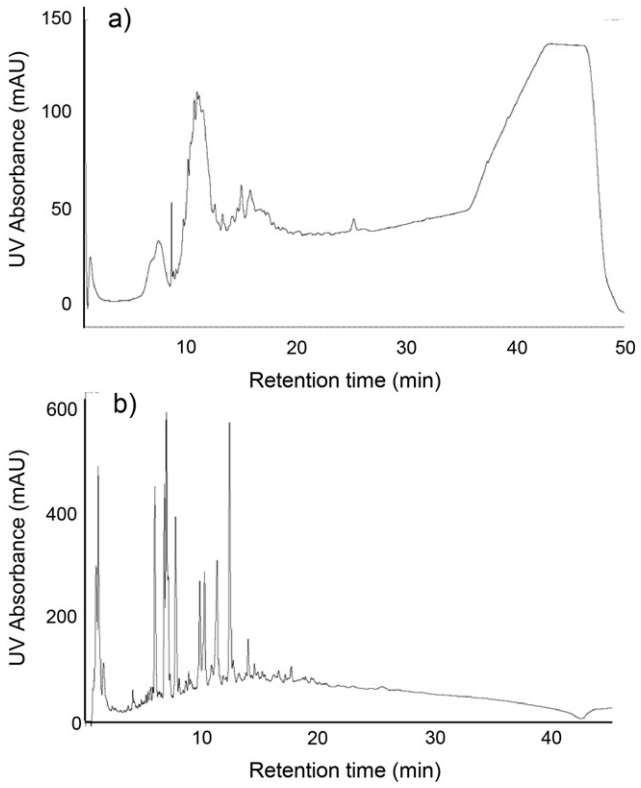

Fig. 6. UV (214 nm) signal obtained from 100 μg of SD1 digest on (a) a PolySULFOETHYL A™ (50 mm × 2.1 mm × 5 μm) column, and (b) Hypercarb™ (50 mm × 2.1 mm × 5 μm) column, both with 30 s fractionation.

(60 min separation space compared to the original, fast, 30 min) an increase in peptide numbers of around 50% was obtained.

Taking together the PGC peak shape (less than 30 s at base), the observed degree of orthogonality to reversed-phase chromatography and the high number of peptide identifications, we were able to conclude that PGC warranted further investigation as a stationary phase for proteomic workflows. To this end we decided to compare its performance to that of an equivalent SCX fractionation strategy since this is currently the most utilized first dimension approach for 2D-LC–MS/MS of complex mixtures.

3.2. Comparison of PGC to SCX fractionation

To date, strong cation exchange chromatography is the most popular choice for first dimension fractionation of a complex peptide mixture for 2D-LC–MS/MS [15]. Fig. 2 shows that for the test compound, glufibrinopeptide, PGC and SCX offer similar levels of efficiency. A full comparison of the two fractionation methods was carried out in order to ascertain which method resulted in the greatest number of peptide identifications from replicate samples. Fig. 6 shows the UV signal for 100 μg of digest loaded onto (a) SCX and (b) PGC column. While a general spread throughout the gradient is observed for the peptides separated on PGC (Figs. 3 and 6b), the same sample shows clustering of peptides which is dependent upon their solution phase charge. Here, in Fig. 6a, most peptides appear to elute in a few regions i.e. around 7, 11 and 15 min. This coincides with solution charge states of +1, +2 and +3, respectively as previously described [22]. While this phenomenon has been successfully exploited to enrich for phosphopeptides [23], it reduces the combined peak capacity for 2D-LC analysis. This clustering becomes more obvious when the number of peptides per fraction is plotted against fraction number for both SCX and PGC (Fig. 7). The grey plot of SCX peptides has the two most prominent peaks for

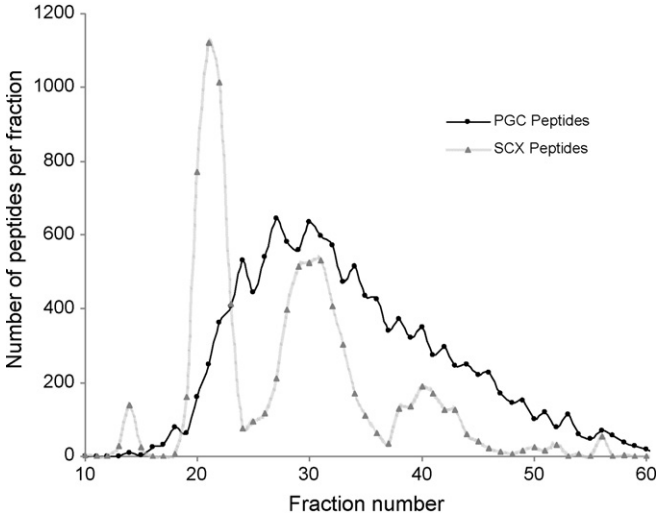

Fig. 7. Number of unique peptides in each PGC and SCX fraction from 10% of original material (equivalent to 10 μg total loading). Solid line = PGC, dashed line = SCX.

Table 1
Total number of unique peptides identified for each fractionation method along with the percentage increase resulting from PGC fractionation (FDR 1%).

	Strong cation exchange	Porous graphitic carbon	Percentage increase in peptide identifications
$n=1$	8667	13,449	55
$n=2$	10,403	14,695	41
$n=3$	10,473	13,625	30

fractions in the region of number 20 and 30 which correspond to elution times of around 10 and 15 min. This seems to correlate with the UV signal shown in Fig. 6a. In contrast, the peptides from PGC fractionation are far more evenly spread throughout the gradient. This spread results in greater peak capacity, which in turn leads to a greater number of unique peptide identifications for PGC as shown in Table 1. In all three replicate analyses, PGC is found to significantly outperform SCX in terms of peptide identifications.

4. Conclusions

From the experiments detailed above, we are able to conclude that PGC performs well as an off-line first dimension stationary phase in a 2D-LC–MS/MS proteomic study involving complex mixtures. The resolution offered by PGC is comparable to that of an equivalent SCX separation with fraction collection at 30 s intervals being optimal. However, peptides were shown to be more evenly distributed throughout the PGC chromatographic run compared to those fractionated using SCX. Consequently, employing PGC rather than SCX as the first dimension in a 2D-LC–MS/MS strategy proved to be a more effective method of analysis. Using PGC, realistic biological quantities of material (approximately 10 μg of digested whole cell lysate) result in the identification of around 14,000 peptides (FDR 1%) using standard chromatographic equipment and a short LC gradient. This represents an increase of around 40% in peptide identifications when compared to a similar SCX fractionation. We propose that PGC represents an excellent alternative to currently employed first dimension stationary phases such as strong cation exchange and has the added advantages of robustness, both chemical and mechanical, and the lack of a requirement for non-volatile buffer salts. Further work will assess the applicability of PGC fractionation to highly enriched (90%) samples of phosphopeptides, once again as a means of off-line fractionation to enable deeper proteome penetrance by LC–MS/MS analysis. The effect of pH adjustment upon selectivity to this subset of peptides will also be investigated.

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