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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