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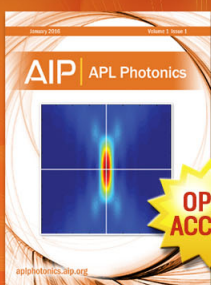
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***In situ* modification of chromatography adsorbents using cold atmospheric pressure plasmas**

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Efficient manufacturing of increasingly sophisticated biopharmaceuticals requires the development of new breeds of chromatographic materials featuring two or more layers, with each layer affording different functions. This letter reports the *in situ* modification of a commercial beaded anion exchange adsorbent using atmospheric pressure plasma generated within gas bubbles. The results show that exposure to He-O₂ plasma in this way yields significant reductions in the surface binding of plasmid DNA to the adsorbent exterior, with minimal loss of core protein binding capacity; thus, a bi-layered chromatography material exhibiting both size excluding and anion exchange functionalities within the same bead is produced. © 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4807391>]

Preparative chromatography, a core technology within the biopharma industry for several decades, plays pivotal roles in the downstream processing (i.e., recovery and purification operations) of modern biopharmaceuticals,^{1–3} but is increasingly being viewed as a serious bottleneck in manufacturing.^{3,4} This is largely because the development of chromatographic hardware and especially media has not kept pace with biological innovations upstream, i.e., rocketing product titers, increasing size, and complexity of emerging bio-products.^{3,4} Modern chromatography media are infinitely superior to their ancestors from the mid 1950s,¹ but in stark contrast to the monumental changes observed in bio-product development over the same period, the basic design (“mono-functional porous polymeric beads”; Fig. 1(a) remains effectively unchanged.⁵ As a result, few commercially available chromatographic materials tailored to the separation of many newer increasingly complex products exist at the present time. This point is beautifully highlighted by the unique separation challenges posed by “biological nanoplexes,” a rapidly growing and diverse product grouping characterized by large physical size, fragility, complex surfaces “plus” chemical similarity to smaller contaminating macromolecular components.⁵ These properties prohibit the efficient manufacture of nanoplexes using routes and chromatographic materials established for therapeutic human proteins of much smaller dimensions.^{6–8} For example, whilst ion exchange chromatography is extensively used for the commercial scale purification of antibiotics and protein-based drugs, its application for biological nanoplexes (e.g., naked plasmid DNA, viral vectors, virus-like particles, mega-molecular vaccines, mega-protein complexes, IgMs) is far less attractive. This is because these species are often as large as, or larger than, the pores of most conventional chromatographic media; thus

their adsorption is confined to the exterior surface of the adsorbent, resulting in exceptionally low target binding capacities per unit volume.^{8–11}

New types of beaded chromatography adsorbents featuring two or more distinct functional regions spatially separated from one another within the same support bead have been the subject of a handful of research reports since 2002;^{5,12–17} the most promising of these describing the manufacture and testing of bi-layered bi-functional supports comprising ion exchange (IEC) functionalized cores surmounted by outer size excluding (SEC) layers.^{5,13–15} The main attraction of the bi-layered SEC-IEC support architecture (Fig. 1(b)) is that it enables efficient separation of certain nanoplex bio-products (e.g., plasmid DNA) from smaller, but chemically very similar “problem” contaminants (protein, RNA) in a “one column—one bead” chromatography process combining size exclusion and ion exchange principles.^{5,13,14} The ideal bi-layered SEC-IEC support should possess inert mechanically reliable “non-stick” exteriors or barriers, that are freely accessible to smaller components (proteins, RNA), but not larger entities, such as long chain nucleic acids, cell debris fragments, nanoplexes, etc., and in order not to compromise mass transport and sorption properties, they must also be very thin, i.e., ideally <1 μm.^{5,18} To date, these criteria have not been met.

This letter details the *in situ* generation of atmospheric pressure plasma within an aqueous solution of “soft” porous hydrogel chromatography adsorbents; the reactive oxygen species (ROS) produced in the plasma are transported to the chromatography beads via gas bubbles and are shown to effectively remove surface functionality while leaving core functionality intact. Given that the technique is applied *in situ*, it significantly expands the range of chromatography materials that can be modified to exhibit both size exclusion and the specific adsorptive properties, i.e., ion exchange, hydrophobic interaction, mixed mode, or affinity adsorption.

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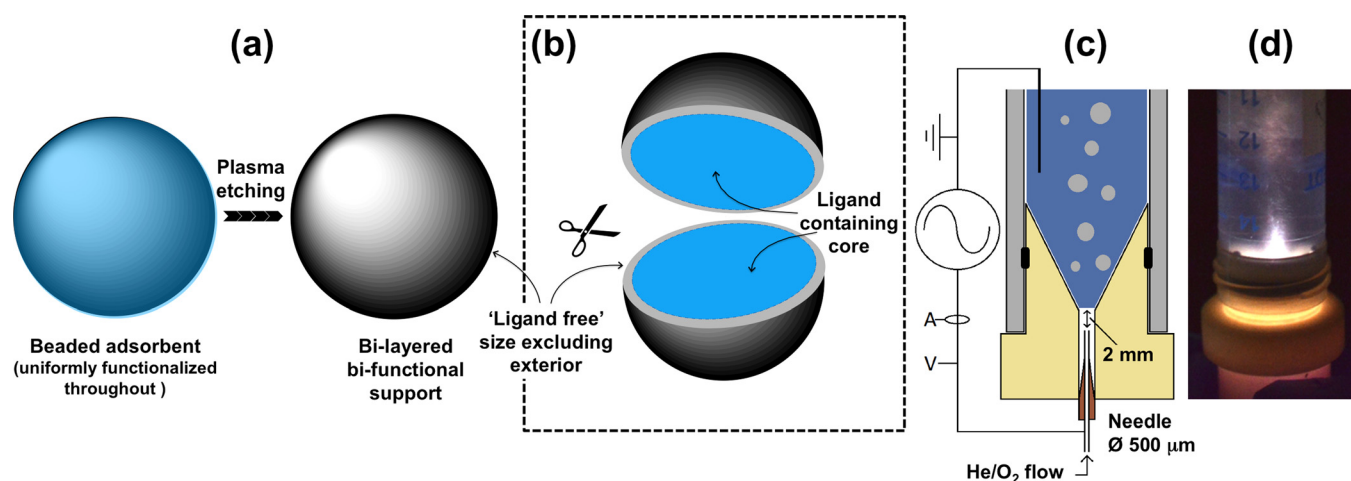


FIG. 1. Schematics illustrating (a) plasma surface etching of a chromatography adsorbent bead to form a bi-layered bi-functional support, (b) sectioning of through the resulting support to highlight the “ligand free” exterior and ligand functionalized core, (c) the underwater fluidized bed plasma treatment reactor operating with He-O₂ at an input power of 3.7 W. Also shown is a photograph (d) of the plasma reactor in operation.

Figures 1(c) and 1(d) show the reactor design used for the treatment of aqueous slurries of support; it consisted of a ceramic base through which a hollow needle of 500 μm diameter was inserted. The sides of the ceramic base are intentionally sloped to encourage the transport of support particles toward the plasma region. A gas flow was directed through the needle at a rate of 0.05 Standard Liters per Minute (SLM), the gas composition used was either oxygen (O₂) or helium (He) with 0.5% O₂ admixture. The output of a high voltage sinusoidal power source, operating at 14.5 kHz, was connected to the hollow needle and a ground electrode placed within the aqueous support slurry. In order to access optical information from the plasma, a second reactor was fabricated employing a quartz housing, thus facilitating the transmission of UV; all critical dimensions (i.e., needle diameter, distance between needle and liquid) in the second reactor matched those in the actual treatment reactor. Slurries of the strong anion exchange chromatography adsorbent, Q Sepharose Fast Flow (GE Healthcare, Sweden), were prepared (total volume 8 ml, settled bed concentrations of 12.5% v/v and 25% v/v with respect to total slurry volume) in 15 ml plastic centrifuge tubes and attached to the ceramic base of the reactor. Following attachment of the ceramic base and inversion of the centrifuge tube, a 1 cm Ø hole was made in the tip of the tube to allow for a ground electrode to be inserted and act as a means for gas to escape. This porous beaded matrix, comprising a cross-linked 6% agarose backbone derivatized throughout with the strong quaternary ammonium (Q) anion exchange group, was selected as representative of the majority of soft hydrogel based commercial chromatographic adsorbents employed for low pressure chromatographic separation of biomacromolecules, such as globular proteins. In these polymeric media, the high water content (typically >90% of the support volume), renders them—in stark contrast to rarer rigid types able withstand prolonged vacuum conditions^{2,5} (e.g., gel-in-a-shell’ or controlled pore glass supports)—poorly suited to vacuum plasma processing.

The selectivity of “surface vs. core” modification following plasma treatment of Q Sepharose Fast Flow was evaluated in simple batch binding tests, conducted in 0.05 M Tris-HCl

pH 7.5 with two differently sized negatively charged macromolecular binding probes; i.e., a 27.4 kbp supercoiled plasmid DNA (pDNA) much larger than the adsorbent’s pores (binding strictly limited to the exterior surface of the adsorbent),^{8–11} and one small enough to access most of the interior pores, the 66.4 kDa globular protein, bovine serum albumin (BSA). Equilibrated adsorbent samples (25 μl) were mixed with 1 ml portions of either 25 μg/ml pDNA or 10 mg/ml BSA. After 0.5 h at room temperature, the adsorbents were recovered by centrifugation in a microfuge before carefully aspirating the supernatants and assaying for residual pDNA or BSA content as described previously.⁵ The results of static pDNA and BSA binding studies are shown in Fig. 2 for adsorbents treated with He-O₂ and O₂ plasma over various time periods and average plasma input powers. Significant losses in “surface” pDNA binding with much lower reductions in “core” BSA binding were observed for all 3.7 W He-O₂ plasma treated samples. The optimum conditions were observed using the shortest treatment time of 150 s, which resulted in a >67% reduction in “surface” pDNA binding with just 8% loss of core BSA binding capacity. Longer He-O₂ plasma treatment times (300 or 600 s) proved detrimental to the selectivity of “surface vs. core” modification. Though the reduction in surface pDNA binding increased to over 70%, the loss of core BSA binding rose significantly, reaching nearly 14% after 600 s of contact. Very similar results were obtained for He-O₂ treatment of two diethylaminoethyl (DEAE) functionalized anion exchange chromatographic media, EMD Fractogel DEAE and DEAE Sepharose Fast Flow adsorbents (data not shown). The mass transfer of various ROS species can be used to explain the above findings. Several studies have demonstrated that plasma generated ROS within bubbles are transferred to the liquid phase;^{19,20} however, the penetration depths of highly reactive species such O, O*, and OH are limited (<10 μm), hence they are only able to interact with the exteriors of adsorbent particles (~90 μm mean Ø) coming into close contact with the plasma bubble-liquid interface. The concentration of longer lived species, especially H₂O₂, increase steadily over time,¹⁹ with the potential to interact with both the surface and core of the adsorbent particles; this finding goes some way to explaining the increased loss of core binding observed at longer

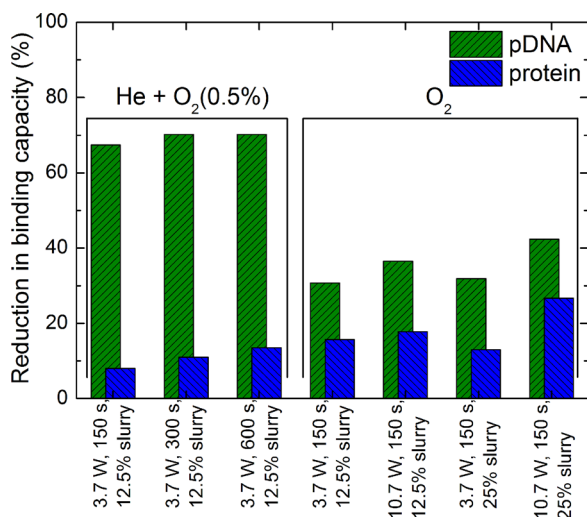


FIG. 2. Effect of He-O₂ and O₂ underwater plasma treatments on reductions of “surface” pDNA (green hatched bars) and “core” BSA (blue hatched bars) binding capacity of Q Sepharose FF.

treatment times. Interestingly, the results obtained for O₂ plasma treatments differ significantly from those obtained using a He-O₂ discharge (Fig. 2). In contrast to He-O₂ plasma treatment, the losses in pDNA (31%–42%) and BSA (15%–27%) binding indicated comparatively poor “surface” over “core” modification selectivity for the O₂ plasma.

The differences observed between He-O₂ and O₂ plasma treatments were not initially consistent with expectation, plasmas generated in either gas mixture are well known to produce an abundance of ROS including OH, O, O*, ¹O₂, O₃, H₂O₂, and O₂⁻.^{19,21} Figure 3 highlights the emission

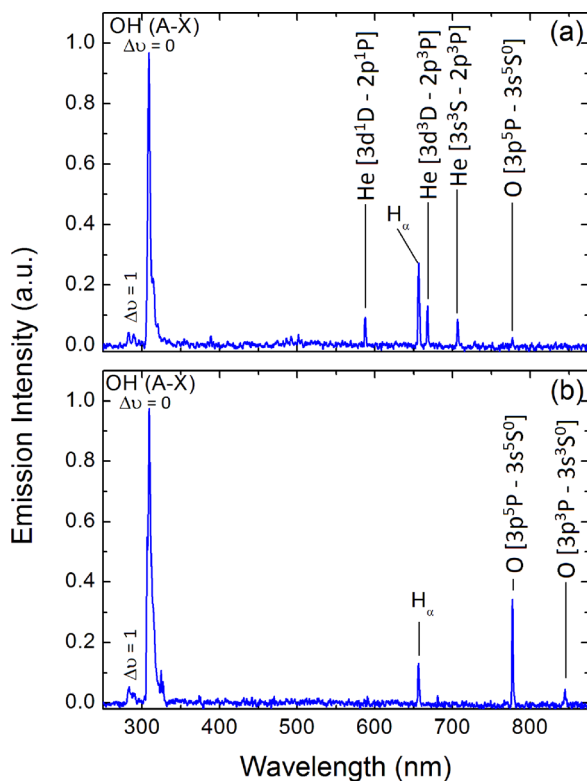


FIG. 3. Low resolution emission spectra from 250–850 nm showing: (a) excited states in a 3.7 W He-O₂ plasma discharge; and (b) excited states in a 3.7 W O₂ plasma discharge.

spectra from He-O₂ and O₂ plasmas; both are clearly dominated by the OH (A²Σ – X²Π) emission. In both cases, the main production pathway for OH(A) is through the dissociative electron excitation of water; with an additional pathway in the He-O₂ discharge of the dissociative excitation of water with metastable He (19.8 eV).^{22,23} Notably, the O₂ discharge indicates a strong emission from O* at 777 nm (⁵P → ⁵S⁰) and 844 nm (³P → ³S⁰), suggesting increased production of the powerful oxidant O*. While optical emission spectroscopy does not provide a clear indication of ground state species, it is safe to assume that both discharges are capable of producing a plethora of oxidizing species, thus the observed differences in adsorbent modification are not explained by a lack of ROS production.

To further probe the differences between the He-O₂ and O₂ discharges, Fig. 4 shows high-resolution optical emission spectra of the OH (A²Σ – X²Π, Δv = 0) transition for both discharges operating at 3.7 W; by fitting the experimental data with a synthetic spectra calculated at a known temperature, the rotational temperature is ascertained. It is well known that a deviation from a Boltzmann rotational population distribution occurs due to the formation process of OH(A); hence a two-temperature fitting routine has been adopted, similar to that used by Bruggeman *et al.*²⁴ As the rotational relaxation time is fast at atmospheric pressure, the temperature T₁ from the two-temperature fitting routine can be considered as a reliable estimate of gas temperature.²³ The best-fit temperature, T₁, in the He-O₂ discharge was

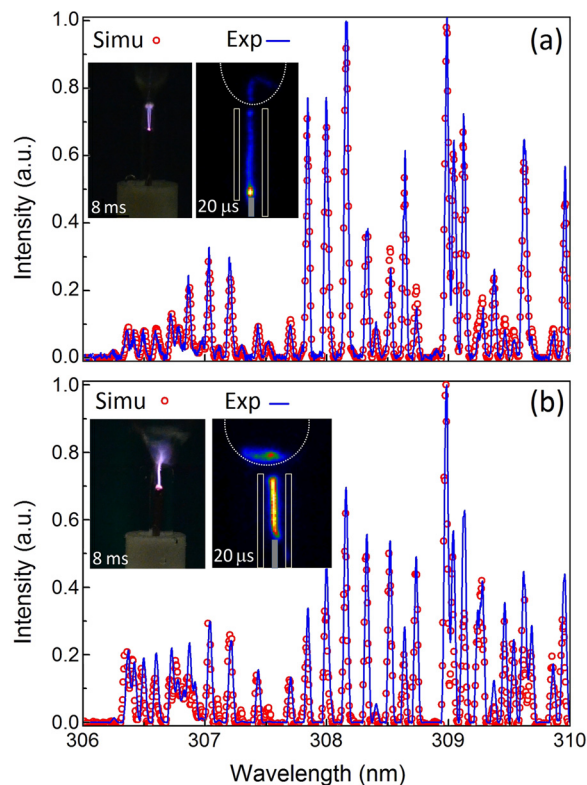


FIG. 4. High resolution emission spectra from 306 to 310 nm showing OH (A-X) emission in: (a) He-O₂ plasma at 3.7 W, best-fit parameters T₁ = 380 K (90%) and T₂ = 6500 K (10%); and (b) O₂ plasma operating at 3.7 W, best-fit parameters T₁ = 1100 K (80%) and T₂ = 4000 K (20%). The solid blue lines indicate the experimental data, the red circles represent best-fit theoretical spectra, and the inserts show images captured using exposure times of 8 ms and 20 μs, respectively.

found to be 380 ± 50 K; in contrast, the best-fit temperature in the O_2 discharge was significantly higher at 1100 ± 50 K. Such discrepancies are typical and are linked to the thermal conductivities of atomic and molecular gases and differing energy transfer mechanisms.²³ Also shown in Fig. 4 are images taken with 8 ms and 20 μ s exposure times, thus capturing ~ 200 discharge events and ~ 1 discharge event, respectively. In both cases, it is clear that a filamentary discharge is produced; this is consistent with the previous studies of plasma generation in bubbles.^{24,25} Crucially, in O_2 , an intense filament was observed to extend directly from the pin electrode to the bubble (or liquid surface after bubble detachment). Given that temperatures in excess of 1000 K are expected, it is highly likely that adsorbent material passing in close proximity to the discharge will experience significant heating, with the potential to cause structural damage. In the He- O_2 case, a filamentary discharge is also observed. However, the peak emission intensity arises at the electrode tip some 2 mm from the capillary exit. As the temperature measurements are not spatially resolved, it is likely that this intense region dominates the OH emission profile and the plasma reaching the bubble has a significantly lower temperature than the 380 K measured.

Analysis of electrical data, shown in Fig. 5, indicates that the peak dissipated power in each discharge differed significantly despite the time-averaged power being held constant. In He- O_2 current, spikes of 8–10 mA were observed in each half-cycle of the applied voltage, resulting in peak instantaneous dissipated powers of ~ 15 W. Conversely, in O_2 a large and sporadic discharge current spike was observed roughly once in every 10 applied voltage cycles, with amplitudes between 100 and 200 mA, leading to instantaneous peak powers of several kilowatts. Increasing the time-averaged

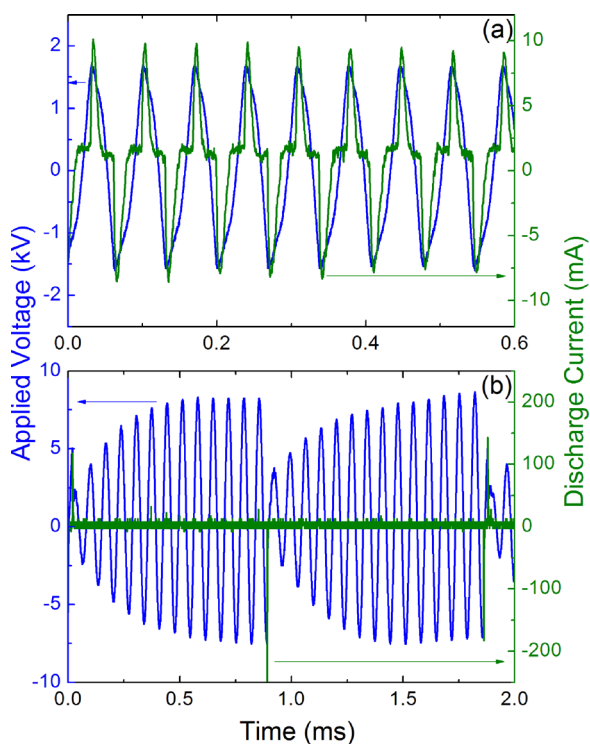


FIG. 5. Current and voltage waveforms in: (a) a He- O_2 discharge operated at 3.7 W; and (b) an O_2 discharge operated at 3.7 W.

input power in the O_2 discharge from 3.7 W to 10.7 W yielded an increase in the number of discharge events in a given time period to approximately once in every three applied voltage cycles. The differences between the He- O_2 and O_2 discharge current waveforms are attributed to the different physical characteristics of each plasma, most notably the electron density and breakdown voltage, which are both significantly higher in O_2 .

In order to assess the impact of plasma treatment on the structural integrity of the adsorbent material, scanning electron microscopy (SEM) was employed. After plasma treatment, samples were prepared by dehydrating in ethanol followed by critical point drying. Figure 6 shows SEM images of untreated and He- O_2 and O_2 plasma treated Q Sepharose Fast Flow media. The appearance of Q Sepharose Fast Flow remained unaffected following treatment with He- O_2 plasma (compare Figs. 6(a) and 6(b)). In stark contrast, numerous deep punctures or holes (ranging from 5 to 25 μ m across) are clearly evident in adsorbent particles previously exposed to the O_2 plasma (Fig. 6(c)). Such damage is a likely result of individual supports coming in direct contact with an

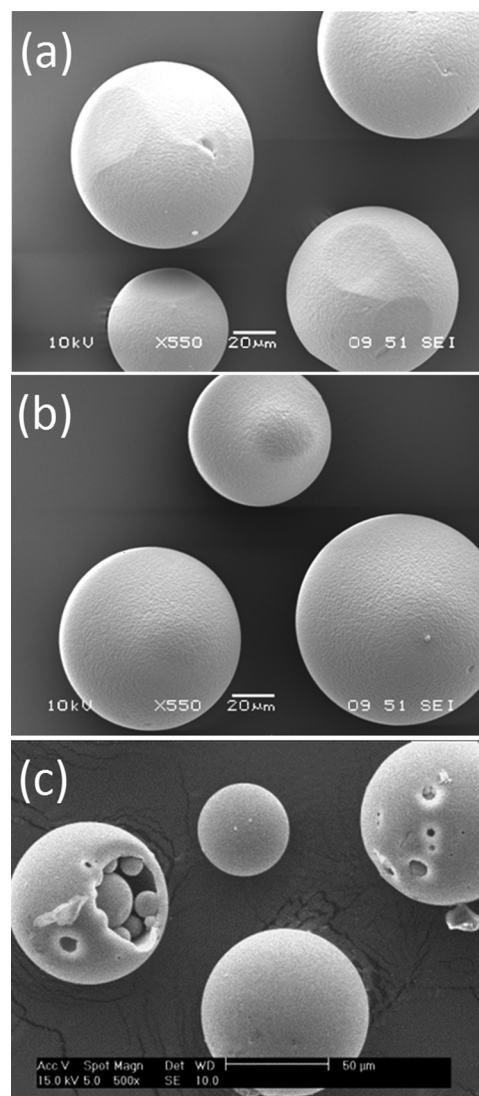


FIG. 6. SEM images of (a) untreated Q Sepharose Fast Flow and Q Sepharose Fast Flow following 150 s of exposure to 3.7 W He- O_2 (b), and O_2 (c) plasmas.

intensely hot plasma filament. The punctures not only increase the amount of surface that pDNA can access and bind to, they also simultaneously reduce the overall core volume of individual adsorbent beads and therefore their BSA binding capacity. The severe damage noted here clearly precludes the use of O₂ plasma for surface etching of soft chromatography materials, and also fully explains the lack of surface vs. core modification selectivity inferred from the static pDNA and BSA binding studies (see Fig. 2).

In summary, this letter reports a technique to modify the surface properties of chromatography adsorbent media directly in aqueous solution. The results show a promising level of selectivity can be achieved between surface pDNA binding and core protein binding, thus forming a bifunctional media and potentially addressing one of the critical bottlenecks associated with packed bed chromatography. Moreover, the work represents one of the first demonstrations of atmospheric pressure plasma processing of materials directly in solution; it is envisaged that the techniques detailed will have considerable application potential not just in the field of packed bed chromatography, but across the whole field of plasma surface engineering.

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