CHO Cells Adhering to Nitrogen-Rich Plasma-Polymerised Ethylene Exhibit High Production of a Specific Recombinant Protein

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In many industrial applications, inadequate cell attachment can be a limitation, especially when serum-free media are used. Nitrogen-rich plasma-polymerised ethylene (PPE:N) exhibits high concentrations of polar groups that can help to promote the attachment of weakly adherent cell types. Tissue plasminogen activator-producing Chinese hamster ovary (CHO) cells, adapted to suspension, were grown in the presence PPE:N flakes and were found to adhere to them. The growth rate was reduced, but cell viability was enhanced and their metabolism was more efficient, with generally higher recombinant protein productivity. Finally, cell adhesion on PPE:N surfaces was found to be independent of integrins, and was probably mediated by certain non-specific interactions with the PPE:N surface.

Introduction

Cell adhesion on a surface is a key event in mammalian cell culture, one that enables sustained viability and is often a premise to growth.[1] Several materials enable cell anchoring, the most widespread being commercially available tissue culture polystyrene (TCPs) (for conventional Petri dishes); TCPs is usually plasma modified so as to incorporate bound oxygen at the surface, thereby rendering the surface hydrophilic and negatively charged. The first phase of cell adhesion is the adsorption of serum proteins (especially vitronectin and fibronectin)[2,3] on the plastic surface, followed by cell attachment to these proteins. Consequently, adhesion may be greatly impaired in the absence of serum. However, efforts toward avoiding serum addition are becoming increasingly frequent because of inconsistent serum composition and its potential for pathogenous content.[4,5] Therefore, materials with improved attachment properties are of great help for serum-free cultures and many investigations have been undertaken in this direction. With the same objective, to develop new high-performance cell-culture surfaces, a novel material, nitrogen-rich plasma-polymerised ethylene (PPE:N) has been developed.[6] PPE:N is deposited using a...
dielectric barrier discharge (DBD) plasma at atmospheric pressure. Characteristics of PPE:N such as a high nitrogen concentration, \([N]\), and a high surface energy, \(\gamma_s\), render it a promising potential candidate for promoting strong cell adhesion, largely due to its highly functionalised surface comprising amines, imines, and nitriles.\(^6\) The ability of cells to adhere and proliferate on PPE:N surfaces has been demonstrated with chondrocytes,\(^6\) mesenchymal stem cells (MSCs),\(^7\) and vascular smooth muscle cells (VSMCs),\(^8\) but most strikingly in the case of the usually non-adherent U-937 monocyte cell line.\(^6\) Moreover, PPE:N is a versatile material, since \([N]\) can readily be varied so as to modulate cell-adhesive behaviour and to control phenotype.\(^7\) Additionally, a wide variety of materials and geometries (meshes, films, particulates composed of polypropylene, polystyrene, poly(ethylene terephthalate), poly(tetrafluoroethylene), inorganic materials, etc.) can be coated with variable thickness and chemical composition (variety and concentrations of functional groups) to suit different applications.

As already mentioned, the advantages of PPE:N have already been demonstrated in tissue engineering by promoting cell adhesion onto biomaterial substrates or by orienting cell phenotype.\(^7,6\) The benefit of its use for other biotechnological processes is now being investigated. One of the possible applications of PPE:N is in micro-carrier culture, which has been used for several decades in largescale culture of anchorage-dependent cells in bioreactors. Common applications are the production of vaccines, viruses, and human recombinant proteins.\(^9–13\) However, depending on the cell line, cell attachment to a micro-carrier can be quite inefficient or weak in serum-free media.\(^10,12,13\) Because of its demonstrated strong adhesive properties, micro-carriers coated with PPE:N might be used for culture of cells that are poorly adherent in the absence of serum.

PPE:N also has a potential use in hollow fiber bioreactors, which are mainly operated to produce antibodies.\(^14–16\) PPE:N coating of the capillaries might enhance cell attachment, especially in the absence of serum. Another interesting application of PPE:N could be in the field of small-scale perfusion bioreactors that are used for on-line monitoring of the metabolic and physiological state of cells \(\textit{in vivo}\) \(^17,18\) and where it is crucial to retain the cells within the growth chamber.

Finally, another important characteristic of PPE:N is its capacity for functionalisation by grafting biologically active molecules to its amine groups. Grafting adhesive peptides such as RGD (arginine-glycine-asparatic acid), polylsine peptides,\(^19\) or growth factors\(^20,21\) might further increase cell adhesiveness. On the contrary, to promote controlled cell detachment, PPE:N might be grafted with poly(N-isopropyl acrylamide) (PNIPAM).\(^22\) PNIPAM-grafted surfaces display temperature-dependent reversible hydrophilic or hydrophobic property alterations that enable the adhesion and growth of cells at 37 °C, but their detachment at lower temperatures. Therefore, it facilitates a trypsin-free cell harvest.

As described above, PPE:N coatings can be used in various applications. However, the suitability of PPE:N-coated surfaces for recombinant protein production cultures still needs to be confirmed. A Chinese hamster ovary (CHO) cell line was chosen for this investigation, one that produces a protein of pharmaceutical interest, namely tissue plasminogen activator (tPA), which is used in the treatment of heart attacks and strokes. This cell type is used extensively in biotechnological processes and is therefore representative of these technologies. CHO cells can be easily adapted to suspension culture, where they then have the particular characteristics to be non-adherent and not to bind to most materials (including TCPS Petri dishes, or glass beads); they do not aggregate and are usually cultured as a dispersed cell suspension. Therefore, the objective of this work was, first, to observe if PPE:N could promote adhesion of non-adherent tPA-producing CHO cells under serum-free conditions. In addition, we also set out to study the effect of the presence of PPE:N on cell behavior, when used as a cell support. Cell viability, growth, metabolism, and protein production in the presence and absence of PPE:N were thus compared.

With these objectives in mind, PPE:N in the form of flakes was added to classical CHO cell suspension culture (the control culture), and we report here the ability of CHO cells to bind PPE:N surfaces, to proliferate, and to synthesise tPA in the presence of this novel material.

**Experimental Part**

**PPE:N Deposition**

The method for depositing PPE:N coatings by atmospheric pressure glow discharges (APGD) has been described by Giraud-Lauriult et al.\(^6\) Briefly, the APGD/DBD apparatus comprised a cylindrical, dielectric-coated stainless steel high-voltage (HV) electrode and a grounded, planar Al electrode on which reposed a 300 × 240 cm\(^2\), 2 mm-thick glass plate that served both as substrate and as a second dielectric layer. The gap between the two was typically 1 mm. The electrode system was housed inside a hermetic, transparent plastic enclosure, in order to maintain a controlled composition of gas flow at atmospheric pressure. The lower (planar) electrode and glass plate were moved under the HV electrode at controlled speed, thereby ensuring a uniform coating thickness on the entire area of the substrate. The precursor gas mixture comprised a flow of nitrogen (\(N_2, 10\) standard l min\(^{-1}\), slm) and ethylene (\(C_2H_4, 10\) standard cm\(^3\) min\(^{-1}\), slcm), and it was introduced into the discharge zone between the HV electrode and the glass plate by means of a special gas diffuser. The duration of plasma-assisted deposition was selected so as to create a PPE:N coating that was some tenths of a micrometer in thickness. Flakes of PPE:N were produced and harvested shortly after deposition by scraping the coating off the glass surface with a sharp (razor) blade; about 10-\(\mu\)g...
quantities of flakes were produced during typical deposition runs. The composition of PPE:N films was determined by X-ray photoelectron spectroscopy (XPS) using a Kratos Axis Ultra system with non-monochromatic Mg Kα radiation. Survey spectra were used to determine the relative elemental concentrations of C, N, and O; for the case of conditions described above, [N] = 29.5 at.-% and [O] = 7 at.-%, the latter derived from reactions of trapped radicals with oxygen when the deposits were exposed to atmospheric air. A very detailed characterisation of PPE:N is presented elsewhere.[23]

**Cell Culture**

t-PA-producing CHO cells (ATCC, CRL9606), adapted to serum-free suspension culture, were kindly provided by Dr. Michael Butler, University of Manitoba. Cells were inoculated into suspension in 24-well polystyrene (PS) culture plates (Becton Dickinson, Franklin Lakes, NJ, USA), at a concentration of 1 x 10⁵ cells·mL⁻¹, in a total volume of 1 mL of Biogro-CHO culture medium, special CellNet formulation (Biogro Technologies, Winnipeg, MB, Canada), supplemented with 7.5 mg·L⁻¹ phenol red, 3.5 x 10⁻³ M glutamine, 2.7 x 10⁻² M glucose, and 0.2% (w/v) Pluronic F68. In half of the wells, 0.33 mg·mL⁻¹ of PPE:N flakes were added. Cultures in the presence and absence of PPE:N were hereafter designated as “PPE:N culture” and “control culture”, respectively.

**Cell Concentration and Viability**

For both the PPE:N and control cultures, three wells of the culture plates were harvested daily to evaluate cell concentration and viability. For the control culture, cell concentration and viability were evaluated with a hemocytometer after dilution by one half with Trypan blue. The remaining cell suspension was then centrifuged (300 g, 5 min), and the supernatant was stored in liquid nitrogen for subsequent analysis.

Regarding the PPE:N culture, an aliquot was first taken to measure cell density and viability, as in the control case. Only free cells (those not adhering to PPE:N) were counted, to evaluate the non-adherent cell concentration, [Cell]Non-adherent. The remaining suspension was then centrifuged (300 g, 5 min) and the supernatant was also stored in liquid nitrogen for subsequent further analysis, as described above. The pellet was resuspended for 5 min at 37 °C in Trypsin/EDTA (0.25% w/v Trypsin, and 0.38 g·L⁻¹ EDTA) then re-suspended in a 20 μm mesh (Spectra/Mesh Nylon Macroporous Filter, Spectrum Laboratories, Rancho Dominguez, CA, USA) to retain the PPE:N flakes, but not the cells, following which cell concentration and viability were evaluated for the filtrate to obtain the total cell concentration, [Cell]Total. The concentration of cells adhering to PPE:N was therefore given by the difference: [Cell]Total = [Cell]Non-adherent. The efficiency of Trypsin/EDTA digestion has been verified by microscopic observation, which revealed complete detachment of cells from the PPE:N flakes, thereby allowing an accurate estimation of cell concentration and viability.

**Glucose, Glutamine, Lactate, and Ammonium Assays**

Glucose, glutamine, lactate, and ammonium concentrations in the culture media were determined using enzymatic assays (glucose assay kit GAHK-20, glucose assay kit G6PDH-2, ammonia assay kit AA0100, all from Sigma-Aldrich Canada, Oakville, ON, Canada; lactate assay kit 735-11 from Trinity Biotech, Bray, Ireland), on media samples harvested daily as described above. Specific nutrient consumption and metabolite production rates (in μmol·10⁶ cells⁻¹·d⁻¹) were calculated using two successive data point intervals.

**t-PA Analysis**

Active t-PA concentration in culture medium was determined by an enzymatic assay using Chromozym t-PA (Roche Diagnostics, Laval, QC, Canada) that can be detected at 405 nm when cleaved by t-PA. In a 96-well plate, 60 μL samples of medium were mixed with 200 μL of 0.4 x 10⁻³ M Chromozym t-PA in 90 x 10⁻³ M Tris with 0.135% (w/v) Tween 80, at pH 8.5. The plate was then incubated for 30 min at 37 °C, following which the reaction was stopped by the addition of 100 μL of 10% (w/v) citric acid, and the absorbance at 405 nm was measured with a microplate reader (Dynatech MRX, Dynex Technologies, Chantilly VA, USA). t-PA analysis was accomplished on media samples harvested daily, as detailed above. These samples were compared to t-PA standards (630 kU·mg⁻¹, EMD Biosciences, San Diego CA, USA), with values ranging from 0.5 to 30 mg·L⁻¹. Specific tPA production rates (μg·10⁶ cells⁻¹·d⁻¹) were calculated using two successive data point intervals.

**Microscopic Observations**

Microscopic observations were performed each day using an inverted microscope (Axiovert S100TV, Carl Zeiss Canada, North York, ON, Canada) in the Köhler transmission mode. Images were acquired with a digital camera and the Northern Eclipse Software (Empix Imaging, Mississauga, ON, Canada).

**Cell Cycle Analysis by Flow Cytometry**

At days 3 and 6, three additional samples of each of the two cultures were harvested. Cells cultured with PPE:N were first pelleted, digested with trypsin/EDTA, and filtered as described in Cell Concentration and Viability. Those cells, as well as undigested cells from the control culture (about 1 million cells) were then centrifuged (300 g, 5 min, 4 °C), fixed by re-suspension in 1 mL of 70% cold ethanol, and kept at −20 °C until analysis. Then the ethanol was removed by centrifugation (300 g, 5 min, 4 °C) and cells were permeabilised with 100 μL of 0.1 M citric acid, 0.5 vol. % Tween 20, under vigorous agitation for 10 min, followed by the addition of 400 μL of 2 μg·mL⁻¹ 4′-6-diamidino-2-phenylindole (DAPI) (Molecular Probe, Burlington, ON, Canada) in 0.4 M Na₂HPO₄. Cells were incubated for at least 15 min in DAPI solution at room temperature and processed within 2 h. DAPI fluorescence was measured with a MoFlo cytometer (MoFlo BTS, DakoCytomation, Carpintera, CA, USA).
USA) under UV laser for excitation (Enterprise II, Coherent, Santa Clara CA, USA). For each sample, 5,000 to 10,000 events were collected, the fluorescence being detected through a 450/30 (FL3) bandpass filter. Signals were amplified in the linear mode, and Summit Software (v. 3.1, Dako Cytomation, Mississauga, ON, Canada) was used to identify quiescent cells (in the G0/G1 phase).

### Western Blotting

At day 6, samples from each of the two cultures were harvested and extracted in RIPA buffer [150 x 10^{-3} mm NaCl, 1% w/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate (SDS), 10 x 10^{-3} mm Tris pH 7.2] with a protease-inhibitor cocktail (1X, Sigma-Aldrich Canada, Oakville, ON, Canada), flash-frozen, and stored at -80°C until subsequent analysis. Once thawed, protein extracts were mixed 3:1 (v/v), with 4X loading buffer and electrophoresed on 7.5% acrylamide/bisacrylamide gel. Proteins were then transferred onto a poly(vinylidene fluoride) (PVDF) membrane and probed for 1 h at room temperature with an anti-integrin β1 (AB 1952, 1/4000; Chemicon, Temecula, CA, USA), then for 1 h with a horseradish peroxidase (HRP) conjugated secondary antibody (Zymed anti-rabbit-HRP, 1/2500, Invitrogen, Burlington, ON, Canada), both in 1 vol.% goat serum, for 1 h at room temperature with an anti-

### Immunostaining and Confocal Laser Scanning Microscopy

All steps were performed at room temperature in 0.3% (w/v) Triton X-100 with phosphate-buffered saline (PBS), unless stated otherwise. At day 6, cells cultured with or without PPE:N were transferred onto a Nylon membrane filter (0.45 μm pore size; Whatman, Clifton, NJ, USA) mounted in a centrifuge tube and the above-described solutions were added onto the filter retaining the cells, and then removed by centrifugation (300 g, 2 min). Cells were first fixed in 4% (w/v) paraformaldehyde in PBS and permeabilised with 0.3% (w/v) Triton X-100 in PBS for 30 min. Afterwards, cells were blocked in 10 vol.% goat serum overnight at 4°C, then incubated with an anti-integrin β1 antibody (AB1952, at 1/400, Chemicon, Temecula, CA, USA), followed by an anti-rabbit antibody conjugated with Cy3 (Pharmacia-Amersham, Baie d’Urfé, QC, Canada), both in 1 vol.% goat serum, for 1 h at room temperature. Actin was stained with Alexa-488 phalloidin (Molecular Probes, Burlington, ON, Canada), diluted 1/1000 in PBS for 1 h, and the nucleus was stained with 0.5 μg · μL^{-1} Hoechst 33258 (Probes, Burlington, ON, Canada) in PBS for 10 min. Each step was followed by three washes of 5 min duration in 0.3% (w/v) Triton X-100 in PBS. Finally, samples were treated against quenching with 11 700 U · mL^{-1} beef catalase and 133.3 U · mL^{-1} glucose oxidase (both from Fluka, Buchs, Switzerland) in PBS for 30 min and mounted in 16.67% (w/v) Mowiol 4-88 (Fluka, Buchs, Switzerland), 33.3 vol.% glycerol, 0.75% (w/v) propyl gallate (Sigma-Aldrich Canada, Oakville, ON, Canada) in PBS. Confocal imaging was performed using an Apochromat 40X/NA 1.2 water-immersion objective, mounted on an Axioplan 2 microscope, equipped with an ISM 510 META confocal laser scanning module (all from Carl Zeiss, North York, ON, Canada). Alexa 488 was excited with a 488-nm argon laser and Cy3 with a 543-nm helium–neon laser, while Hoechst 33258 was exposed to two-photon excitation using a mode-locked, pulsed Ti:sapphire laser (VerdiV10/Mira 900, Coherent, Santa Clara, CA, USA) operating at 780 nm. Images were recorded using a BP 510/520 IR bandpass filter for Alexa 488, a BP 565/615 bandpass filter for Cy3, and a BP 435-485 IR bandpass filter for Hoechst 33258.

### Results and Discussion

#### Cell Adhesion on PPE:N Flakes

In the case of the PPE:N culture, 95 ± 4% of the cells were found to adhere to PPE:N flakes after 1 d, and multi-cellular structures had started to form since the second day (Figure 1A and C). Cells then proliferated, thereby enlarging the cell–PPE:N aggregates; evidently, the outermost cells were not in contact with the PPE:N surfaces. The proportion of aggregated cells around PPE:N flakes was very significant (Figure 2) and constant throughout the 7 d of culture, namely an average of 91 ± 4.7%. In parallel, viability of the attached cells was found to be high, with 87 ± 3.4% of cells viable after 7 d, while viability of unattached cells was only of 60.7 ± 11.8%.

On the other hand, control cells (grown without PPE:N flakes) did not adhere to the surfaces of the wells; they formed only loose aggregates (Figure 1D), and a high cell density was attained at the end of the period of culture. This was expected since this behaviour is typical of this CHO cell line, which is known to be non-adherent and is usually cultured in suspension without aggregate formation.

Therefore, PPE:N seems to promote the binding of tPA-producing CHO cells, as similarly observed by others with a different non-adherent cell type. Its high concentration of...
nitrogen, \([N]\), more particularly of primary amines, \([\text{amine}]/\text{C}_0\text{NH}_2\), is likely responsible for this cell-adhesion property.\(^{23}\) For instance, PPE:N is known to promote the adhesion of U-937 macrophages when containing \([N] > 25\%\) (\(\text{``}[N]_{\text{crit}}\text{''}\)), whereas PPE:N with lower values of \([N]\) does not.\(^6\) Furthermore, PPAN (nitrogen-rich plasma-polymerised acetylene), a similarly \(N\)-rich material, but with a much lower \([\text{amine}]/\text{C}_0\text{NH}_2\) value,\(^{23,24}\) has not been found to promote the adhesion of U-937 cells. Indeed, for the case of U937, the very strong link between cell adhesion and a “critical” (minimum value) of primary amine concentration, \([\text{amine}]/\text{C}_0\text{NH}_2\)\(^{\text{crit}}\), as well as modified gene expression induced in adhering U937 cells, is the object of a very recent, detailed report.\(^{24}\) Several other studies also support the hypotheses expressed above. For instance, grafting amine groups to polyethylene films was found to promote cell adhesion, contrary to other polar functional groups (\(-\text{COOH}, -\text{CH}_2\text{OH}, \text{or the uncharged -CONH}_2\)).\(^{25}\) Similarly, self-assembled monolayers (SAMs) of alkanethiols bearing different functional groups, including methyl (\(-\text{CH}_3\)), hydroxyl (\(-\text{OH}\)), carboxylic acid (\(-\text{COOH}\)), and amine (\(-\text{NH}_2\)), were tested for cell adhesion. Human umbilical vein endothelial cells (HUVEC) adhered well on \(-\text{NH}_2\) and \(-\text{COOH}-\text{rich} surfaces, but poorly on \(-\text{CH}_3\) and \(-\text{OH} ones.\(^{26}\) Consequently, it is highly likely that the observed adhesion of \(\text{CHO}\) cells to PPE:N is due to the surface chemistry of the latter, probably to its \(-\text{NH}_2\) content, but this is yet to be fully confirmed.

**Cell Growth and Viability with and without PPE:N**

Growth and viability in control and PPE:N cultures were compared (Figure 3A). In the control case, \(\text{CHO}\) cells grew exponentially from day 1 to reach \(2.25 \pm 0.47 \times 10^6\text{ cells \cdot mL}^{-1}\) on day 5. During this period the specific growth rate \((\mu)\) was \(0.51 \pm 0.065\text{ d}^{-1}\) while maintaining a high cell viability (\(\approx 90\%\)). However, from days 5 to 7, viability dropped to \(63.2 \pm 5.1\%\) and the viable cell concentration was reduced to \(1.35 \pm 0.51 \times 10^6\text{ cells \cdot mL}^{-1}\). Regarding the PPE:N culture, the exponential cell growth rate was observed to be lower \((\mu = 0.36 \pm 0.027\text{ d}^{-1})\), and the maximum cell concentration was about half of that in the control case. However, total cell viability at the end of the 7-day PPE:N culture period was higher \((83.6 \pm 3.4\%)\) than in the control case \((63.2 \pm 5.1\%)\). Indeed, after 7 d the cell concentrations in the two conditions were quite similar, namely \(1.35 \pm 0.51 \times 10^6\) and \(1.01 \pm 0.18 \times 10^6\text{ cells \cdot mL}^{-1}\) in the control and PPE:N cultures, respectively.

To further characterise the cell growth, cell cycle analyses were undertaken by flow cytometry (Figure 3B). A higher proportion of cells were found to be in the \(\text{G}_0/\text{G}_1\) phase when grown on PPE:N flakes, which confirmed the differences observed in the cell-specific growth rates.

Consequently, under the conditions tested, cells proliferated more slowly when aggregated around PPE:N flakes.
than in single-cell suspension. Similar results have already been reported for CHO, namely that they were demonstrated to grow less rapidly when aggregated than when dispersed.\textsuperscript{[11,27]} Indeed, slower cell growth can be explained by limited nutrient transport to the center of aggregates\textsuperscript{[27,28]} but also by the tighter packing in aggregates that leaves less room for cell division (mitosis). Mitosis probably occurs mainly at the outer layers of the aggregates.

**Metabolic Behaviour of Cells**

The glucose concentration decreased rapidly (Figure 4) during the first 5 d, from $21 \pm 0.29 \times 10^{-3}$ to $1.49 \pm 0.13 \times 10^{-3}$ M in the control culture, and from $21 \pm 0.37 \times 10^{-3}$ to $3.68 \pm 0.32 \times 10^{-3}$ M in the PPE:N culture, indicating that the total glucose consumptions were comparable in the two cases. Interestingly, the lactate concentration reached $54.9 \pm 8.4 \times 10^{-3}$ M in the control, but only $35 \pm 0.5 \times 10^{-3}$ M in the PPE:N culture conditions. This suggests that the presence of PPE:N flakes had considerable influence on the cell metabolism. Indeed, the molar yield, $Y_{\text{lac/gluc}}$ (mol/mol), between days 2 and 5 (the period corresponding to exponential growth) was higher for the control than for the PPE:N cultures, namely $1.94 \pm 0.41$ versus $1.18 \pm 0.07$ mol/mol, respectively. Concerning glutamine, its consumption by cells was higher in the control culture case, namely $2.1 \pm 0.24 \times 10^{-3}$ M in 5 d, compared to $1.4 \pm 0.24 \times 10^{-3}$ M in the presence of PPE:N. Thereafter, between days 5 and 7, the glutamine concentration stabilised in both cultures. In parallel, the concentration of ammonia reached a maximum of $3.29 \pm 0.37 \times 10^{-3}$ M after 4 d in the control culture, and after 5 d in the PPE:N culture, namely $2.72 \pm 0.11 \times 10^{-3}$ M. Similar molar yield values, $Y_{\text{amm/gln}}$ (mol/mol), were calculated for the two cases, $0.95 \pm 0.27$ and $0.85 \pm 0.16$, respectively, between days 2 and 5.

In general, therefore, nutrient consumption and metabolite production were found to be lower in the presence of PPE:N. However, since cell concentrations differed among the two cases, specific rates of production and consumption had to be calculated (Figure 5) to better assess metabolic behaviours for the two culture conditions. While the specific lactate and ammonia production and glutamine consumption rates were similar and almost superimposable, the specific glucose consumption rates were found to differ significantly. In the control culture, it was initially very high but then decreased progressively from $12.3 \pm 3$ to $0.2 \pm 0.3 \mu\text{mol} \cdot 10^{6} \text{cells}^{-1} \cdot \text{d}^{-1}$. In contrast, in the case of PPE:N culture, the value first increased between days 1.5 to 4.5 from 0 to $14.7 \pm 1.6 \mu\text{mol} \cdot 10^{6} \text{cells}^{-1} \cdot \text{d}^{-1}$, but then dropped rapidly to $0.196 \pm 0.4 \mu\text{mol} \cdot 10^{6} \text{cells}^{-1} \cdot \text{d}^{-1}$, concurrently with glucose depletion in the medium.

**Figure 4.** Concentrations of (A) glucose; (B) glutamine; (C) lactate; and (D) ammonium in the culture medium were evaluated daily for up to 7 d for CHO cell cultures with and without the presence of PPE:N flakes. Results shown represent mean values ± standard deviations, $n = 3$. 

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Production of t-PA

The overall production of human recombinant t-PA was higher in the control culture, with the concentration reaching $10.26 \pm 0.43 \text{ mg L}^{-1}$, while the corresponding value in the PPE:N culture was $6.5 \pm 0.36 \text{ mg L}^{-1}$ (Figure 6A). This result was mainly due to the lower cell concentration in the presence of PPE:N, but the specific t-PA production rate was higher in this latter case (Figure 6B). In other words, in the presence of PPE:N, CHO cells exhibited a higher recombinant protein productivity.

Characterisation of Cell Adhesion on PPE:N Flakes

In order to shed additional light on CHO interaction with PPE:N, the adhesion mechanism was investigated. Usually, cell adhesion involves either non-specific physical interactions or specific cell receptors.\cite{29} In the latter case, cells adhere to matrix proteins (such as fibronectin) that are either secreted by the cells themselves or supplied by the culture medium. These proteins first coat the culture surface, subsequently enabling cell attachment and spreading via integrin receptors.\cite{30,31}

Integrins are heterodimers comprising an $\alpha$ and a $\beta$ subunit. Among $\beta$ sub-units, $\beta 1$ is most often implicated in cell–matrix interactions, and it was consequently studied by Western blotting (Figure 7). Two bands were detected, one at 137 kDa corresponding to the extracellular mature protein, the other at 118 kDa corresponding to the immature intracellular inactive protein.\cite{32} When CHO cells were subjected to control culture, both bands were detected and the former protein (137 kDa) was present in higher concentration than the latter (118 kDa). By contrast, in the presence of PPE:N, only the 118-kDa band was observed, implying that cell adhesion to PPE:N did not involve integrin $\beta 1$.

In addition, cells were stained for actin and integrin $\beta 1$ (as illustrated in Figure 8). In both cultures, cells exhibited a rounded morphology and actin remained cortical, rather than organised in stress fibers that typifies cell adhesion to the matrix.\cite{33} The distribution of integrin $\beta 1$ correlated with the Western blotting results. In the control culture, a localized “punctual” staining was observed (immature protein), with occasional stronger staining at the cell–cell interface (transmembrane, mature protein). In the presence of PPE:N, no staining was noticed at the cell–cell or cell–PPE:N interfaces except in the case of some rare flattened cells (see arrow in Figure 8C), suggesting that these cells possibly began to spread. Finally, PPE:N itself was found to fluoresce at the wavelength used to detect integrin $\beta 1$ (see Figure 8B, upper right panel). This was probably due to non-specific binding of primary and/or secondary antibodies to the PPE:N flakes.
The observed round morphology, combined with the absence of mature integrin $\beta_1$ in the case of PPE:N culture, indicated that adhesion most probably occurred here in a non-specific way. To further test the hypothesis of a non-integrin adhesion, RGD peptides were also used (added directly into the culture medium at $0.5 \text{ mg/mL}$). This sequence, found in numerous matrix proteins and recognised by many integrins,\[34\] is known to prevent integrin-mediated attachment of cells to these proteins.\[35\] The inability of RGD peptides to inhibit cell adhesion to PPE:N surfaces (data not shown) reinforced our previous observations. On the other hand, because of the high concentration of primary amines on its surface,\[6,23,24,36\] PPE:N possesses a positively charged surface at physiological pH, which is known to promote cell adhesion.\[37–39\] All of these indications, taken together, point to a non-specific cell-adhesion mechanism.

Finally, there are several sets of evidence that PPE:N may have influenced the expression of adhesion protein in CHO cells. First, it can be observed on the Western blot (Figure 7) that the pattern of integrin $\beta_1$ expression differed between the two cultures. In parallel, PPE:N promoted cell–cell adhesion, as cells not only adhered to the polymer surfaces but also aggregated, which was not observed in the absence of PPE:N (i.e., in the control culture). These results are compatible with previous studies showing that the surface composition can strongly influence the involvement of integrins in cell adhesion. For example, MC3T3-E1 cells cultured on glass surfaces with different functionalities ($-\text{OH}$, $-\text{COOH}$, $-\text{NH}_2$, and $-\text{CH}_3$) were found to adhere by way of different types of integrins.\[40,41\]

**General Discussion**

In previous studies, PPE:N has already been demonstrated to be a very useful material for biomedical applications. It has been shown to promote cell adhesion, to influence cell
behaviour (by promoting cell resistance to apoptosis), and to favourably alter phenotype.\cite{18,19} It has now also been shown to present great potential in biotechnological processes, as an adhesive physical support for cells, which can be used in different types of reactors (stirred suspension, hollow fiber, minibioreactor, etc.). Furthermore, the presence of certain reactive functional groups on PPE:N may enable one to design specially functionalised surfaces on which cell adhesion, cell detachment, and phenotype selectivity may be controlled. PPE:N was found not to be detrimental for cells, which exhibited an excellent viability and active metabolism in presence of this novel material. The only possible drawback in using PPE:N is the somewhat lower rate of cell proliferation observed here in the case of PPE:N culture. However, the shapes of PPE:N flakes used in this work were irregular, and previous studies have shown that microcarriers of regular shape and size are more likely to promote proliferation observed here in the case of PPE:N culture.\cite{17,18} Moreover, the final cell density also depends on the “cell/support surface ratio”,\cite{19,20} which was not optimised in this present study. Optimising this ratio may well have another advantage, in that it would reduce the trend towards stacking of multiple layers of cells, known to be detrimental for the production of recombinant protein.\cite{21} Thus, higher cell densities and TPA concentrations may well be achieved in attempts to optimise these particular culture parameters, a future objective in our ongoing program.

Cell adhesion on PPE:N surfaces did not appear to be mediated by integrin, but is possibly due to the high primary amine content, [−NH₂], of PPE:N. The influence of the NH₂ groups on cell adhesion can be investigated either by NH₂ derivatisation or by using a similar material to PPA:N, but with lower primary amine content (for example, PPA:N).\cite{22,23} This has been done elsewhere for another type of intrinsically non-adherent cells, U-937 monocytes, and results confirmed the importance of the −NH₂ chemical group for the promotion of cell adhesion.\cite{24}

One of the limitations of this study was the inability to deposit PPE:N on 3D supports with the particular DBD apparatus used here. Consequently, scraping a PPE:N-coated glass surface to obtain flakes was the only possibility for utilising this novel material in the context of a suspension culture. By doing so, the geometry of the particles is highly variable, but the chemical composition of the material is unlikely to be altered significantly due to contact with the liquid or possible damage due to the scraping.

Conclusion

The use of PPE:N flakes as support surfaces for CHO cell culture has been demonstrated to result in cell proliferation, high viability of cells, and efficient production of recombinant protein. At the same time, PPE:N was found not to significantly affect the metabolism of cells, but to modify their adhesion behaviour by promoting the aggregation of viable cells, and to alter the integrin β1 expression profile. The remarkably high observed cell adhesion, presumably attributable to elevated concentrations of reactive N-bearing functional groups (such as positively charged protonated primary amines), strongly suggests that PPE:N surfaces have a promising future in the design of new types of cell carriers that can be used in various biotechnological and biomedical applications.

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