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Direct Plasma Deposition of Collagen on 96-Well Polystyrene Plates for Cell Culture

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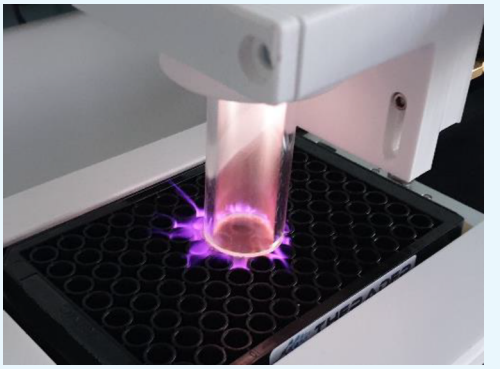
ABSTRACT: A cold atmospheric plasma unit was used to deposit a biologic, in this case collagen, onto a surface. A collagen coating was applied to 96-well polystyrene plates at a range of powers to determine the effects of the plasma power on the coating structure and viability. Plasma characterization was carried out using voltage, current, and power measurements. Coating characterization was completed using gravimetric measurement, cell growth, water contact angle, as well as spectroscopic analysis and compared to commercial collagen-coated plates. Cell culture studies were also undertaken. The plasma coating matched the performance of the commercial plate but dramatically reduced production time and cost. This method could allow for automated inline production of collagen-coated plates for cell culture applications.



INTRODUCTION

The term plasma was first used in 1928 by Irving Langmuir to describe electrical discharges in gases.[1] Plasma has been used industrially for many decades to modify surfaces. Vacuum plasma systems, which rely on low pressure and high frequency to generate plasma are used for surface modification, etching, and sterilization as well as applying coatings[2] and are integral to all semiconductor manufacturing processes. Other types of systems can operate at atmospheric pressure such as dielectric barrier discharge plasma (DBD plasma)[3] and corona discharge.[4] The first DBD plasma was created in 1857[5] long before Langmuir coined the term plasma. The advantage of these systems over vacuum plasma is they can be placed in line for production rather than waiting for vacuum plasma systems to be loaded, pulled vacuum, and then be unloaded. This is an attractive option for industry allowing plasma processes to be automated in a production line.

Plastic microplates are widely used in biological research and analyses and began with machining of plates from solid blocks of acrylic in the 1950s. Currently molding plates using polystyrene dominates the market.[6] Benefits include ease of molding process, rigidity, and clear optical properties compatible with a range of analytical techniques. Plastic microplates are inert, which is not always ideal for attachment of cells or use in cell biology, which has driven the adoption of plasma treatment to modify the surface.[7] The most common plasma treatment of microplates is for tissue culture treatment (TCT), using either atmospheric or vacuum plasma systems to activate the surface of the plastic plate to reduce its hydrophobicity. Having a more hydrophilic surface enhances cell adherence to the surface with proliferation on the plate. Without this treatment, cells would not attach well and would clump together as the surface is not ideal for growth.

Despite the success of TCT plates, microplates for diverse functions require additional treatments to render them more biocompatible, and a range of coatings are now available across different plates. These treatments improve attachment in the first few hours and thus improve overall cell growth.[8,9] Collagen coating is commonly applied to enhance cell growth as it allows cells to grow on a more natural surface than glass or plastic.[10] The current collagen coating process is slow and costly, with a plasma activation treatment of the plate surface carried out before coating with a functional group. This plasma treatment of a surface is used to promote and improve attachment of proteins to surfaces, often with cell culture applications.[11,12] Dilutions of collagen are then made and tested before being dispensed into the wells and left to adhere. The plates require a minimum of 1 h curing at room temperature, after which the remaining collagen solution must be aspirated carefully without damaging the applied coating. Each plate is then washed three times with PBS or the relevant media for immediate use or left to dry further for

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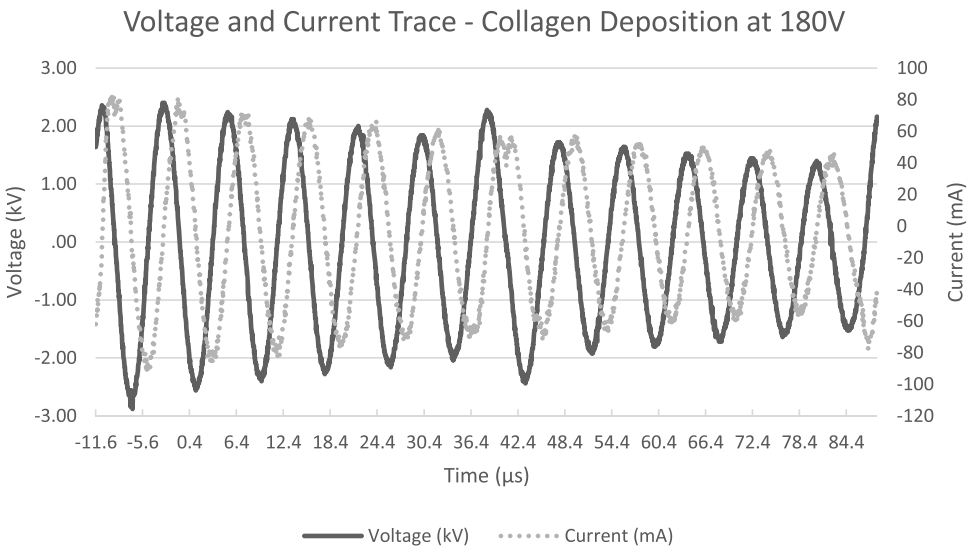

Figure 1. Example of current and voltage at 180 V input voltage.

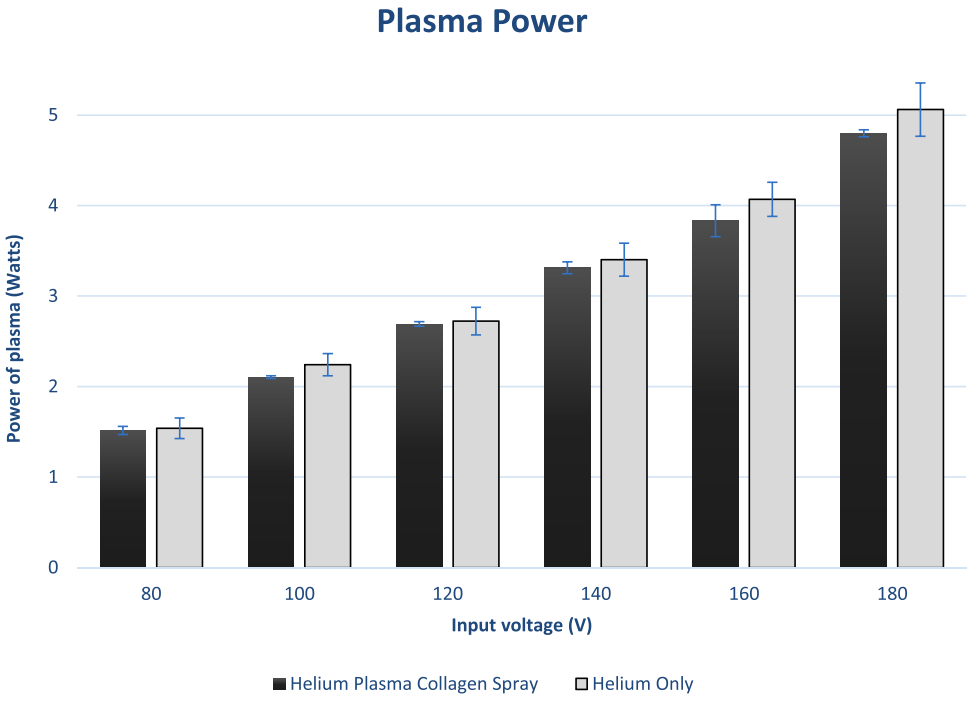

Figure 2. Plasma power over a range of input voltage levels.

storage. This lengthy process requires a clean room environment and aseptic technique to be applied at all stages.

The routine use of coated microplates in research and diagnostics and the process limitations in their production provides the rational for this study, where the concept of direct plasma deposition of collagen was considered.[13] It was hypothesized that direct plasma deposition technique would allow a thin layer of collagen to be evenly applied to the surface, with process advantages of speed and adherence. This study employed a custom-fabricated plasma deposition unit to deposit nebulized collagen. A series of collagen coatings were prepared and applied to plastic microplates. The surface characteristics of the coated microplates were characterized, and the functionality of the coated plates were evaluated as a function of the process parameters applied. Where experimentally possible, the plates prepared with the novel direct plasma deposition process were compared to a commercially available collagen-coated microplate.

RESULTS

Electrical Parameters. The electrical characterization revealed a maximum output voltage (peak to peak) of 5.28 kV and a max current of 174 mA, as shown in Figure 1 representing an example of the voltage and current reading as a function of time.

Initial readings for voltage and current were taken for a range of input voltages, allowing the data to be converted into an average watt output power using eq 1:[14]

$$W = F \int_{t}^{t+T} I(t)V(t)dt \quad (1)$$

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The equation uses the current and voltage measurements over one cycle to determine the plasma output power, where $T = 1/F$, V is the measured voltage, and I is the measured current. The equation was applied to the example shown in Figure 1 and yielded a power of 4.746 watts. This is comparable to other nonthermal plasma system studies carried out using similar equipment designs.[15−17]

Plasma Power in Watts. Voltage and current data were collected for each of the applied input voltages for both a plasma only system and when the system was depositing collagen. Readings were taken in triplicate, and the average calculated readings were plotted (Figure 2). It was observed that increased input voltage yields a steady increase in plasma power from a low average of 1.5 watts at an 80 V input to 5 watts on average at 180 V input power. A clear effect was detected upon the addition of collagen to the system. Although not dramatic at first, there was a noticeable reduction in the output power of the plasma at each setting, averaging a 4% reduction and a maximum power reduction of 6%. This was expected as the liquid partially quenches the plasma discharge and absorbs some of the free energy of the plasma. The reduction in the power is accompanied by a significant reduction in the variation, as shown by the error bars, most notably at 180 V. This implies that the plasma coating process is very controlled and can be consistent when depositing on plate surfaces.

Uniformity of Surface Treatment. Water contact angle measurements were used to probe the uniformity of the surface treatments as well as the change to the surface response following deposition. This test is often used on both TCT plates and commercially available collagen plates to monitor their hydrophilicity or hydrophobicity. An immediate drop in water contact angle (WCA) was recorded following plasma treatment, with the contact angle dropping from a high of above 90° on a blank plate to a low of 34° (100 V). After the lowest point, a rise in WCA is observed for settings from 120 to 160 V before declining again at 180 V. This could indicate that a lower power provides a more uniform, low contact angle coating, which would be ideal for cell culture.[18,19]

Table 1 shows the measured contact angle for standard plate types for comparison. The plasma-deposited coatings averaged 39°, while a contact angle below 60° is required for a TCT plate.[18,19] This data showed that the plasma coating was not identical to the commercial collagen but was lower than the TCT plates. Therefore, additional investigations were undertaken using detailed surface science and cell culture studies.

Table 1. Average Water Contact Angle

Average Water Contact Angle	
Plate Type	Water Contact Angle (°)
Blank Polystyrene	93
TCT Plate	46
Thermo Fisher Collagen Coating	24
Plasma-Deposited Collagen Coating	39

Effect of Plasma Deposition Process on a Collagen Structure. The chemical structure of the deposited collagen was analyzed using FTIR. Attempts to collect spectra directly from the polystyrene plates were unsuccessful, as the contribution from the thin collagen layer was not distinguishable from the background plastic. A number of NaCl slides were therefore coated over the full range of powers from 80 to180 V. A control sample of wet collagen was deposited onto a NaCl disc to compare the coating to the traditional coating method. All coatings carried out using plasma deposition were dry instantly, while the wet collagen deposition method was left to dry for 2 h and retested after 72 h to ensure it was fully dry.

Once scanned, the spectra were processed, and the features of interest were identified. The expected amide groups A, B, I, II, and III were present in all deposited layers. This confirms that a protein-like coating was present, and these were further inspected for changes or evidence of damage to the collagen structure. As shown in Figure 3, the air-dried sample produced a spectrum that is typical of collagen, with clear peaks at 1658 for Amide I, 1553 for Amide II, and 1241 cm^{-1} for Amide III. The Amide A and B peaks can also be seen at 3325 and 2918 cm^{-1} range, respectively.[20] Comparing the spectra of the plasma-deposited materials to the air-dried collagen produced no measurable shifts in peak positions for any of the Amide groups even at the highest power applied where the potential for cell damage is greatest. This indicates that the collagen did not undergo any measurable chemical changes when passing through the plasma.

Effect of Plasma Deposition of Collagen on Cell Growth. Based on the demonstrated high similarity between the plasma-deposited coatings and the commercial collagen coating (Figure 3 and Table 1), a cell culture study was undertaken to compare the cell growth characteristics of both commercial and plasma-deposited collagen plates. A further series of 96-well polystyrene plates (Thermo Fisher 96-well plates, product no. 265301) were coated with collagen at the same range of powers used previously. These plates were plasma-coated using Gibco rat tail collagen, as this is the same collagen Thermo Fisher uses to coat their commercially available collagen-coated plates, thus maintaining comparability for the cell growth challenge study.

Vero cells (ATCC CCL-81), an adherent epithelial kidney cell line, require a treated surface to grow well.[21] This cell line was selected for this study due to the requirement for an optimal surface and thus would clearly show if there was a significant difference between the plasma coating and the commercially available collagen-coated plates in the study. The plates were seeded with cells, incubated, and cell proliferation was estimated using the WST-1 assay at selected time points. Figure 4 compares cell proliferation on the plasma-deposited collagen plates against a commercially available collagen-coated plate and a blank polystyrene plate. The cell count was taken at three different time intervals, 24, 48, and 72 h. On the blank polystyrene plate, the cell numbers initially decreased over the first 48 h and only began to increase above the initial seeding level at the 72-h time point. All of the plasma-deposited collagen-coated plates significantly outperformed the blank polystyrene control plate at all time points, with a p-value of $P < .001$. Cell growth increased significantly at each time point, and there was no significant difference between the deposited coatings. The proliferation assay testing showed that all plates prepared using the plasma deposition process performed equally or better than the commercial collagen control plate at all time points. Optical microscopy confirmed this finding, as shown in Figure 5. The images from left to right show cells growing on a blank plate, a commercially coated collagen plate, and a plasma-deposited collagen plate after 24 h. On the blank plate, cells were observed to clump together as the surface is not appropriate for their growth. There is little

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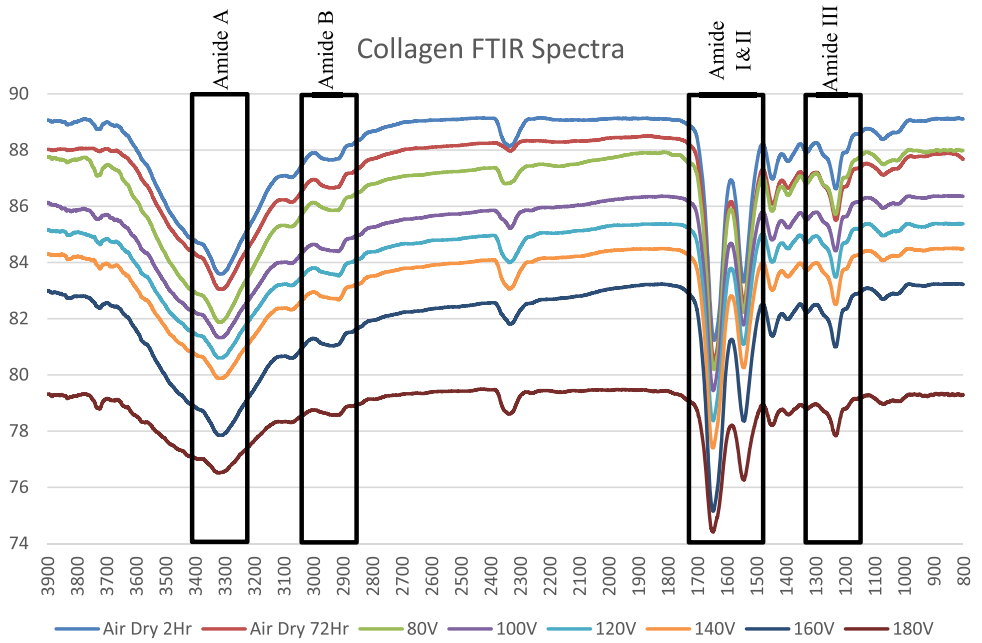

Figure 3. FTIR of collagen plasma deposited at different power levels.

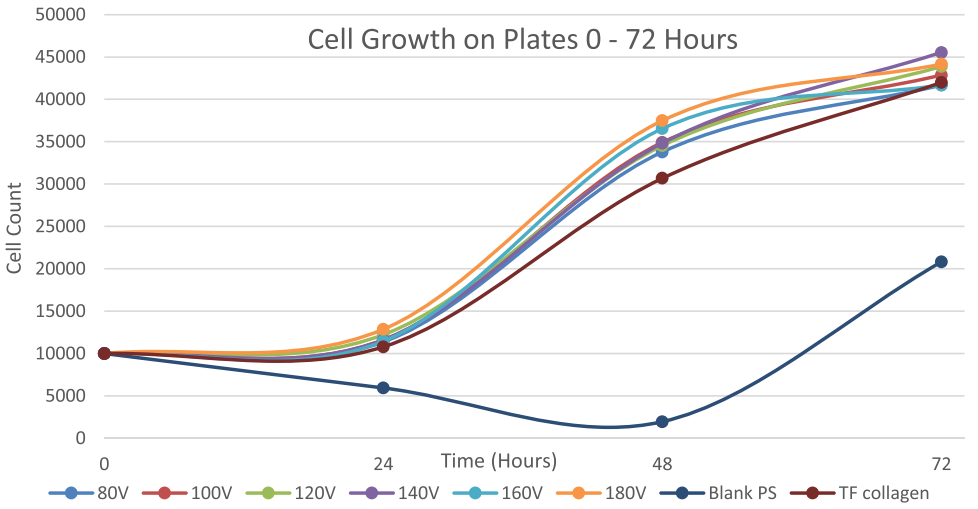

Figure 4. Vero cell growth as measured using the WST-1 assay at various time points.

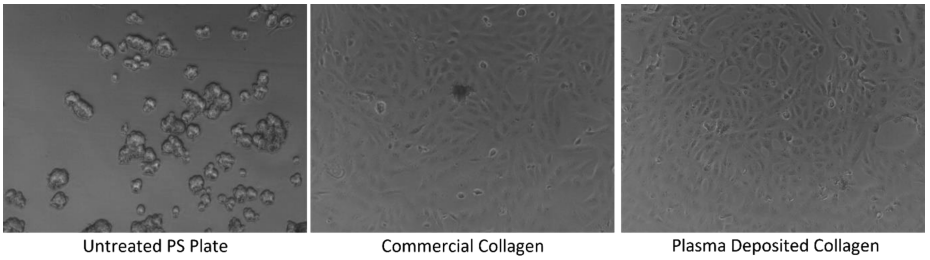

Figure 5. Optical microscopy images of cell growth at 24 h.

visible difference in the cells' shape and appearance between the commercial collagen and the plasma-deposited collagen plates with improved cell adherence over the area of the well, absence of clumping, and use of the plate surface to grow and proliferate.

Weight Gain Analysis. To determine what the effective amount of collagen deposited onto a surface was, a weight gain study was performed. Glass discs were used as the test deposition surface as they are easy to clean, stable, and nonreactive. The deposition was carried out for 5 min per glass disc to allow enough time to deposit a measurable amount of collagen. The flow rate was 40 uL/min, and the collagen used was Gibco rat tail collagen at a concentration of 3.2 mg/mL. This would give a maximum weight change of 0.64 mg per

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disc. The discs were 50 mm in diameter and weighed before and after the deposition to determine the weight change. Four readings were taken per disc both before and after deposition to find the average weight gain. The deposition voltage range was that as applied previously to determine the optimum process for collagen deposition. A no plasma, listed as 0 V, deposition was also carried out to determine its effect on the efficiency, and this data is summarized in Table 2.

Table 2. Collagen Deposition Rate at Various Applied Plasma Powers

Voltage (V)	Collagen Used (mg)	Deposited Average (mg)	Deposition Efficiency (%)
180	0.64	0.22	34
160	0.64	0.19	29
140	0.64	0.19	30
120	0.64	0.22	34
100	0.64	0.19	29
80	0.64	0.21	33
0 (no plasma)	0.64	0.25	40

The data shows that the heaviest deposit was produced at 0 V. This can be explained by the higher moisture content of being deposited without plasma. The 0 V sample retained considerable moisture, which was visually observed as a wet deposit, while the plasma-deposited samples were all dry immediately after deposition.

Using this data, the effective deposition rate of collagen onto a surface could be determined. By knowing the time, flow rate, and weight of the collagen and comparing the input weight to the output, the amount of collagen deposited on the surface can be calculated. Some losses can be attributed to losses to the atmosphere, as this is an atmospheric plasma system. Another is to the inside of the chamber on the plasma head, as it is designed to direct the deposition onto a target surface, any excess can build up on the wall of the chamber.

Assuming equivalent deposition efficiency on the 96-well microplates, the amount of collagen consumed in the process would be 2.6 $\mu g/cm^2$, and the amount deposited on the PS plate using the plasma process would generate 0.87 $\mu g/cm^2$. This collagen consumption is between 47 and 73% less than the amount of collagen suggested in the literature for standard collagen coating of microplates; Sigma-Aldrich recommends a coating protocol, which advises using 6−10 $\mu g/cm^2$, and the coating procedure advised by Gibco for their collagen, which states using 5 $\mu g/cm^2$ to coat plates with.[26,27] The industry coating methods only give guidelines on the concentration of liquid to be pipetted into each well, but after incubation, excess liquid must be removed. This removal process would remove any collagen that was not attached out of the well, and the efficiency of the wet chemical deposition method is not widely reported. It is possible that much of the collagen is extracted with the removed liquid, and the actual concentration present on the surface of both methods may be similar.

DISCUSSION

Plasma has been used for decades for activation of surfaces and for sterilization in various applications. However, due to the high-energy nature of many plasma systems, it has not traditionally been used for deposition of temperature-sensitive biologics due to the potential for thermal and oxidative damage. Recent studies have challenged that dynamic. Various studies have shown that low-temperature helium atmospheric pressure plasma discharges can be used to deposit functional protein coatings[22−24] with minimal evidence of degradation. Detailed studies have shown that the choice of low-energy helium plasma discharges and transient contact times results in minimal degradation of amino acids and proteins.[25,26]

In this study, it has been shown that plasma deposition can deliver biologically functional thin film coatings of a protein on to cell culture consumables. The low power and short exposure time of the plasma has allowed the protein to be deposited without denaturing it or changing its chemical structure, which can be clearly seen by the FTIR spectra. In addition, all of the coatings were shown to enhance cell proliferation in a similar manner to conventional collagen-coated microplates. The plasma-deposited coating was shown to be effective across a range of applied powers, which indicates that the process is robust, stable, and offers a wide process window. Given the similarity between the commercial collagen-coated plate and the plasma-coated plates in water contact angle and FTIR analysis, it is not surprising that the cell growth figures show consistency, repeatability, and can match the current wet chemical process.

This suggests that plasma may be a viable alternative to the current labor-intensive, aseptic techniques used where the process is carried out in cleanrooms. Switching to a plasma production line could allow the plates to be coated at a faster rate, but it gives less plate to plate variation as it eliminates human error. For the testing carried out in this paper, a custom-built plasma unit was used on a lab bench with a CNC and took 2 min to coat a blank plate, and it was immediately dry and ready for use. In a large production line, the unit could be scaled to improve times drastically, as well as deposition efficiency.

Due to the fact that these plates usually have to be prepared in cleanrooms using aseptic techniques, plasma presents another advantage in the fact that the plasma would be sterilizing the surface as it coats, which would reduce the bacterial contamination. The reduced cycle time would also ensure that the plate is only exposed to the atmosphere for a short amount of time meaning the likelihood of contamination is also greatly reduced.

In industry, another notable change is the amount of collagen deposited onto the surface. During the weight gain study, deposition efficiency was shown to be 32% on average. Despite this, the process consumed between 47 and 73% less collagen than the standard coating protocols recommended. This could save resources and reduce costs if the amount of collagen that was being deposited could be lowered and controlled more accurately with an automated system such as the plasma deposition unit in this paper.

This study has shown that on 96-well polystyrene plates, a consistent and effective collagen coating can be produced at a range of plasma powers. Further work is needed to investigate translation to a range of plates, the number of wells, and the impact of receiving a surface material from simple polystyrene to polypropylene or cyclic olefin copolymer (COC). The success of this approach with collagen as a one protein structure provides insight to the structures that may be suitable for this class of surface preparation process. However, it is likely that the applied deposition power could be crucial for adherence properties, which can be adjusted to provide an operating range suitable for diverse biologic coatings.

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CONCLUSIONS

The plasma deposition of collagen without denaturing the collagen or affecting its function as a surface for the growth of cells post deposition has been demonstrated. This study provides insights to how plasma deposition of collagen could replace the time-consuming and labor-intensive multistep and a wet chemical process with a coating process is applied in minutes and has the ability to be automated and scaled up. Varying the power led to no detectable adverse effects in the final product as a collagen cell culture plate. The insights gained from testing provide the option for other proteins to be deposited with greater control in the future.

EXPERIMENTAL SECTION

Materials and Methods. Gibco type I rat tail collagen, 3.2 mg/mL was sourced from Thermo Fisher, Denmark. Industrial grade helium was sourced from Irish Oxygen, Cork, Ireland. Untreated, optically clear flat-bottomed, black, 96-well polystyrene microplates and equivalent commercially collagen-coated microplates were sourced from Nunc, Thermo Fisher, Denmark.

Plasma deposition was carried out using a purpose-built deposition system (Figure 6) comprising a Redline G2000

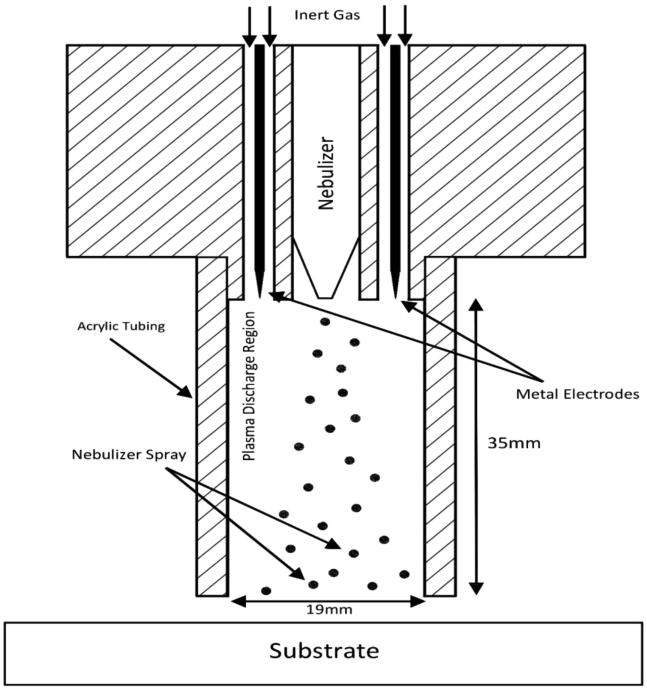

Figure 6. Schematic of a plasma deposition device.

High Voltage power supply connected via a step-up transformer to a custom-built Teflon deposition unit that encased two metal electrodes. The generator was operated at an output voltage range from 80 to 180 V and in 20 V increments while keeping a constant frequency of 19.8 kHz and with a 45% duty cycle. A pneumatic nebulizer (T2100, Burgener Research, Ontario, Canada) was placed between the electrodes, and this was connected to a syringe pump to provide a constant flow of collagen solution at 40 μL/min. The liquid was nebulized using a gas flow of approximately 2 slm. In addition, a separate helium flow of 8 slm was provided to the metal electrodes to create a plasma discharge. The plasma discharge was combined with the nebulized droplet spray in an acrylic tube (19 mm inner diameter × 35 mm length), and the substrates to be coated were placed at the outlet of the tube. This is shown schematically in Figure 6. In order to coat the microplates, the plasma head was mounted on a computer numerically controlled table and moved in a raster pattern over the target surface that required 2 min to coat the entire plate.

Electrical Characterization. The applied voltage was measured using a Testec TT-HVP 40 high voltage probe with a ratio of 1 V per kV, which was directly connected to the high voltage output of the G2000 power supply. Current readings were measured using a Pearson 4100 current monitor with a ratio of 1 V per amp placed around the HV cable, and readings from both voltage and current were collected using a Tektronix TDS 2014C digital oscilloscope, and the data was captured and analyzed using Microsoft Excel.

Surface Chemistry. Contact angle measurements were conducted using a Kruss TVA100, which uses a top view method to determine contact angle measurements. This facilitated direct measurement of the contact angle within the well of a microplate. Measurements were taken using a 1 μL droplet, and measurements were taken at equilibrium; after 60 s, the droplet was placed on the surface and was analyzed using the Kruss Advance software package. Top view analysis (Figure 7) differs from conventional drop analysis where two LEDS are applied, and the reflection from the top of the drop is used to determine the contact angle. This approach allowed measurements to be taken from the bottom surface of the wells in each microplate.

Fourier transform infrared (FTIR) spectroscopy was conducted using a Perkin Elmer Spectrum 2000 instrument operating in single beam mode using 64 scans and a 2 cm^{-1} resolution.

Gravimetric analysis was carried out using a Sartorius Secura 125-1S analytical balance with an accuracy of 0.01 mg.

Cell Culture. Vero cells (ATCC CCL-81), an adherent epithelial kidney cell line, were used for testing, and cell proliferation was estimated using the WST-1 assay as per the following protocol.

The cell lines are expanded in 175 cm flasks in complete culture medium (RPMI) containing 10% conditioned medium. On the first day, the cells were detached from flasks using trypsin and counted. Prior to seeding, plates were sterilized under UV light for 15 min. Ten thousand cells were then seeded per well of a 96-well plate in 100 μL culture medium. After 1 h, the culture medium was removed from the top quadrant (four rows (A, B, C, and D) in columns 1−6) and replaced with 100 μL fresh medium. After 24 h, the culture medium was removed from the top four rows (A, B, C, and D) and replaced with 100 μL fresh medium containing a 10% WST-1 solution. The plates were incubated at 37 °C for a further 4 h. Medium was then transferred from each well in the top four rows into new assay plates, which were placed into a microplate reader (Tecan model), and data was collected at OD450 nm using OD630 nm as a reference. The original plate with cells was incubated at 37 °C and 5% CO_2 until the next time point. This process was repeated for the other quadrants of the plates at the 48- and 72-h time points using the same methods previously described.

Cell culture assays were performed under contract by Bayside BioSciences Inc., San Jose, CA, USA.

Weight Gain Analysis Study Calculations. Collagen concentration of 3.2 mg/mL

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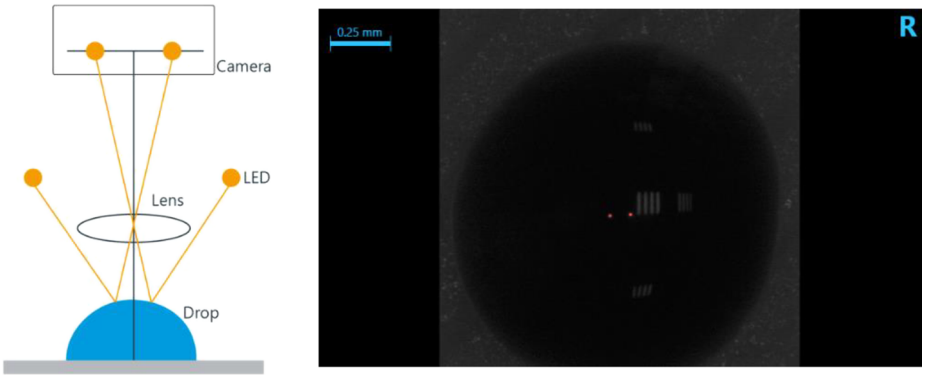

Figure 7. Kruss TVA 100 contact angle measurement.

Collagen flow rate of 40 μL/min
Time per disc of 5 min
Time × flow × concentration
4 min × 40 μL/min × 3.2 mg/mL = 0.64 mg total collagen used
Effective deposition rate
Collagen input into system/observed weight gain = efficiency
Average deposition rate efficiency of 32.5%
Amount of collagen per cm^2
Polystyrene plate total surface area = 12 cm × 8 cm = 96 cm^2
Coating time of 2 min
2 min × 40 μL/min × 3.2 mg/mL = 256 μg of collagen used
256 μg collagen/96 cm^2 = 2.66 μg/cm^2 used
Actual amount deposited
2.66 μg/cm^2 × 32.5% (average system efficiency) = 0.87 μg/cm^2

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Notes

The authors declare the following competing financial interest(s): D. O Sullivan and L. O'Neill are employed by Theradep and they're engaged in commercialization of plasma technology.

ABBREVIATIONS

PTFE	polytetrafluoroethylene
PS	polystyrene
TCT	tissue culture treatment
DBD	dielectric barrier discharge
FTIR	Fourier transform infrared spectroscopy
WCA	water contact angle

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