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Fabrication of free standing collagen membranes by pulsed-electrophoretic deposition

Fabrication of Free Standing Collagen Membranes by Pulsed-Electrophoretic Deposition

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Abstract. This work reports an important new development in the production of collagen membranes, based on pulsed electrophoretic deposition (P-EPD), suitable for a wide range of biomedical applications. Collagen membranes are of great interest as a biomaterial and in a range of other industries, though current production techniques suffer from limitations with scaling up, homogeneity, and complex shapes. P-EPD can be used to rapidly create detachable, large-area, homogeneous products with controlled thickness in a wide variety of shapes. We provide a new understanding of the influence of a range of parameters (pulse width, voltage, duty cycle, solvent additions) and their effects on membrane structure. Characterisation by AFM, SEM, and cryoSEM revealed the ability to produce dense, structurally defect-free membranes, and significantly, we show and discuss the ability to produce thicker membranes by sequential deposition without seeing a corresponding increase in cell electrical resistance. We anticipate this novel, rapid, and controllable method for production of collagen membranes to be of interest for a wide range of fields.

Keywords: Electrophoretic deposition, Collagen, Membranes, Coatings

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1. Introduction

Collagen, one of the most abundant proteins found in vertebrates has been widely used in a number of forms as a biomaterial as it has a low immunogenicity, high biocompatability, and can act as a scaffold for cell proliferation and wound healing[1]. Collagen membranes are used in a range of roles such as wound dressings[2], guided tissue regeneration[3], and barrier layers[4], and are usually formed by solvent casting or decellularisation of animal tissue. Current production methods however suffer from significant issues; solvent casting requires extensive drying times, even layer production can be difficult at scale, and there can be uneven loading of incorporated drugs leading to undesirable release profiles[5]; decellularised membranes are significantly constrained in size and shape due to limitations of the original source material, and can have biocompatibility issues from cellular component remnants and from chemicals used during processing[6]. As such, there is an urgent need to explore different methods to produce dense, reproducible collagen membranes of even thickness, that can be scaled easily and can produce larger sizes and more complex shapes.

Electrophoretic deposition (EPD) is a colloidal processing method widely used in ceramic processing as a rapid, low cost, scalable, and highly reproducible technique for producing coatings and free standing films that is currently being explored for use in polymer and biological systems such as chitosan [7, 8, 9, 10, 11, 12] and hydroxyapatite [11, 13, 14, 15, 16, 17]. In electrophoretic deposition, a particulate material is dispersed in a liquid medium where it gains a surface charge [18]. An electric field is then applied to the suspension causing the particles to migrate towards the oppositely charged electrode, where they then deposit forming a dense homogeneous layer [19]. A number of different theories have been suggested for the formation of a deposit during EPD, and these can be divided generally into three categories (a) charge neutralisation, (b) electrochemical coagulation, and (c) particle accumulation [20]. The overall effect of each of these mechanisms on deposit formation is currently not clear, and different mechanisms may predominate in different electrophoretic systems.

To date, there have been a small number of papers that have examined the effects of an electric field on collagen monomers [21, 22, 23, 24], however there have been no reports on the production of films or coatings from insoluble collagen by EPD. The use of EPD to produce collagen membranes allows for the potential for production of structures that are not possible through current methods including non-planar layered structures [25], membranes with aligned fibres [23], or collagen-based actuating robots [22], as well as offering the possibility of production of collagen films with significantly greater density than produced through conventional solvent casting [26, 27]. Further, EPD can be used to produce coatings over large surface areas with variable membrane composition or structure [16]. The drawback of the use of EPD to produce membranes and deposits is that a suitable liquid carrier medium must be found that results in a stable suspension with a high particle charge [28].

Collagen molecules are made up from a number of different amino acids, giving it

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a pH dependent surface charge when suspended in an aqueous liquid, with amino acid protonation at acidic pH values leading to a positive ζ-potential value and deprotonation at alkaline pH values leading to a negative ζ-potential [29]. Under the influence of an electric field, a charged collagen molecule or fibre will migrate towards the cathode when in an acidic environment or towards the anode when in an alkaline environment [24]. When used as a biomaterial, collagen is often prepared in acetic acid in order to allow the fibres to swell [1, 30, 31]. This leaves the collagen fibres with a positive surface charge, allowing for their manipulation via electric fields, and migration of the fibres toward the cathode.

While most EPD of ceramic systems uses organic liquids as a suspension medium, allowing for high applied potentials, EPD of biomaterials requires use of a non-toxic suspension medium, such as an aqueous system[32]. This is potentially problematic, as while use of aqueous systems in EPD is not unknown, their use is associated with a number of issues, particularly the electrolysis of water. When a potential above 1.23V at 25°C is applied across an aqueous liquid, electrolysis occurs, leading to the release of hydrogen and oxygen gas at the cathode and anode respectively[33]. The evolution of these gasses at the electrodes cause bubbles to form which disturb the deposit as it forms, damaging it and reducing the rate of deposition, and which can become trapped within the deposit[34].

There have been a number of different approaches tried in the literature to avoid the problem of gas evolution at the electrodes in aqueous media, including the use of hydrogen absorbing Palladium electrodes[35], the addition of a barrier membrane located between the electrodes as a substrate for deposition[36], the addition of hydroquinone which reacts with the oxygen generated[37], the use of AC electric fields[38, 39, 40], and the application of pulsed electric fields (Pulsed-EPD)[34, 41, 42]. While Pulsed-EPD has been used extensively as a method of increasing current densities when using organic liquids, Besra demonstrated that it could be used to suppress gas bubble formation in aqueous media, theorising that during Pulsed-EPD the gas nucleation site changes between pulses, allowing gas molecules generated by electrolysis to diffuse away, and preventing coalescence into macroscopic, deposit damaging bubbles[34, 42].

In this study, the application of Pulsed-EPD to the formation of collagen membranes was explored to determine if the technique could be used to produce macroscopically defect-free, free standing collagen membranes. A number of parameters associated with Pulsed-EPD were varied and their effect on deposited films was characterised. Further, the microstructure of collagen membranes produced by Pulsed-EPD was examined in both a dry and hydrated state to examine the macro and micro-scale effects of this fabrication method.

2. Experimental methods

Collagen was rehydrated from insoluble bovine achilles tendon collagen (C9879 Sigma Aldrich, UK) by soaking in 0.05M acetic acid at 4°C for 48hr. It was then homogenised

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on ice for 30 minutes at 10,000rpm using an Ultra-Turrax VD125 homogeniser (VWR International Ltd, UK) until a milky appearance was achieved and no large particles were apparent. Ethanol was then added to 50% volume unless otherwise stated under further homogenisation. Unless otherwise stated, the final concentration of the collagen suspension was set to 0.1% (w/v). The ζ-potential of the suspension was determined using laser Doppler electrophoresis with a Zetasizer Nano-ZS (Malvern Instruments).

EPD experiments were carried out at constant voltage using a custom built cell as seen schematically in figure 1a, consisting of two 316L steel electrodes separated by silicone rubber spacers, with deposition area 1cm x 2.5cm. The electrodes were connected to a TGA1241 Arbitrary Waveform Generator (TTI) which was used to produce a pulsed voltage in which the pulse length, duty cycle, and voltage could be independently varied, shown schematically in figure 1b. The duty cycle (DC) of the pulse is defined as $DC = T_{on}/(T_{on} + T_{off})$.
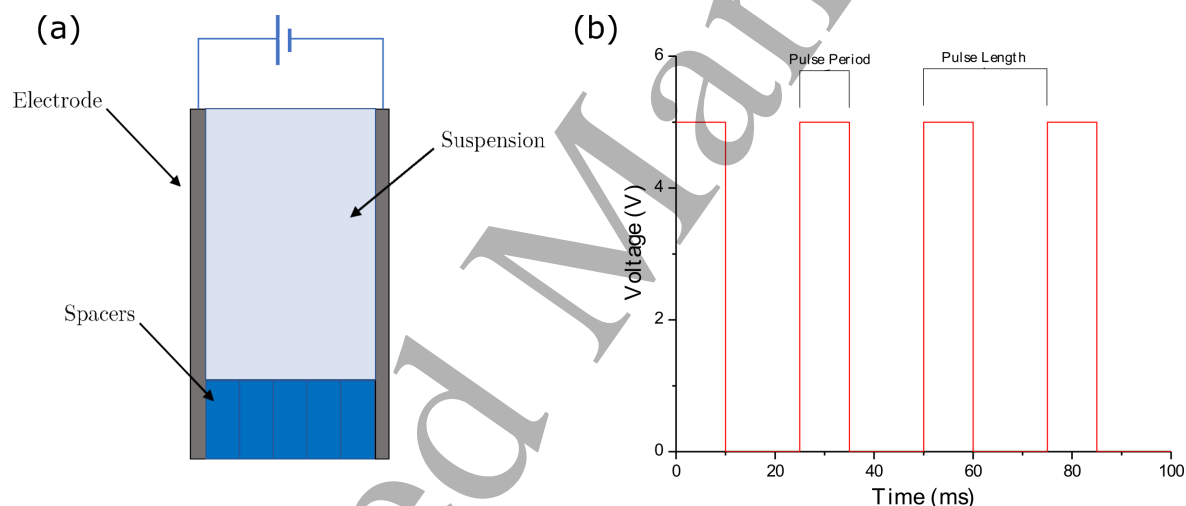

Figure 1. (a) A schematic diagram of the EPD cell used, (b) Schematic of a constant voltage pulse with period 10ms and 40% duty cycle

Unless otherwise noted, depositions were carried out for a total T_{on} of 1.5 hours. Deposited films were evaluated either as is, or were dried overnight in a fume hood, and dry masses of films were measured using a Sartorius CP124S balance (0.0001g $\pm$0.0001).

Free-standing collagen films were prepared as above, followed by separating dried collagen films from the electrode through mechanical-cleavage, which involved mechanically separating the film using a razor blade [43], or through use of a release layer.

Cast films were prepared by syringing 5ml of collagen suspension into a silicone mould (Lakeland Ltd.), before drying overnight in a fume hood. Samples were released by inversion of the moulds.

Films for density measurements were prepared from 0.25% (w/v) collagen in 0.05M acetic acid with 50% (v/v) ethanol. Films prepared by P-EPD were deposited with a pulse period of 0.025s, a duty cycle of 40%, and a voltage between 6-10V. After

deposition collagen films were dried and the mass was recorded. Cast collagen films were produced by pouring the same collagen suspension into moulds and allowing them to air dry overnight. Dimensions were measured using a digital calliper (Mitutoyo Absolute Digimat, 0.01mm±0.02), and the thickness was measured with a digital micrometer (0.001mm±0.001).

AFM was carried out by placing samples on a silicon substrate before imaging with a Dimension 3100 (Bruker Ltd) in light tapping mode using RTESP Silicon AFM tips (Veeco: resonant frequency 200400Hz, spring constant 2040N·m^{-1}) at room temperature. Scans were performed at random locations at a scan rate of 0.5 HZ and 512 samples per line. Image analysis was performed with Gwyddion software [44].

Samples for SEM were mounted on stubs before being sputter coated using a gold target at 25mA for 4 minutes. Samples were imaged on a JEOL JSM-5800LV SEM (JEOL UK Ltd) at 15kV accelerating voltage in secondary electron imaging mode. Samples for cryoSEM were frozen with slushy nitrogen at -195°C and shattered under vacuum, before being sputter coated with a platinum target in situ. Samples were then transferred to a Zeiss EVO HD15 (Carl Zeiss Ltd) where they were imaged at 25kV accelerating voltage in backscattered electron imaging (BSD) mode.

3. Results and discussion

3.1. The effect of pulse parameters

Figure 2 shows the surface morphologies of collagen films prepared with a range of pulse width, duty cycles, and voltages, with a total T_{on} of 5 minutes for a 0.1wt% collagen slurry, deposited onto the cathode.

Figure 2a shows that the number of bubbles formed in the deposit initially decreased with a reduction in the pulse time, before increasing again when the pulse time was reduced below 1ms. The pulse widths employed in figure 2 are much lower than those found for pulsed deposition of other systems in the literature, such as work by Besra on Pulsed-EPD of alumina [34], indicating that the deposition of collagen by Pulsed-EPD is highly sensitive to the evolution of gas at the electrodes . Pulsed-EPD has been theorised to reduce the formation of bubbles in deposits by leading to a change in the gas generation site with each separate pulse [34], as such we can explain the increase in bubble formation below 1ms by theorising that when the pulse length becomes short enough there is not sufficient time for gas molecules to diffuse away from the gas generating site before the site becomes active again during a later pulse.

Figure 2b shows deposits formed with a range of duty cycles. We can see that there is a reduction in the number of bubbles produced as the duty cycle decreases, but also that when the duty cycle reached 20% the formation of the film was disrupted, with no coherent deposit being obtained.

Figure 2c shows the effect of voltage on deposit formation. A clear change can be seen in both the quantity of bubbles produced and in the quality of the deposit. At 5V
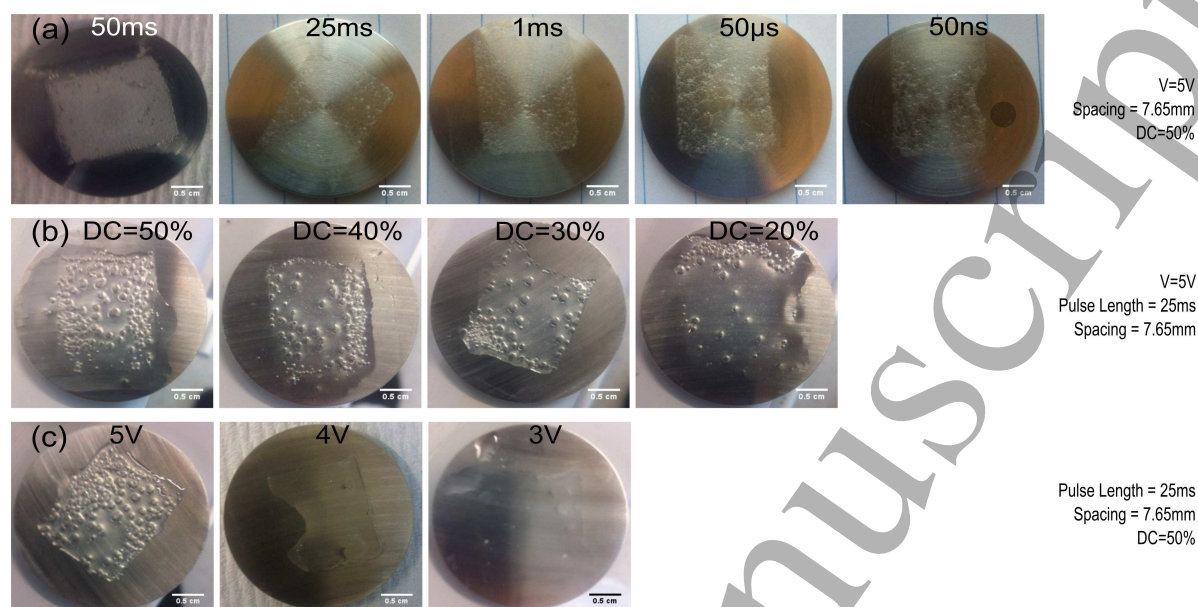
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Figure 2. (a) Images of collagen films formed at a range of pulse lengths by Pulsed-EPD, (b) Images of collagen films formed by Pulsed-EPD with a range of duty cycles, (c) Images of collagen films formed by Pulsed-EPD with a range of voltages.

there is a large number of bubbles present in the deposit, at 4V there are no bubbles present and there is a coherent deposit, and at 3V no deposit was formed.

3.2. The effect of solvent additions

To avoid evolution of gas at the electrodes, many EPD systems employ organic liquids as the suspension medium. To investigate the effect of addition of an organic liquid to the collagen suspension, ethanol was chosen as it is completely miscible with water and is non-toxic. Figure 3a shows the ζ-potential of collagen with respect to the proportion of ethanol in the mixed acetic acid:ethanol suspension. The ζ-potential was seen to rise linearly with increasing ethanol proportion up to 50% ethanol. Above this ethanol proportion ζ-potential readings could not be determined. Collagen films were then deposited by Pulsed-EPD with varying proportions of ethanol to explore the effect of ethanol on the deposited films, seen in figure 3b. At 25% ethanol, the deposited films show the effects of bubble formation, with a large amount of visible damage to the surface of the film, at 50% ethanol the deposited film is free from damage caused by evolved gas, giving a defect free deposit, and at 75% a defect free film was formed but the film appeared much less dense and was less robust. The reduction seen in the level of gas evolved with increase in volume fraction of organic medium is an expected result, as by reducing the amount of water that can be electrolysed that is in contact with the electrode then we can achieve a reduction in the amount of gas evolved.

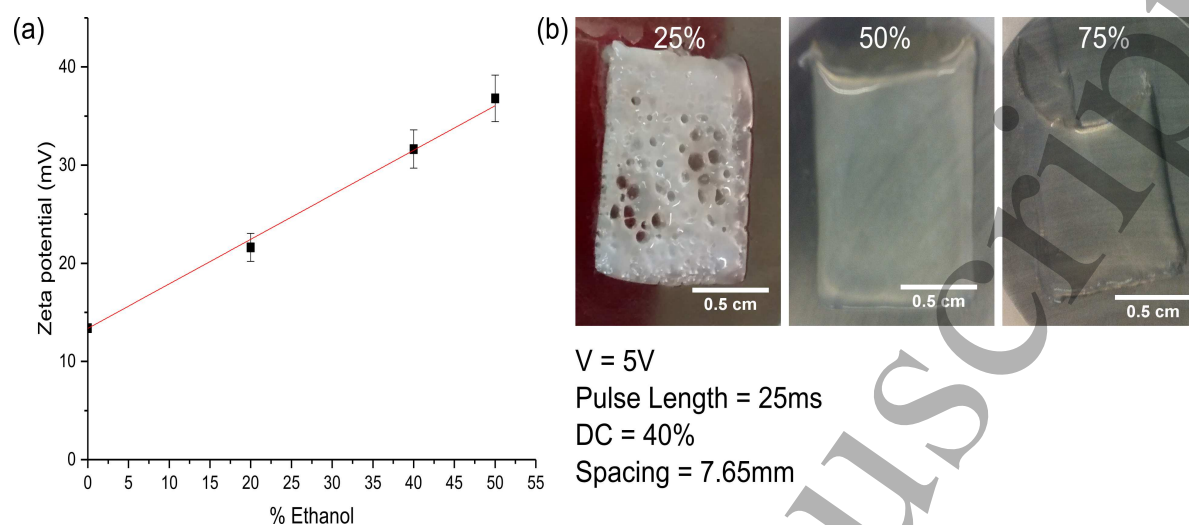**Figure 3.** (a) ζ-potential of collagen as a function of suspension ethanol percentage, (b) Images of collagen films formed by Pulsed-EPD with a range of ethanol suspension vol%.

3.3. The effect of pulse width

To determine if altering the pulse width affected the mass of collagen deposited on the electrode for a given T_{on}, the deposited mass was measured for a range of pulse widths while the T_{on} was fixed, seen in figure 4. A 0.1wt% suspension of collagen was deposited for T_{on} of 5 minutes. Pulsed-EPD at 10V lead to a mass of approximately 0.001g being deposited, with this decreasing to 0.0006g when the potential was set to 5V. The smaller deposited mass at a lower applied potential is expected as a lower applied potential will reduce the velocity of the suspended particles and hence reduce the rate of deposit formation. Additionally, at both applied potentials, altering the pulse width had no effect on the mass of collagen deposited. This implies that there is little difference in the mass transfer rate when the pulse width is altered, probably because the suspended particles continue moving due to their momentum while the applied potential is 0.
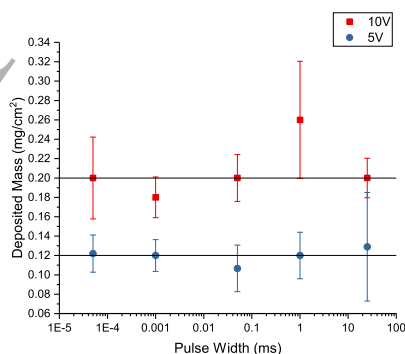

Figure 4. Graph showing mass of collagen deposited for different pulse widths, with a T_{on}=5minutes. [DC=50%, electrode separation = 7.65mm]

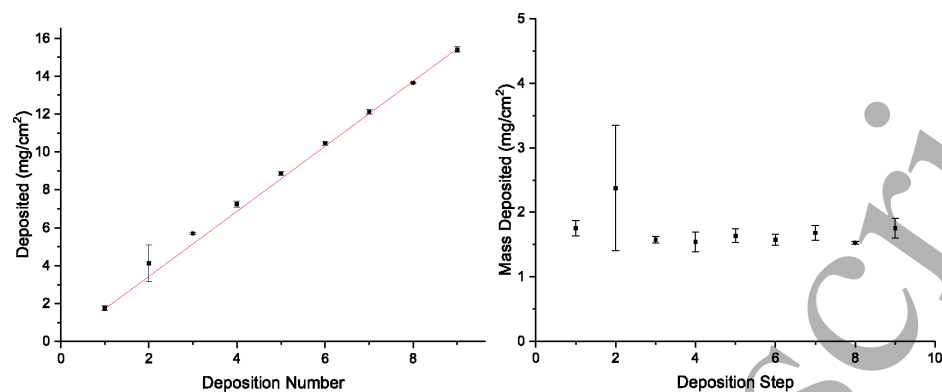*Fabrication of collagen membranes by P-EPD* 8

Figure 5. (a) Graph of total deposited mass of collagen with number of sequential depositions , (b) Graph of mass deposited per deposition step for sequential depositions. [Pulse length=25ms, DC=40%, Electrode spacing=7.65mm, V=5V, 3 samples]

3.4. The effect of multiple depositions

In EPD of ceramic systems it has generally been accepted that as material is deposited at the electrode the EPD cell resistance increases [19], reducing the rate of deposition, and decreasing the mass that can be deposited in each subsequent deposition step . It has been suggested however that this decrease in the rate of mass deposition is controlled by a complex mechanism involving the electrostatic interactions between ions and the charged deposit, and the retention of the charge carrying species in the deposit, which is dependent on a range of variables such as the pore size and Debye screening length of the depositing particles [34]. To determine the effect of deposited collagen on the deposition of further layers, collagen was deposited sequentially onto an electrode, with the electrode being dried and massed between each deposition step. The results from this, seen in figure 5, show that the mass of collagen deposited was unaffected by previous deposition steps. Additionally, we found that when the collagen suspension was replaced after each deposition, without drying, the mass that could be deposited in each step did not change (data not shown). From figure 3b we can see that when a collagen deposit is initially produced during EPD it forms a highly hydrated gel. We theorise that this initially deposited gel has sufficient porosity to allow transport of conductive species through, as there is a conductive pathway that results in a minimal increase in the EPD cell's resistance. During drying the deposited gel collapses, producing a much denser film approximately 100 times thinner that cannot be rehydrated to the original deposit thickness. We have found that the deposition behaviour, and hence electrical conductivity, is unaffected. It is possible that the densified collagen membrane is still permeable enough to allow for ion transport unimpeded, as has been found with small molecules in some reconstituted collagen membranes with nano-scale pores [45]; or that the hydrated collagen membrane, when an electric field is applied, undergoes structural changes that affect the double layer repulsions between the collagen fibres [46], changing the porosity of the membrane and producing a pathway for conductive ions; or the collagen membrane could become rapidly charged upon application of a
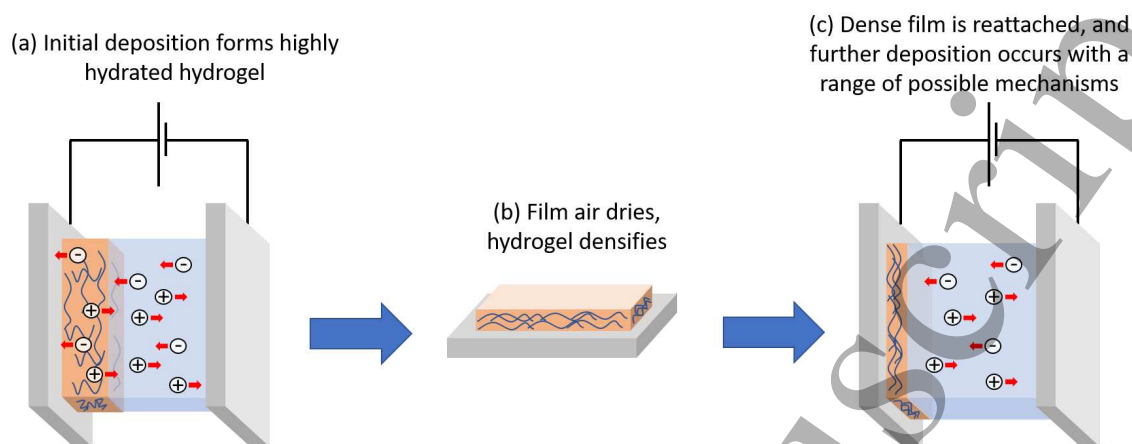
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Figure 6. (a) Film is initially deposited as a highly hydrated hydrogel, with sufficient space between collagen fibres for ion transport to continue, (b) After deposition, the film and electrode are removed from the EPD cell and allowed to air dry. As the hydrogel dries, it forms a dense, non-porous, film. (*c*) The electrode and film are reattached to the EPD cell and new collagen suspension is added. The film charges, allowing for deposition to continue without seeing an increase in the resistance.

electrical potential [47]. This is shown schematically in figure 6. Through deposition of multiple layers of collagen, thicker collagen membranes can be produced as desired.

3.5. The effect of voltage on film density

The effect of varying the deposition voltage on the density of collagen films produced by P-EPD was examined by changing the applied voltage during deposition. Additionally, the density of collagen films prepared by casting was determined and compared with the density of films produced by P-EPD. This is shown below in Figure 7.
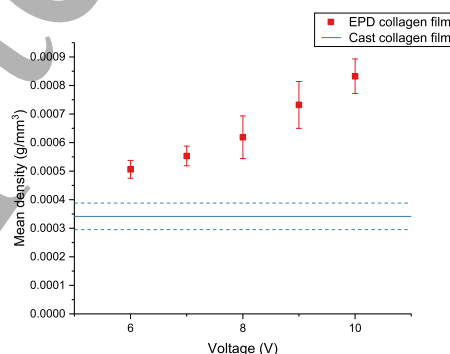

Figure 7. Graph showing density of collagen membrane against voltage for collagen membranes produced by P-EPD. Also shown is density of collagen membranes produced by solvent casting.

From Figure 7 we can see that all the collagen membranes produced by P-EPD show a higher density than those produced by solvent casting. The density of P-EPD collagen

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membranes also shows a dependence on the applied voltage, with increasing voltage
leading to denser films. Pulsed-EPD has been previously shown to produce ceramic
coatings and deposits with high density [34]. It has been theorised that application of
a pulsed or varying electric field allows particles to rearrange at the surface into a more
favourable packing arrangement, similar to the effect of tapping a particle filled container
[48], as well as the preferential deposition of individual particles rather than clusters
during P-EPD, further improving the particle stacking [49, 50]. This effect, combined
with the increased force applied during particle stacking during film formation, explains
the increase in density seen with an increase in applied voltage, and can be used to
control the thickness of collagen membranes produced through P-EPD.

3.6. Deposit microstructure

To determine the effect of Pulsed-EPD on the microstructure of collagen films we
compared the films produced by Pulsed-EPD with collagen films prepared by solvent
casting. Dry films were examined by AFM, and SEM, and hydrated films were examined
with cryoSEM. Figure 8a shows an AFM height map of a Pulsed-EPD collagen film.
Visible on the surface are a number of circular and ring like structures. These structures
resemble the "exploded" micrometer scale surface bubbles seen by Ammam *et al.* during
DC-EPD of polypyrrole which were caused by evolution of gas bubbles during deposition
that were then coated by depositing material [51]. As the effect of Pulsed-EPD is
to reduce the size of bubbles formed during EPD, preventing coalescence into larger,
macroscopically damaging bubbles, rather than to prevent bubble formation at all it is
likely that many of these larger circular structures are likely caused by the evolution of
micro-bubbles during the deposition process, with the ring structures the remnants of
collapsed bubbles [42]. As gas bubbles form during deposition, they can become trapped
by incoming collagen fibres, sealing the shape of the bubble into the film. If the gas
then escapes, the space can collapse inwards due to the pressure of incoming particles,
leaving an annular structure on the surface of the membrane. This is in agreement with
the theorised mechanism of changing gas evolution sites during Pulsed-EPD, as micro-
bubbles have been able to nucleate but none of these visible micro-bubbles were able to
coalesce into larger membrane damaging macro-bubbles. These annular structures are
absent in AFM maps of collagen films formed by solvent casting, shown in Figure 8c,
indicating that their appearance is due to the use of Pulsed-EPD.

The surface roughness of films formed by Pulsed-EPD and casting of collagen was
calculated from AFM height map data, shown below in Table 1. Collagen films produced
by EPD were produced with 1, 2, or 3 layers to measure the effect of multiple depositions
on roughness.

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Table 1. AFM roughness measurements

Film	Collagen layers	Scan Size (μm^2)	R_q (RMS) (nm)	Std Dev (nm)
EPD film 1	1	25	191.4	53.35
EPD film 2	2	25	211.1	66.94
EPD film 3	3	25	146.0	36.54
Cast film	1	25	458.1	176.6

The data shows no significant difference in surface roughness between collagen films produced by EPD, and no dependence of surface roughness on number of deposited layers. The roughness of the cast film was found to be higher than that of the films deposited by EPD, with a greater variation in roughness also seen. Though performed on a limited number of samples, the data suggests that collagen films formed by EPD have a lower surface roughness than films formed by solvent casting.

SEM investigation of the surface of the Pulsed-EPD collagen film, seen in figure 8b shows a similar structure, with the overall surface of the film being planar with small circular lumps caused by micro-bubbles or due to collagen globules. Figures 8c and 8d show the surface of a collagen film formed by solvent casting, in which a broadly similar morphology can be seen, though the characteristic ring structures produced by collapsing bubbles are absent as expected. The surface similarity between the cast and Pulsed-EPD films is confirmed by figure 8d which shows a textured surface morphology with a number of collagen globules present.

The existence of the interior micron and sub micron pores was confirmed by cross sectional cryoSEM, seen in figure 8g. This porosity is likely to be due to bubbles that have nucleated on the surface of the membrane during deposition, which have then been overlaid with further collagen fibres, leaving a void internally within the membrane. This indicates that there is limited movement of the collagen fibres once they have come into contact with the deposit as the pores have remained unclosed during the drying process. The pores formed through this mechanism are too small however to have more than a local effect on the microstructure of the membrane. Figures 8f and 8g also show no signs of damage at the edges of the membrane due to the mechanical separation from the electrode.

CryoSEM was also used to examine the general microstructure of the membranes, with figures 8f and 8g showing the interior microstructure of a swollen membrane. The membrane is clearly of an even thickness across its length, and in figure 8g the membrane can be seen to be comprised of many individual collagen fibres in a dense lamellar structure. Use of EPD has been shown to lead to high packing densities when suitable parameters are chosen [52, 53, 39, 27], as continued application of the electric field applied a force, causing the particles to rearrange to a close packed structure. A similarly dense collagen membrane structure was produced by Tanaka, who produced collagen laminates by centrifugation of collagen on a slide[54]. The force applied during centrifugation increased the density of the resultant membrane, similarly to
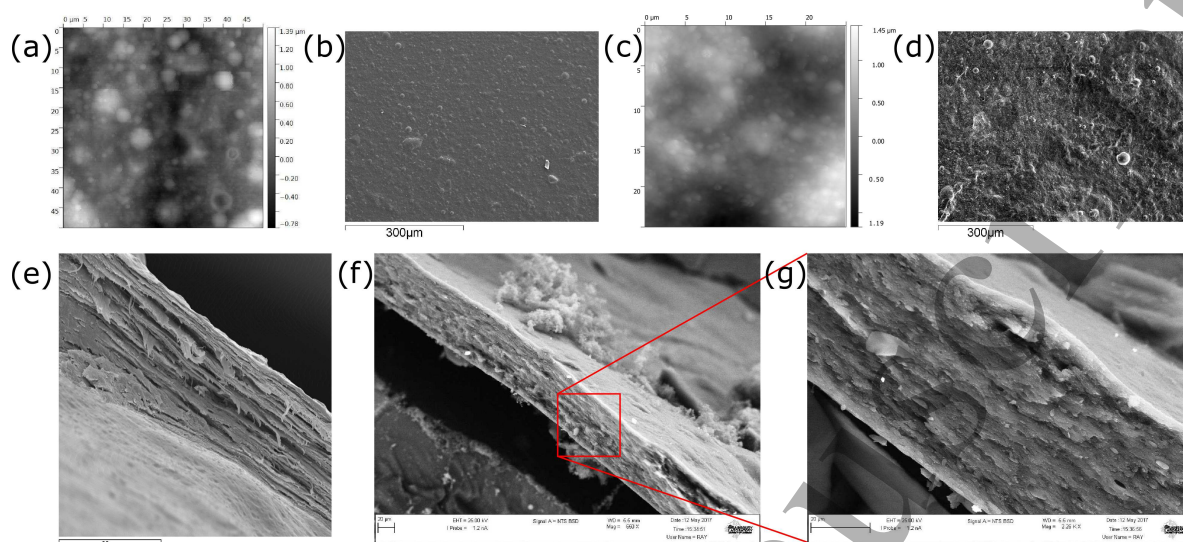
Figure 8. (a,b) AFM and SEM-SEI micrographs of the surface of a collagen film deposited by Pulsed-EPD, (c,d) AFM and SEM-SEI micrographs of the surface of a cast collagen film. (e) SEM micrograph of a cross section of a cast collagen film, and (f,g) BSD cryoSEM micrographs of the edge of a freeze-shattered collagen film produced by Pulsed-EPD

the densification of the collagen membrane due to the application of an electric field during EPD. This close packing of collagen fibres is in agreement with the density measurements in Figure 7, which showed a higher density was achievable in collagen membranes produced through P-EPD when compared to membranes produced through solvent casting.

3.7. Preparation of free-standing collagen membranes

In order to produce free-standing collagen membranes after deposition, membranes were deposited as described before mechanical cleavage was applied by running a razor blade between the electrode and the membrane. Figure 9e shows a collagen membrane removed by this method. No damage caused by separation was visible and the film maintained mechanical stability, integrity, and flexibility once removed from the substrate.

4. Conclusions

By careful choice of deposition parameters such as voltage, pulse width, and duty cycle, it was shown that deposition of macroscopically defect free films of collagen can be produced from an aqueous suspension by Pulsed-EPD. The deposition rate was found to be greater at higher voltages, and deposition of multiple suspensions onto a single electrode was found to increase the mass linearly. Investigation of the microstructure showed minor differences in surface morphology between collagen membranes formed by solvent casting and by Pulsed-EPD, and the internal structure of hydrated collagen membranes was found to be densely packed, with a small number of nano-scale voids.
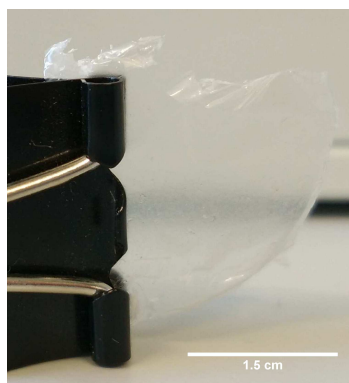
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Figure 9. Image of a free standing collagen film separated by mechanical cleavage.

Membranes produced by Pulsed-EPD were found to show significantly higher densities than cast collagen membranes, and the density was found to increase with an increase in applied voltage. The thickness of the membranes produced by P-EPD could be controlled through the number of deposited layers, or the magnitude of the applied electric field. Free-standing membranes could be produced by mechanically separation from electrodes easily after drying with no visible damage. Overall, P-EPD shows great promise as a method for rapidly producing collagen membranes.

5. Acknowledgements

Declarations of interest: none

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