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ORIGINAL PAPER

Using electrophoretic deposition to identify protein charge in biological medium

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Abstract Protein adsorption is the first step involved in establishing a suitable integration between a biomaterial and host tissue. It is therefore of highest interest to know the electric charge of proteins present in the relevant medium to be able to predict the behaviour of cells on given surfaces. In this study, electrophoretic deposition (EPD) was used as a simple method to identify the charge of proteins present in biological medium. In the model experiment carried out here, EPD was conducted using a biological medium containing 10% fetal calf serum (FCS) and the charge of the protein was determined by examining the migration of the protein in the EPD cell under a certain applied voltage. In addition, the suitability of EPD of proteins to deliver tailored surfaces for enhanced bioactivity or for controlled deposition of protein films on metallic surfaces was explored.

Keywords Electrophoretic deposition · Proteins · Coatings

Decheng Meng and Lydia Francis share first co-authorship status.

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1 Introduction

In tissue engineering, the integration of the bioactive material used as scaffold with the host tissue is extremely important. A strong bonding at the tissue/material interface will positively affect the growth of new tissue as well as prevent any immunogenic reaction towards the substrate [1]. Protein adsorption is the first step involved in establishing this integration between the biomaterial surface and host tissue. Studies by Kauffman et al. [2] have reported that within few seconds of implantation of a scaffold in the body, a series of physico-chemical changes takes place at the biomaterial surface which is followed by the adsorption of proteins.

In relation to a number of relevant factors such as surface chemical composition, surface morphology (topography) [3] and pH of the surrounding medium, surface charges appear to be the main physical factor influencing the binding of proteins to the scaffold. This electrostatic interaction (strength and type) between the proteins and the material surface can also be controlled by varying the concentrations of electrolyte solutions and the pH of the medium [4]. By varying these conditions, proteins with different surface electric charges can also be bound onto a scaffold.

Fetal calf serum (FCS) present in biological media is isolated from the foetus of an unborn calf and is used for the culturing of cells [5]. Bovine serum albumin (BSA), the major component of FCS, has a molecular weight that ranges between 68,000 and 72,000 Da [4]. In the human body, albumin is also one of the first proteins to adhere non-specifically to an implant. Both human and bovine albumins have been reported to have 18 amino acids in common. The charge on this protein is dependant both on the ionic strength and pH of the solution. Patil et al. [6]

reported that at relatively low pH values (4.78) the interactions of albumin with the biomaterial would be hydrophobic interactions due to the compact hydrophobic nature of the protein. However, when the pH of the solution is 7.00, the zeta-potential charge of the protein changes to negative and binds to the implant surface through electrostatic interaction. Investigations by Krajewski et al. [4] have also suggested a close relationship between the adsorption of proteins and the zeta-potential, which is influenced by the pH of the medium. A higher increase in protein adsorption is usually observed with increase in the negative value of the zeta-potential [4].

Cellular attachment to an extracellular matrix normally occurs through receptor proteins known as integrins. These integrins have a 'RGD sequence' (Arg-Gly-Asp) and (G) RGD(S) (Gly-Arg-Gly-Asp-Ser) due to which the cellular attachment takes place [7]. This RGD sequence exists also in some attachment proteins present in serum, e.g. thrombospondin, vitronectin, fibronectin, collagen and osteoponin, which are present in smaller amounts when compared to albumin. Once these proteins attach to the implant they induce the cells to anchor on the surface [7]. With the increase in the protein adsorption capability of a surface the initial cellular attachment is also higher [8].

The migratory behaviour of cells and their attachment on charged biomaterial surfaces can affect the subsequent cellular behaviour [9]. For example, poly(methyl methacrylate) beads of 10–100 μm in diameter with positive and negative charges were used to study cell attachment and cell behaviour on surfaces with different charges [9]. From these studies it was observed that a single cell (osteoblast) appeared to have a 'stand-off' position on a negatively charged substrate and did not appear flattened when compared to a positively charged surface. The cells on a positively charged surface appeared flattened and communicated with each other through a wide pseudopodium, as schematically shown in Fig. 1. Thus, it was demonstrated that the cellular migratory behaviour is influenced by the charge on the surface, which again is dependent on the charge carried by the adsorbed protein present on the surface of the scaffold.

The present discussion indicates that it is of highest interest to know the charge of proteins present in the relevant medium to be able to predict the performance of the cells on given surfaces. In order to identify the charge of proteins present in a biological medium, electrophoretic deposition (EPD) is proposed as a simple qualitative method. EPD has been shown to be one of the most effective methods to manipulate charged particles and biomolecules as well as biological entities in liquid media [10–15]. In this technique, charged particles or large molecules suspended in a liquid medium migrate under the influence of an electric field (electrophoresis) and are

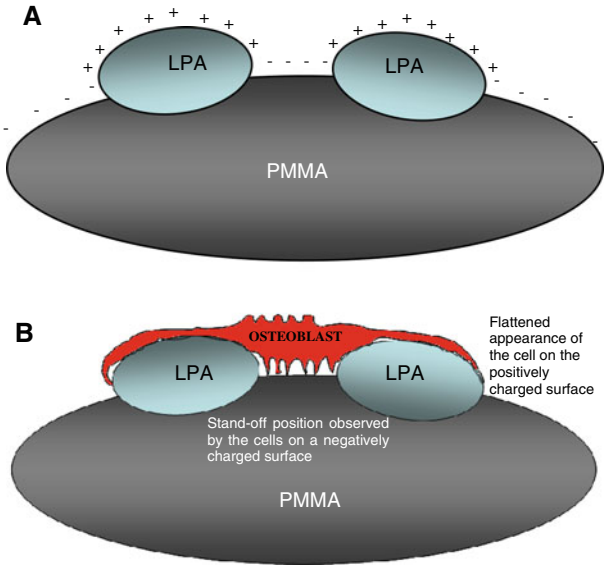

Fig. 1 **a** Diagrammatic representation of a bead surface showing regions of opposite surface charge, **b** diagram showing a single osteoblast migrated onto both the positively and negatively charged areas (adapted from Davies et al. [9])

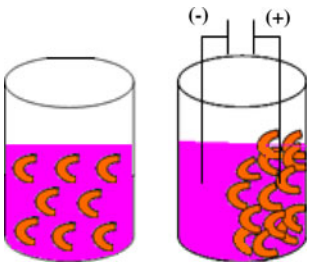

Fig. 2 Diagrammatic representation of the migration of negatively charged proteins present in a biological medium towards the anode under the influence of an electric field

deposited onto an electrode with the opposite charge, as schematically shown in Fig. 2. Thus, the aim of this study was to show the identification of the surface charge of proteins in biological medium by applying EPD, so that a better understanding of protein adsorption on given surfaces could be obtained. This knowledge can be used to improve the ability to control the orientation and stability of proteins with the objective of developing tailored biomaterial surfaces for enhanced bioactivity. Moreover, successful EPD of proteins on biomaterial surfaces could be exploited to functionalize such surfaces for enhanced biocompatibility of implants and scaffolds [13].

2 Materials and methods

The EPD experiments were performed in a custom made EPD cell using a biological medium (DMEM, Gibco, 10%

Fetal calf serum, Biosera). The electrodes were made of stainless steel (316L) foil with dimension of 20 × 10 × 0.02 mm^3. The separation distance between the electrodes was 20 mm. Electrodes were connected to a Thurlby Thandar Instruments (TTi) EL561 power supply (Cambridgeshire, UK) and the current intensity during EPD was recorded by using a TTi 1906 Computing Multimeter. A constant voltage was applied when the electrodes were lowered into the DMEM medium. The electrodes' area immersed in the DMEM medium was 17 × 10 mm^2. Electric fields of 150, 250 and 500 mV mm^{-1} were applied. Before deposition, the electrodes were cleaned in acetone, washed with distilled water and dried with compressed air. The deposition time for all three conditions was 5 min. The current density was calculated by dividing the current intensity measured in each case by the area of electrode immersed in the suspension. 100 current readings were taken every 3 s over 5 min. The pH was measured using a JENWAY 3510 (UK) pH meter.

After deposition, the electrodes were withdrawn from the DMEM medium slowly in order to avoid the influence of the drag force between the medium and the deposited wet serum coating, which could lead to disruption of the coating. Finally, the electrodes were left to dry at room temperature in air. The electrode with the protein deposition was visualised using SEM (JEOL 5610LV).

3 Results

The DMEM medium appears as a pink solution, as shown in Fig. 3a. Applying an electric field of 100 mV mm^{-1} to the

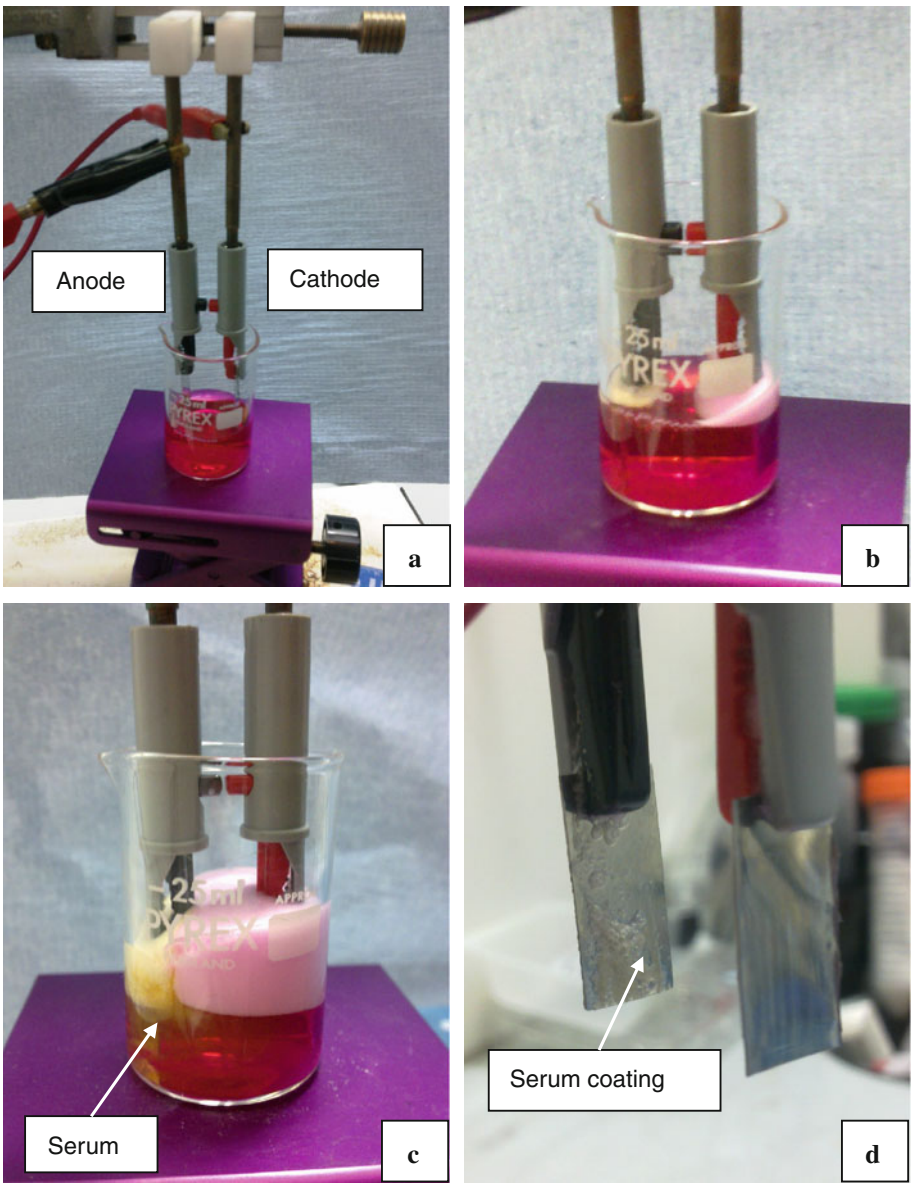

Fig. 3 Photographs of the EPD cell showing: **a** DMEM medium before the experiment; **b** the observation during EPD at 300 mV mm^{-1}; **c** the observation during EPD at 500 mV mm^{-1}, serum separated out from the medium; **d** serum coating on the anode after EPD at 500 mV mm^{-1} for 5 min

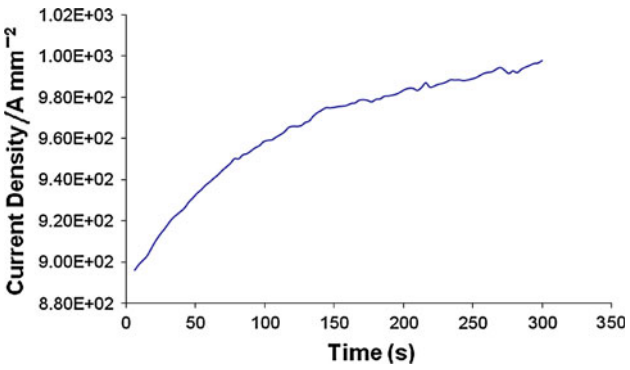

Fig. 4 Current density (A mm^{-2}) versus time during the EPD process at 500 mV mm^{-1}

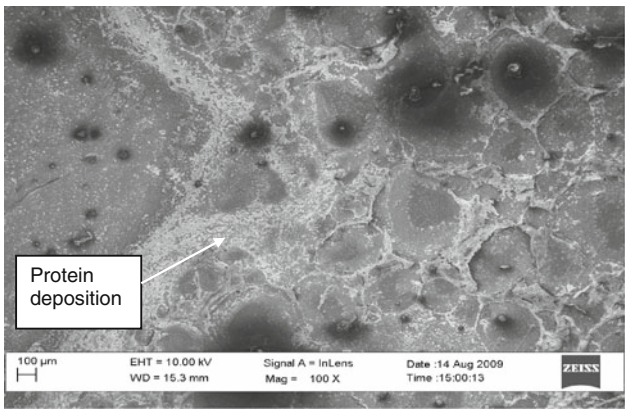

Fig. 5 SEM image showing the presence of a protein layer deposited on the stainless steel substrate (anode) during EPD (500 mV mm^{-1} for 5 min). The protein deposition is indicated by the *arrow*

EPD cell, only limited water electrolysis was seen to take place, no other reaction was observed. When the electric field was increased to 300 mV mm^{-1} (Fig. 3b), it was possible to observe that deposition started to take place, as the serum, which is yellow in colour, migrated to the anode. This simple experiment thus proved that the proteins are negatively charged in DMEM medium. At 500 mV mm^{-1} (Fig. 3c), numerous bubbles were produced due to water electrolysis, and the medium was separated into two 'layers': the yellow serum layer was attracted to the anode (left electrode in Fig. 3). After EPD for 5 min, the coated electrode was removed from the EPD cell, dried at room temperature in air and weighted. It was found that a mass of 0.2 mg of serum was deposited onto the electrode for EPD at 500 mV mm^{-1} for 5 min (Fig. 3d).

Figure 4 shows the variation of current density versus time during the EPD procedure at 500 mV mm^{-1} for 5 min, it was observed that the current density increases as the experiment progresses.

SEM examination of the deposition electrode confirmed that a relatively thick protein layer (e.g. in Fig. 5 for EPD at 500 mV mm^{-1} for 5 min) was obtained on the anode.

4 Discussion

Protein attachment on a biomaterial surface is largely dependent on factors like surface chemistry and topography [3, 6, 16]. Although other variables contribute to the adsorption of proteins, such as pH and protein concentration in the medium, proteins can be adsorbed due to electrostatic interactions. Studies conducted by Patil et al. [6] have shown the adsorption of BSA on ceria nanoparticles with different surface charges. In that study, they proved that attractive forces existed between the positively charged ceria nanoparticles and the negatively charged BSA proteins, which were responsible for strong protein adsorption. Similarly, when negatively charged ceria nanoparticles were used, repulsive forces between the proteins and ceria nanoparticles resulted in no protein adsorption. The studies also showed that ceria nanoparticles with higher negative zeta-potential exhibited higher cellular uptake when compared to the positively charged oxide particles [6]. In the present study, by using a simple EPD experiment, the polarity of surface charge of proteins in serum was determined, and the possibility of coating a metallic substrate with a protein layer by EPD was explored. Clearly, the method can only be considered successful if the deposited proteins retain their activity. Alkan et al. [17] have reported the effect of electrolysis on enzyme activity. Urease was used in that study, and the activity of the enzyme was measured using Berthelot method. It was reported that the activity of proteins during electrolysis is affected by the nature of the electrolyte. The possibility that DMEM, the medium used in this study, inhibits protein activity upon electrolysis, remains to be investigated. Alkan et al. study [17] also reported that the variation of pH during electrolysis in deionised water caused denaturation of the enzyme, however, the pH remained in the neutral range during the present EPD process.

A correlation between the pH of the biological medium and the surface charge of the proteins has also been reported [6]. At the start of the experiment, the pH of the biological medium was recorded to be 7.9, after 10 min when an electric field of 500 mV mm^{-1} was applied, the pH changed to 7.45. It is important to identify the changes in pH and their possible effect on protein adsorption. In our case, EPD enables to confirm the expected protein charge at pH $\sim$7. Since the major component of FCS is bovine serum albumin and as reported in the literature [4], the isoelectric point (IEP) of the protein occurs at pH 4.75, at pH 7.00 the protein surface charge is thus negative and the biomolecule migrated towards the anode. These findings are in agreement with the results of Krajweski et al. [4], confirming the close relationship that exists between the surface charge of the proteins and the adsorption behaviour

influenced by the pH of the solution. A highly acidic pH is also known to favour albumin adsorption; however, in the human body during restoration of bone, the surrounding medium can become highly acidic due to the activity of osteoclasts and macrophages, which are known to remove the damaged bone [4]. An acidic environment should be less conducive for osteointegration, because the coating of proteins on the surface of the implant hinders this process. By identifying the charge of proteins by EPD, the surface charge of the scaffold/bioactive material can be tailored for cellular attachment studies.

From Fig. 4, it is confirmed that the current density increased over the duration of the experiment. This result explains why the pH of the medium changed upon EPD. It should be also highlighted that the application of EPD to deposit protein layers on metallic surfaces represents a convenient alternative to develop bioactive surfaces for enhanced tissue attachment, for example in orthopaedic applications and tissue engineering [13, 18]. However, the retention of the deposited protein activity must be confirmed in each case.

5 Conclusions

Electrophoretic deposition was used to identify the charge of proteins present in a biological medium containing 10% FCS. Molecules migrated towards the anode in the EPD cell. A mass of 0.2 mg of serum was electrophoretically coated onto the electrode at 500 mV mm^{-1} and 5 min of deposition time. EPD may represent a convenient technique to develop protein coated metallic surfaces for application in the biomedical field, including biosensors and orthopaedic implants. Depending on the protein under consideration, its remaining specific activity after the EPD process should be tested.

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