The platelet electrome: evidence for a role in regulation of function and surface interaction

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The platelet electrome: evidence for a role in regulation of function and surface interaction

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Abstract

Introduction. Platelets protect the body from injury through formation of blood clots, changing from a normal, quiescent state to become "activated" in response to external stimuli such as chemical cues, shear stress and temperature. This causes changes in shape, increased adhesion, and alteration of the electrical properties such as membrane potential V_m and zeta potential ζ. These phenomena have been regarded as largely unconnected; for example, changes in ζ have been attributed solely to alteration of surface lipid concentration. However, recent reports suggest that cells can alter ζ electrostatically by alteration of V_m in red blood cells. We hypothesised that if platelets also modulate ζ via V_m, this may provide an alternative mechanism to alter cell-cell interaction.

Methods. We investigated platelets stored at different temperatures (4°C, 22°C, 37°C) for 24h, which is known to alter platelet behaviour and electrical properties, and compared these with analyses of freshly-harvested platelets. These four conditions exhibited unique sets of electrical properties (V_m, ζ, membrane conductance G_{eff} and cytoplasm conductivity σ_{cyto}) as well as surface exposure of the adhesion molecule P-Selectin. These were analysed to identify correlations between electrical parameters and platelet activation state.

Results. Many parameters exhibit pairwise correlation across all four conditions, in particular between ζ and G_{eff}, and also between V_m and σ_{cyto}. Furthermore, when the electrical behaviour of platelets stored at 4°C (known to activate the cells) was removed from the analysis, additional relationships were observed among the remaining conditions, including those connecting ζ and V_m to the amount of P-selectin binding.

***Conclusion*.** Results suggest that V_m may mechanistically alter the availability of cationic molecules at the cell surface, a process never reported before, with implications for our wider understanding of cell-molecule and cell-cell interaction.

INTRODUCTION

Platelets are small (2-3µm diameter) fragments of bone marrow megakaryocytes. They constitute approximately 5% of total cells in normal circulating blood. Their function is to adhere to one another and to surrounding tissues in response to extracellular cues indicating the occurrence of damage to blood vessels. This aggregatory behaviour, in collaboration with initiation of the coagulation pathways, results in the formation of clots which act to stem bleeding. The process whereby platelets change their behaviour (activation) to stick to other cells (adhesion) is complex, involving a number of biomolecular and physical changes. These include translocation of cell surface adhesion molecules, modulation of adhesion receptor affinity, change of shape from oblate discoid, through the production of protrusions, or dendrites, to a thin disc with a protruding cell body[1]. Whilst these chemical and morphological changes have been studied in some detail, changes in bioelectronic properties have been largely overlooked. Bioelectronics is a long-established discipline[2] most commonly (though not exclusively) concerned with the effects of the membrane potential V_m developed between the cell interior and exterior, by which neuronal and muscle functions are initiated, transmitted or propagated[3,4]. The membrane potentials of most non-excitable cells range between -40mV and -70mV depending on cell type, maintained by the action of the K+/Na+ ion exchanger. However, many cells do not follow this pattern; for example, cancer cells are depolarised compared to non-cancerous tissues from the same site[5,6], and V_m has been found to play an important role in developmental biology[7]. Platelet V_m has been identified as significant to platelet function; platelets[8] were shown to depolarise from -63.8mV immediately after venepuncture, to -35 mV when held at 0°C for 60 minutes, which gradually returned to normal after resuspension in 37°C medium for 30 minutes. Depolarised platelets exhibited increased aggregation induced by ADP, collagen and adrenaline. Conversely, Krötz et al.[9] reported that hyperpolarisation inhibited platelet adhesion. This suggests V_m may modulate, or be modulated by, platelet function.

A less commonly explored electrical aspect of cells is the zeta potential ζ. This is the voltage due to charge at the cell surface, but measured at the end of the hydrodynamic shear plane, about a nanometre outside the cell surface. ζ is used in materials science to establish whether colloidal solutions are electrostatically stable: whether electrostatic repulsive force due to surface charge is sufficient to overcome attractive van der Waals forces. Given that platelets need to become adhesive on activation, ζ has a potentially significant role to play. Coller[10] first showed that platelets do indeed change ζ in response to activation stimuli, in line with understanding of colloidal stability, becoming better able to adhere and aggregate when activated. This was assumed to be solely due to the reconfiguration of surface chemistry, in particular the lipid groups found on the surface between

quiescent and active states, with phosphatidylserine relocating from the inner leaflet of the membrane to the outer leaflet[11].

However, recent research suggests another mechanism which may play a role. Hughes *et al*[12] demonstrated that in red blood cells (RBCs), many electrical parameters interact: one example of this is that V_m is connected to ζ by a constant Ξ, with other associations observed with cytoplasm conductivity and surface conductance. These interconnections were described as a *cellular electrome*. In this study, we apply this approach to human platelets, to measure the platelet electrome and its implications for platelet function. We compared multiple cellular electrical properties immediately after harvesting with those stored at three different temperatures for 24h in order to place the cells in different physiological (and hence electrical) states. Our results suggest that internal electrical effects may affect both the adhesive behaviour of platelets and their interaction with extracellular proteins, with significant implications for cell-cell interactions.

MATERIALS AND METHODS

Cells: Studies were conducted in concordance with the principles of the Declaration of Helsinki. Study approval was received from the Ethics Committee of the University of Surrey. To obtain PRP, whole blood from five volunteers was collected in BD vacutainer glass ACD (acid citrate dextrose) solution B tubes (Becton Dickinson, UK). Whole blood was transferred to 15 mL falcons and centrifuged at 90 xg for 60 minutes[13] The PRP layer was the removed using graduated Pasteur pipettes (StarLab, UK) avoiding the buffy coat layer. Cells were either tested immediately, or stored for 24 hours in sealed Eppendorf tubes at 4°C (refrigerator), known to alter platelet electrical properties[8]; 22°C (incubator), which has been reported to permit storage of viable platelets for up to five days[13]; or 37°C (water bath), representing normal physiological temperature.

ζ-potential: ζ was measured using a Malvern Zetasizer Nano SZ (Malvern, UK) following injection of 800 µL of PRP into a disposable cuvette. Six measurements were taken per sample.

Dielectrophoresis: Measurements were taken using a DEPtech 3DEP[14, 15] (Heathfield, UK) by adding 10% PRP to 90% isosmotic medium comprising 8.5% (w/v) sucrose and 0.5% (w/v) dextrose, and then adjusted using PBS to yield an overall conductivity of 200mSm^{-1}. Effective membrane capacitance and conductance (C_{eff}, G_{eff}) and cytoplasm conductivity (σ_{cyto}) were extracted by modelling using best-fit to the Clausius-Mossotti model[14]. Four technical repeats were taken per sample.

V_m: A stock solution of $DiOC_3(5)$ (Stratech Scientific, UK) was made to 1 mM in DMSO, with a working stock solution in PBS at 1.5 µM. Pellets were resuspended at a concentration of 10^6 cells per mL and

incubated for 25 minutes, then washed twice with PBS for 5 minutes at 237 x g. Fluorescence measurements were performed on BD FACSCelesta™ (BD Biosciences, US) with BD FACSDIVA™ software (BD Biosciences, US)). Cells were gated forward scatter versus side scatter.

P-Selectin: Platelets were incubated with 10µM/ml of CRP-XL (Cambcol Laboratories, UK) at 0 and 24 hours for 5 minutes. 10 µL of PRP was incubated in HEPES buffer saline for 5 minutes. 1 µL of PE-Cy™5 Mouse Anti-Human CD62P (BD Biosciences, US) was added and the PRP underwent further incubation for 20 minutes at room temperature in the dark, as per manufacturer instruction. Additionally, there were two controls, an unlabelled sample and a sample labelled with an isotype control (PE-Cy™5 Mouse IgG1 κ Isotype Control (BD Biosciences, US), added at the same concentration as the staining antibody) to measure the non-specific binding. 100 µL of 0.2% formyl saline was added into the well containing the platelets with 40 µL of this final volume transferred to round bottomed test tubes (VWR, UK) containing 160 µL of formyl saline. Samples were read using a BD FACSCelesta™ flow cytometer (BD Biosciences, US) with BD FACSDIVA™ software (BD Biosciences, US) and events were kept below 2000 per second, with the total number of events per sample set to 10,000.

Data Analysis: Data were averaged across the four experiments, then compared in a pairwise fashion to identify linear correlations using the correlation coefficient r^2. For DEP parameters the data were averaged before modelling, providing a single robust data value; for flow cytometry and ζ measurements, correlations were determined using the mean values. Mean data from the four donors were collated for parameters G_{eff}, σ_{cyto}, K_s, P-selectin (both before and after stimulation with CRP, plus control for measurement of non-specific binding), ζ and DIOC5 (a correlate of V_m); and four conditions; control cells were assessed immediately after harvesting, and compared to platelets which were stored at 4°C, 22°C and 37°C for 24 hours in order to ensure they had reached equilibrium at the stored temperature. The five parameters were then analysed in pairwise fashion in order to identify correlative behaviour. The ten possible combinations of parameter pairs were examined to identify linear correlations, with r^2 values being recorded of each pair of parameters. Furthermore, having first considered linear correlations between data from all four cell conditions, we then performed a re-analysis with the data taken from cells stored at 4°C excluded from the analysis. The rationale for this is that since storage at 4°C results in platelets swelling and changing shape by growing dendrites[16-18], then correlative behaviour which is dependent on shape will be shared between the other three conditions, but not the cells stored at 4°C

RESULTS AND DISCUSSION

The aim of these experiments were threefold; to identify whether correlations between electrical parameters in platelets resemble those observed in RBCs; to examine the implications for platelet storage from the perspective of electrical (and hence, ionic) concentration; and to examine whether such relationships, if they exist, have potential to explain observed platelet behaviour during interactions with their environment. Data are shown in table 1. Typical correlations can be seen in Figure 2; correlations, featuring an example case where the 4°C data conforms to a trend observed with the other cells, and another where the 4°C data are markedly different, can be seen in Figure 2.

Correlative changes in parameters suggest the RBC "cellular electrome" model is applicable to platelets. We first analysed all four data sets together. In line with the observations of Hughes *et al*[12], we identified similar linear relationships (r^2>0.92) between V_m and σ_{cyto} across all conditions. This is in line with expectations[12], as both arise from ion concentrations in the cytoplasm. Similarly, we identified near-perfect (r^2=0.99) correlations between ζ and G_{eff}, both of which arise due to charge on the cell surface. Interesting, as observed in RBCs, both surface parameters are affected by V_m, but in opposite directions; depolarisation of V_m results in depolarisation of ζ, but an increase in G_{eff}.

As described above, it is known that platelets stored at 4°C data are significantly morphologically different to those stored at higher temperatures. Since these changes may affect the way in which the cell interior and exterior interact, we also looked for correlations among the remaining three, morphologically similar populations. Intriguingly, in this second study we identified a near-perfect (r^2=0.97) negative correlation between G_{eff} and V_m, with increasing values of G_{eff} associated with a more depolarised V_m; another negative correlation (r^2=0.85) was observed between G_{eff} and σ_{cyto}, with rising conductivity producing reduced conductance. Both of these correlations have been also observed in red blood cells[12,19,20]. A weaker correlation was observed between ζ and σ_{cyto} (r^2=0.72). These correlations differ from those observed when the 4°C data were included, since the correlating parameters link properties related to the cell surface (ζ, G_{eff}) to those related to the cell interior (V_m, σ_{cyto}).

This suggests that under normal conditions, platelets exhibit similar electromic behavior to that observed in RBCs. The prior study[12] suggested that the mechanism by which the surface and interior electrical properties are coupled is capacitive in origin. The value of effective membrane capacitance C_{eff} was slightly higher (20.2 mFm^{-2}) than in the other three conditions (17.7-18.3 mFm^{-2}), but not by enough to suggest significant changes in membrane morphology. However, the surface properties of platelets are also known to alter due to scramblases relocating inner leaflet phospholipids to the outer membrane leaflet[21]. If we extrapolate the value of ζ using the three non-4°C values, it predicts

a value of ζ=-6.2mV if the cell were completely depolarised, suggesting that the drop in ζ cannot be purely as a result of capacitive action, though this could be responsible for the changes in the other three cases.

Antibody surface binding is related to membrane potential. Cell surface P-selectin levels were measured concurrently with the other measurements. When P-selectin levels were compared to the electrical measurements of the four conditions grouped together, P-selectin binding showed very strong correlations to G_{eff} (positive correlation, r^2=0.99) and ζ (negative correlation, r^2=0.90). When the 4°C data were excluded, strong correlations were also observed to V_m (r^2=1) and σ_{cyto} (0.93). P-selectin levels (though not other measures) were also recorded following stimulation with CRP in order to contextualise the levels observed in unstimulated cells. Exposure of platelets with CRP raised surface exposed P-selectin levels detected by 10-100 fold, consistent with stimulation of activation and demonstrating that cells were not activated at time of measurement.

There are two potential explanations for the correlations between P-selectin exposure levels following platelet stimulation and the electrical parameters; either the amount of P-selectin recruited to the platelet plasma membrane varies according to membrane potential suggesting a mechanistic link between platelet regulation mechanisms and membrane potential; or that CD62P antibody binding is itself affected by the electrical properties. It is known that IgGs carry a negative charge[22], meaning that the concentration at the cell surface and hence available for binding ($c_i(0)$) deviates from the bulk concentration c_{oi} by an amount governed by the Poisson-Boltzmann equation:

$$c_i(0) = c_{oi} exp\left(\frac{-z_i e \Psi_{st}}{kT}\right) \quad (1)$$

Where ψ_{st} is the Stern layer potential, z_i the valency, e the electric charge, k the Boltzmann constant and T the temperature, as shown in Figure 3. Whilst it is generally assumed that ψ_{st} relates only to the fixed charge on the cell surface, work by Hughes *et al.*[12] suggested that this due to the surface charge *plus* a portion of membrane potential, such that surface ion concentration can be altered by V_m. This would explain why CD62P levels vary linearly with electrical properties which relate to the cell surface potential (G_{eff}, ζ), and why the detection CD62P levels vary with membrane potential for the conditions excluding 4°C; it suggests that for these cells, the P-selectin levels on the cell surface may remain constant whilst the availability of antibodies to bind to the protein changes.

Furthermore, levels of non-specific binding *also* showed correlation and G_{eff} (r^2=0.97), ζ (0.89) and σ_{cyto} (0.95); when 4°C was excluded, good correlations were also observed with V_m (r^2=0.77) and P-selectin (r^2=0.78). The high degree of correlation with surface parameters G_{eff} and ζ suggest that for non-specific binding, antibody availability at the surface is the primary factor governing non-specific

binding, implying that such a mechanism may be a contributory factor for altering CD62P concentration; however, the lower correlation between non-specific binding and P-selectin suggests that alteration of the amount of P-selectin in the membrane may also be a process related to V_m[9].

Implications for platelet storage. One notable aspect of the work is that platelets stored for 24 hours at all three temperatures had different properties to the control cells at time zero hours. All exhibited a reduction in σ_{cyto} (by ~15% for 4°C and 22°C, ~25% for those at 37°C), and similar losses in membrane potential (reduced by over 35% for 4°C and 22°C, and nearly 50% for 37°C). As described above, surface chemistry appeared unaffected for cells stored at 22°C and 37°C, but changes in G_{eff} and ζ appeared to suggest a change in surface charge for those stored at 4°C. Interestingly, when we examined P-selectin surface exposure after activation with collagen-related peptide (CRP), we found very similar levels in control cells (13.8 ±3.1 kRFU) and those stored at both 22°C (13.4 ±3.0 kRFU) and 4°C (12.8 ±6.1 kRFU), but substantially lower levels (7.4 ±3.9 kRFU) for those stored at 37°C, suggesting reduced ability to activate. This may suggest that membrane potential, or cytoplasm ionic content, may regulate the activation process. This raises interesting questions about platelet storage, and the response of platelets to different storage temperatures. Notably, none of the three storage temperatures produces cells resembling the same cells prior to storage. It is known that 22°C offers the optimum platelet storage temperature[13,23]. We have already discussed the changes observed in 4°C storage, but it is also notable that those stored at 37°C exhibited the lowest V_m after 24h; since platelets spend their functional life at or near 37°C one might expect cells stored at this temperature to most closely resemble control cells, but this is not the case. Of the three storage temperatures, this was the only one to exhibit significantly lower platelet activation when treated with CRP, potentially suggesting that higher temperatures are required to maintain cells in a quiescent state; alternatively it may suggest that other factors found in normal physiology not reproduced in our storage system may be required in order to maintain the same response. Notably, within the healthy circulation platelets are constantly exposed to endothelium-derived factors such as nitric oxide and prostacyclin which serve to suppress platelet activation, factors that are rarely included in studies of platelet function *ex vivo.* The storage media used in this study did not include an energy source and platelet consumption of glycogen reserves may also be affected by temperature. Understanding these relationships may shed further light on the response of platelets to temperature and allow more optimised platelet storage.

Wider biological aspects. In our previous work[12] we speculated that if V_m were to influence ζ, it would alter extracellular ion balances at the cell surface, potentially conferring a similar function to voltage-gated ion channels on conventional ion transporters by altering ion availability. We can speculate similarly on the results presented here.

If we consider the relationship between both specific and non-specific binding events with V_m and σ_{cyto}, we notice strong relationships between both binding types and intracellular ion concentration. Since we have established across two cell types that V_m and ζ are related where membrane capacitance is constant, it stands from equation (1) that V_m should also vary ion concentrations outside the cell – and as antibodies are cationic, it would also alter the antibody availability for surface binding; whilst antibodies have a high binding affinity, it does require both molecules to come into contact, and it is this process which may be altered electrostatically. This may has significance across biology where surface markers are labelled for flow cytometric study, since changes reported by such methods be due to a reduction in target binding rather than a reduction in target molecule concentration. It may even offer insights into other cases where cells mechanistically alter Vm for unexplained benefit, such as in cancer metastasis[5] and drug resistance[24]. Similar effects have been observed in the literature, such as V_m dependence on the extent of Annexin V and lactadherin binding in apoptotic cells[25], and V_m dependence on the amount of ion uptake in plants[26].

More widely, we can also consider the effect of platelet-platelet interaction. It has been reported in the literature that platelet aggregation is related to membrane potential[9], though no mechanism bas been presented for this. However, if V_m alters ζ, which governs how colloids of like charge repel one another at close range, then we would expect aggregation to be affected by V_m.

It may also be the case that temperature modulation of platelet response may be responsible for unusual platelet behaviours observed in other mammals. For example, it is known that the platelets of hibernating mammals remain quiescent even at low temperatures[27], whilst camel platelets have been observed to maintain functional in blood temperatures of 50°C[28], given that temperature is observed to affect the electrical properties of cell, it may be that changes in the way in which this occurs may be responsible for the different physiological responses in these animals.

CONCLUSION

In our previous work we demonstrated that red blood cells exhibit mechanistic connections between multiple bioelectric phenomena, including the membrane potential, ζ-potential and passive electrical properties. Here we show that similar behaviour can also be observed platelets, specialised cells which are known to alter properties with storage temperature. We demonstrate that the electrome model does indeed translate to these cells, but also that the effect of this is that V_m has a demonstrable modulating effect on cell-antibody binding and may also have an effect on cell-cell interaction (of particular note in platelet function). This is a significant result for the bioelectricity

community, demonstrating an extracellular electric field effect arising from V_m with implication across multiple cell types and biological functions.

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TABLES

Table 1. Mean parameters (*n*=4) for cells analysed immediately after donation ("control") plus cells stored for 24h at either 4°C, 22°C or 37°C. SEM is given for non-DEP parameters; σ_{cyto}, C_{eff} and G_{eff} are the best-fit parameters to averaged DEP spectra.

	σcyto (S/m)	Ceff (F/m^2)	Geff (S/m^2)	ζ (mV)	P-Selectin (RFU)	Non-specific binding (RFU)	P-selectin on CRP activation (kRFU)	DIOC5 (kRFU)
Control	0.25	0.0183	0.00012	-8.69 ± 0.3	129 ± 14	11.3 ± 3.3	13.8 ±3.1	27.1 ± 6.0
4°C	0.215	0.0202	0.00025	-4.63 ± 1.6	1693 ± 692	11 ± 1.8	12.8 ±6.1	17.6 ± 3.2
22°C	0.215	0.0177	0.00013	-8.62 ± 0.2	588 ± 353	12.3 ± 2.3	13.4 ±3.0	16.9 ± 2.5
37°C	0.1875	0.0177	0.00017	-7.01 ± 0.4	706 ± 115	14 ± 0.7	7.4 ±3.9	14.4 ± 2.4

Table 2. Maximum correlation coefficients (r^2) values for linear fits to pairwise combinations of mean (*n*=4) parameters from Table 1. Correlations were made for combinations of parameters from all four conditions (cells analysed immediately after donation, and cells stored for 24h at either 4°C, 22°C or 37°C) as well as correlations with the 4°C data excluded. Strong correlations ($r^2 > 0.9$) are highlighted in bold, weaker correlations ($0.6 < r^2 < 0.9$) in pale.

Pair	Including 4°C	Excluding 4°C
P-selectin vs ζ	0.90	0.47
P-Selectin vs. G_{eff}	0.99	0.62
P-Selectin vs. σ_{cyto}	0.17	0.93
P-Selectin vs. V_m	0.28	1
ζ vs. V_m	0.18	0.46
ζ vs. G_{eff}	0.99	0.98
ζ vs. σ_{cyto}	0.15	0.72
V_m vs. G_{eff}	0.62	1
V_m vs. σ_{cyto}	0.92	0.92
G_{eff} vs. σ_{cyto}	0.15	0.85

FIGURES

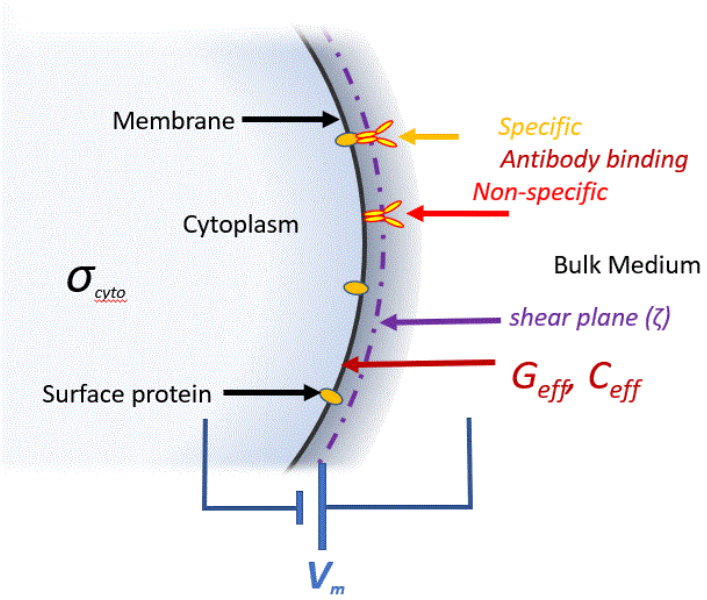

Figure 1. A schematic of the cell, showing the different parameters investigated in this study.

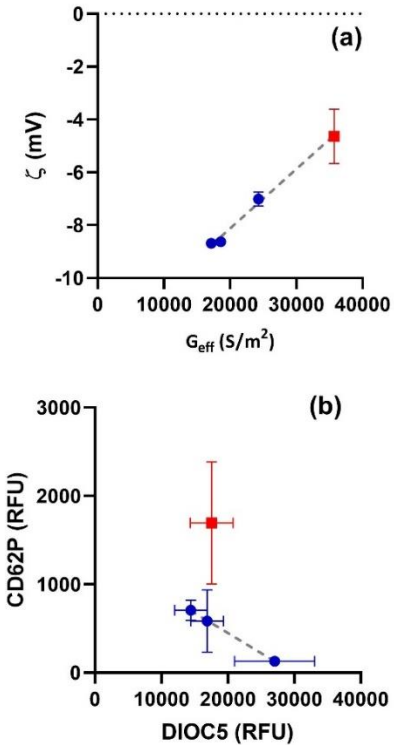

Figure 2. Examples of pairwise linear correlation of measured parameters, with the 4°C point indicated by a red square and the control, 22°C and 37°C points shown with blue circles. (a) ζ and G_{eff} demonstrate a linear correlation (r^2=0.99) with all four data points; however (b) V_m vs P-selectin shows a linear correlation (r^2=1) when the 4°C sample is excluded.

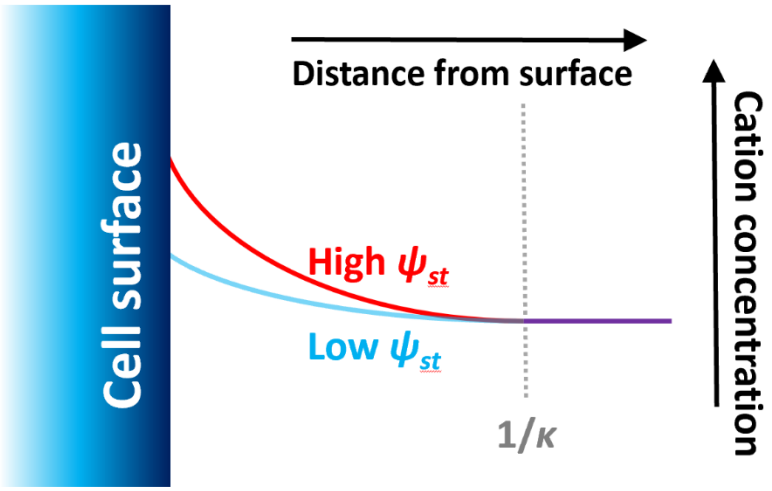

Figure 3. Schematic showing cation concentration inside the electrical double layer. The double layer is considered to begin a distance 1/κ (the Debye length, typically a few nm) from the surface; beyond this is the bulk medium. As the negatively-charged cell membrane is approached, the cation concentration will increase (the anion concentration, now shown here, will decrease). The extent to which this occurs depends on the surface potential ψ_{st}; a more polarized surface will attract more counterions. Since CD62P is cationic, it would be expected to follow this behaviour.