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Comparative electrokinetic properties of extracellular vesicles produced by yeast and bacteria

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ABSTRACT

Extracellular vesicles (EVs) are nano-sized, biocolloidal proteoliposomes that have been shown to be produced by all cell types studied to date and are ubiquitous in the environment. Extensive literature on colloidal particles has demonstrated the implications of surface chemistry on transport behavior. Hence, one may anticipate that physicochemical properties of EVs, particularly surface charge-associated properties, may influence EV transport and specificity of interactions with surfaces. Here we compare the surface chemistry of EVs as expressed by zeta potential (calculated from electrophoretic mobility measurements). The zeta potentials of EVs produced by *Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae* were largely unaffected by changes in ionic strength and electrolyte type, but were affected by changes in pH. The addition of humic acid altered the calculated zeta potential of the EVs, especially for those from *S. cerevisiae*. Differences in zeta potential were compared between EVs and their respective parent cell with no consistent trend emerging; however, significant differences were discovered between the different cell types and their EVs. These findings imply that, while EV surface charge (as estimated from zeta potential) is relatively insensitive to the evaluated environmental conditions, EVs from different organisms can differ regarding which conditions will cause colloidal instability.

1. Introduction

Through chemical and biological signals, microorganisms communicate with one another, resulting in a complex web of intercellular and interkingdom relationships [1–3]. One mode of such communication is mediated by extracellular vesicles (EVs). EVs are nano-sized membrane-bounded particles (20–500 nm), produced and emitted by cells into their surrounding environments [4–6]. Interest is growing in both medical and geological-biological sciences to understand the roles that EVs from microbes may play in intercellular and interkingdom communication in environmental systems [1,3].

The distribution and roles of EVs in the environment are extensive. EVs are produced by cells across all domains of life and have been found in many environmental compartments, including soil, seawater, and river water [3]. Moreover, EVs have been found in various biochemically harsh environments (e.g. extreme temperatures, high salt), confirming their ability to persist in stressful conditions [7–12]. The functionalities of EVs include shuttling electrons between cells, protecting extracellular DNA from degradation, promoting plant immunity, and advancing microbial pathogenicity [7,13–17]. Despite their ubiquitous presence in the environment, even in harsh conditions [7,9], and range of environmental functions [3,5,18], current understanding of the

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mechanisms by which EVs are transported between organisms is minimal.

Any functionality of EVs in the environment depends first on their transport, transformation, and resilience. Although strides have been taken to explore microbial EVs and their roles in interkingdom communication, few studies have examined the mechanisms by which EVs are transported throughout various environmental systems or the interactions with surfaces they encounter. Many previous EV studies focused on specific binding sites or isolated surface elements, as opposed to evaluating the systems of integrated surface phenomena arising from EV surface chemistry that may affect their attachment to environmental and physiological surfaces [19–21]. In fact, even a single organism can produce several subpopulations of vesicles with varied surface properties and likely differing functions [4,5]. Studying interactions in the context of a single receptor/ligand pair limits our ability to understand what these heterogeneous nanoparticles are doing as a population in the environment. Therefore, studying EV properties in the context of their overall properties as a population may provide insight into their general role in the environment, especially when considering the added complexities of broad ecological systems. For example, aquatic ecosystems (e.g., surface water, groundwater, agricultural runoff) vary greatly in their respective environmental conditions, which contribute to the complexity of evaluating EV transport. Parameters like ionic strength, ionic composition, and suspended organic matter affect colloidal surface chemistry and thus must be considered when studying EVs as colloids.

Methods developed in colloid chemistry, and more recently in the field of environmental nanotechnology for engineered, and incidentally- and naturally- occurring abiotic nanoparticles, have helped to describe the chemistry and physics governing how very small particles move through and interact with each other and their surroundings. The insights and methods from these fields hold promise for improving our understanding on the environmental behavior of cellularly-produced EVs [22–26]. Both physical (e.g., size and shape) and chemical (e.g., electrostatic attraction and repulsion) properties of colloids influence their ability to remain suspended in a fluid without aggregating or depositing on a surface. Far field transport of particles can be approximated primarily based on physical factors, such as particle size and density, and the characteristics of fluid flow. However, when particles approach a surface (including another mobile particle surface) their attachment behavior is increasingly influenced by chemical factors, such as those arising from particle surface charge and steric interactions between adsorbed moieties. Surface property characterization of colloids such as EVs, therefore, provides information on the potential for particles to aggregate or deposit in environmental and physiological systems. A particle's surrounding environment influences its surface properties, however, and thus its interactions with other surfaces. The zeta potential of a particle, typically calculated from electrophoretic mobility measurements in the context of a system's electrokinetic properties, is often used as a proxy for particle surface charge [27]. Zeta potential has been characterized for numerous colloidal systems in a variety of environmental conditions, including pH, different ions, and organic matter [28–30]; for EVs, zeta potential is often reported, but has rarely been interrogated as a function of similar conditions. Similar studies have been completed for human choriocarcinoma cell-derived (JAr) EVs [31] and for *Escherichia coli* EVs [32] which demonstrate that EVs are sensitive to changes in pH, ionic strength, and ionic composition. However, these reports did not compare between EVs from different cell types or between EVs and their respective parent cells. By teasing out relationships between environmental conditions and surface charge, predictions can then be made as to the potential transport patterns of EVs, as shown in several existing reports [33–36].

In this paper, we explore the electrokinetic properties of EVs to establish a foundation for EV colloidal research in environmental systems. Specifically, we investigate variations in changes in surface charge as a function of pH, ionic strength, ionic composition, and the presence of organic matter for EVs from three different microorganisms: a yeast (*Saccharomyces cerevisiae*), a Gram-negative bacterium (*Pseudomonas fluorescens*), and a Gram-positive bacterium (*Staphylococcus aureus)*. We selected these organisms due to our prior familiarity with them, as well as their common presence in the environment and the wealth of existing literature on these organisms' EVs [1,37–39]. We further evaluate size measurement data from dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) for EV samples from each of the three investigated parent cell microorganisms. Collectively, the previously described measurements allow us to probe physical (e.g., size) and chemical (e.g., zeta potential) differences in the EV populations secreted by the three organisms. Additionally, the electrokinetic properties of EVs are compared with those of their respective parent cells with the expectation that these might be similar. Ultimately, the purpose of this study is to determine an initial range of surface charges for colloidal vesicles from different organisms and under differing environmental conditions. From the experimentally determined zeta potential trends, we then examined previous research into the specific surface elements on EVs to connect composition with surface charge. Beyond this paper, knowing the extent of surface charges will allow for initial predictions of the surface interactions of EVs in the natural environment. For example, existing frameworks from the field of colloid chemistry to forecast the likely interactions between a particle and its surroundings can be blended with current research on specific surface moieties to identify the factors that most significantly impact fate.

2. Materials and methods

2.1. Organisms and growth conditions

Saccharomyces cerevisiae strain YEF473 (a gift from D. Lew, Duke University, Durham, NC) was grown in 3 L of liquid yeast nitrogen base (YNB) media (Sigma Aldrich; St. Louis, MO) supplemented with 0.79 g/L complete synthetic media (Sunrise Science Products; San Diego, CA) and 20 g/L dextrose at 30 °C overnight, with shaking to stationary phase. *Pseudomonas fluorescens* Migula ATCC 13525 was grown in 500 mL of liquid King's Broth (KB) media (2% proteose peptone, 8.6 mM K_2HPO_4, 1.4% glycerol, 6 mM $MgSO_4$) at 30 °C overnight, with shaking to stationary phase. *Staphylococcus aureus* strain Newman was grown in 3 L of Nutrient Broth (NB) media (0.5% proteose peptone, 0.3% beef extract) at 37 °C overnight with shaking, to stationary phase. Sample growth curves for all organisms are shown in Fig. S1. Unless specified, chemicals and reagents were purchased from Sigma Aldrich; St Louis, MO.

2.2. Vesicle isolation

To remove cells, cultures were centrifuged at 10,000 x g (Eppendorf 5804 R, Rotor: Rotor FA-45–6–30) for 30 min at 4 °C. The supernatants from the cell cultures were passed through a 0.45 μm polyvinylidene fluoride (PVDF) filter (Millipore Sigma, P: HVLP02500) using a vacuum filtration unit to completely remove cells. Vesicles in the cell-free supernatants were then concentrated using a cross-flow filtration chamber and method, similar to that described in [40]. The supernatants were filter-concentrated using a 750 kDa molecular weight cutoff polysulfone membrane (GE, P: UFP-750-E-4×2MA) to approximately 30 mL at a concentration of 10^{10} particles/mL (initial culture volume varied per strain to ensure this final vesicle concentration). The concentrated EV solution was washed six times with 30 mL of diluted PBS, as described in McNamara et al. (2018) [40]. PBS buffer was diluted threefold with nanopure water to ensure a low initial ionic strength for testing. EVs were stored at 4 °C and processed within two weeks.

2.3. Cell isolation

To isolate cells, a 1 mL aliquot of each culture was centrifuged at 10,000 x g (Eppendorf 5804 R, Rotor: Rotor FA-45–6–30) for 30 min at 4 °C. The cell pellet was washed three times with 1 mL 1X phosphate

buffered saline (PBS) (CMF-DPBS, Gibco P: 10010023) and resuspended in 1 mL 1X PBS until processing.

2.4. Analytical methods

Size measurements were performed by conventional dynamic light scattering, DLS, (Malvern ZetaSizer ZS, Malvern; UK) and by Nanoparticle Tracking Analysis (NTA) using a ZetaView Instrument (Particle Metrix; Germany). For size-distribution profiles and concentrations of EVs obtained using NTA, the analyzer was standardized, both for size and zeta potential, using manufacturer-supplied 102 nm polystyrene beads diluted at 1:250,000 in nanopure water to achieve 100–250 particles per field of view. Three technical replicates, each with 11 fields of view, were recorded for calibration. A typical observed mean size was 108 nm, and the mode size was 105 nm. Calibrations were only accepted if the mean and mode sizes were within 10% of the reported values (in this case, 102 nm). After successful calibration, the stage was washed with 10 mL of nanopure water delivered by a plastic syringe, and washes were repeated until fewer than 5 particles per field of view were observed. Preparations of EVs were made in serial 10-fold dilutions. Solutions were injected via a plastic syringe into the ZetaView stage. Solutions were analyzed that contained approximately 100–250 particles per field of view (linear range of detection). For each biological sample, 3 technical replicates consisting of 11 independent fields of view were analyzed for size. For each experiment, at least 3 independently derived EV preparations were analyzed.

For all analytical measurements, EVs were diluted with nanopure water to a concentration of approximately 10^{10} particles/mL at a final concentration of approximately 10 mM total ionic strength as added by PBS. Similarly, cells were diluted to 10 mM total ionic strength as added by PBS to match the ionic strength of the EV samples. The starting pH value varied between 6 and 8, depending on initial solution composition, and was adjusted using 0.1 M HCl or 0.1 M NaOH.

For the indicated experiments, solutions of 11.25 mM sodium chloride (NaCl) or 1.125 mM magnesium chloride ($MgCl_2$) were used to dilute the vesicle suspension to final ionic strengths of 20 mM NaCl and 11 mM $MgCl_2$. To adjust the organic content of the EV suspension, Pahokee Peat standard humic acid (HA) (International Humic Substance Society [IHSS], cat. #1S103H) was dissolved in water and added to a final concentration of 10 mg/L humic substances. The lowest pH attainable for experiments was about pH 4 due to chemical precipitation and significant alterations to the ionic strength of the total solution. For both size and zeta potential experiments, EV-free negative controls with the appropriate media conditions were analyzed as well; however, the measurements were not above the detection limits for any instrument and thus are not included here.

Zeta potential values were calculated using the Henry Equation [41] from electrophoretic mobility measurements in triplicate, which were obtained using 1 mL aliquots in disposable folded capillary cells (Malvern; P: DTS1070) in the Malvern ZetaSizer ZS (Malvern; UK) at room temperature. The sample settings for refractive index (1.330) and absorption (0.060) for liposomes were used.

2.5. Statistical comparison of zeta potential trends

The effect of changes in pH, ionic strength/composition, and the presence of organic content in EV suspensions on the zeta potential of vesicles from different organisms, was evaluated with two-way analysis of variance (ANOVA), followed by pairwise testing with Tukey's HSD for the effects founds to be significant. Prior to running ANOVA, data were examined if they met normality assumptions via Shapiro-Wilks test and a qqplot was visually examined. All statistical analyses were performed in the coding platform R using the stats package [42].

2.6. Imaging

EVs obtained were prepared for imaging by deposition of 5 μL of each sample on formvar coated grids (200 mesh; 100491–092; VWR) for 2.5 min at room temperature, followed by staining with 1% uranyl acetate. All imaging was performed by transmission electron microscopy (TEM) using an FEI Tecnai G^2 Twin microscope (Thermo Fischer Scientific; US). Images were then processed using the NIH's Image J software to determine the sizes of the captured EVs. Images can be seen in the supplementary information (Fig. S2).

3. Results and discussion

To our knowledge, no studies exist containing a side-by-side, in-depth comparison of the surface properties of EVs from different microorganisms. We selected *P. fluorescens* and *S. aureus* and their respective media conditions due to the extent of existing research on their EVs and to evaluate a range of sizes and surface chemistries among bacterial EVs [1,37,43,44]. *S. cerevisiae* was selected to compare surface properties of EVs from different kingdoms. Because the literature exploring yeast EVs is much less extensive, with more variety in media conditions [43,45,46], the yeast media was selected based on prior familiarity with this growth medium.

3.1. Size evaluation of EVs

The size of colloidal particles has direct implications for their transport capabilities [47–49]. Different sizes of suspended particles result in varied transport mechanisms. Conversely, if only evaluated with respect to size, we would expect particles to be transported similarly if the average size of the suspended particles in the suspension is similar. For this report, obtaining size measurements for EVs served two purposes: to confirm the presence of EVs and to compare the size distributions of the suspensions of EVs from the three different organisms. DLS and NTA methods were used to evaluate the size distributions of the EV suspensions (Table 1). The sizes of the EVs measured using NTA are reported as normalized particle size distributions (Fig. 1).

The DLS and NTA measurements indicated that the EV populations from all three organisms were similar in size. This was consistent with the purification procedure since larger vesicles were filtered out up to the 0.45 μm cutoff, and small vesicles may have been removed up to the pore size of the tangential flow filter (750 kDa). We note that these methods each have limitations that influence the measurement of nano-sized particles. Conventional DLS uses an ensemble measurement of light scattering which must be deconvoluted to arrive at a distribution; large particles interfere with this conversion [50]. This results in a bias towards larger particles in the measured size distribution. Moreover, the light scattering intensity, which is reported here, scales with particle size via a power function, contributing to the bias toward larger particles [50]. On the other hand, NTA detects the Brownian motion on a particle-by-particle basis, which typically makes larger particles harder to track and introduces a bias toward smaller EVs [51]. Hence, the actual size of these EVs is likely somewhere in between the values reported here for all the EVs. Previously reported measurements of EVs from the same organisms (*S. cerevisiae, S. aureus, P. fluorescens*) fall in the range of the sizes reported in this study (Supplementary Table S1). Our evaluation of EVs using different downstream analytical sizing methods suggests that both NTA and DLS provide similar results. In addition, we note that upstream EV isolation and purification methods appear to impact EV measurements [40]. The absence of a universally accepted isolation method makes comparisons between different studies very challenging, illustrated for size measurements here (Table S1) but likely also pertaining to other downstream analyses that might change as a function of isolation protocols.

The observed similarity of sizes for EVs from yeast and bacteria has significant implications for their transport mechanisms and, ultimately,

Table 1
Size measurements for EVs in 10 mM PBS.

Method	Measurement[a]	Organism		
		P. fluorescens	*S. aureus*	*S. cerevisiae*
DLS (Zetasizer)	**Z-Average Diameter**	109.61 ± 11.71	118.96 ± 31.53	315.21 ± 166.88
NTA	**Average Diameter**	135.4 ± 52.8	150.6 ± 69.5	127.8 ± 54.16

[a] All measurements reported in nm. Error is standard deviation for three measurements for DLS and NTA.

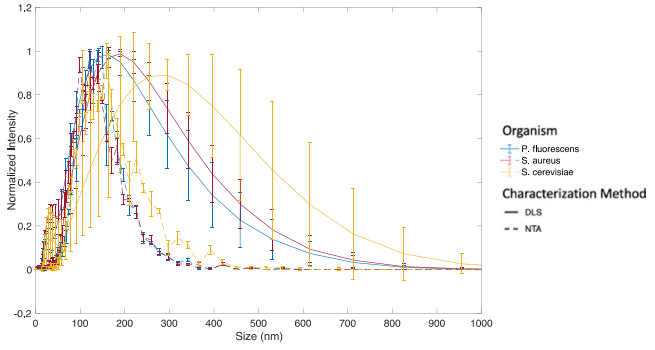

Fig. 1. Normalized particle size distributions for *P. fluorescens*, *S. aureus*, and *S. cerevisiae* EVs. EVs were evaluated using nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS) in biological triplicate. The samples are normalized to the total particle counts for the most populous size bin for each respective sample.

for field sampling in the environment. For similarly sized suspensions of EVs, the expected fate in the extracellular environment should be identical. Hence, if differences in transport are observed, other factors beyond size must be influencing EVs as colloids, such as surface charge or steric considerations. In addition, this similarity in size has implications for challenges in separating EVs in mixed cultures for future work with environmental samples. The results of this study suggest that EVs from different organisms would have similar sizes, which would make isolating a target organism's EVs difficult.

3.2. Zeta potential

Zeta potential is reported as a proxy for surface charge of colloidal particles. By evaluating how zeta potential changes as a function of environmental parameters, we not only probe the stability of colloidal particles (that is, the likelihood of particles to stay suspended in their liquid media as opposed to aggregation, deposition, or settling), but also how this stability depends on the environment of the particles. Conditions particularly relevant to microbial environments in which EVs are found include pH, ionic strength, ionic composition, and the presence of

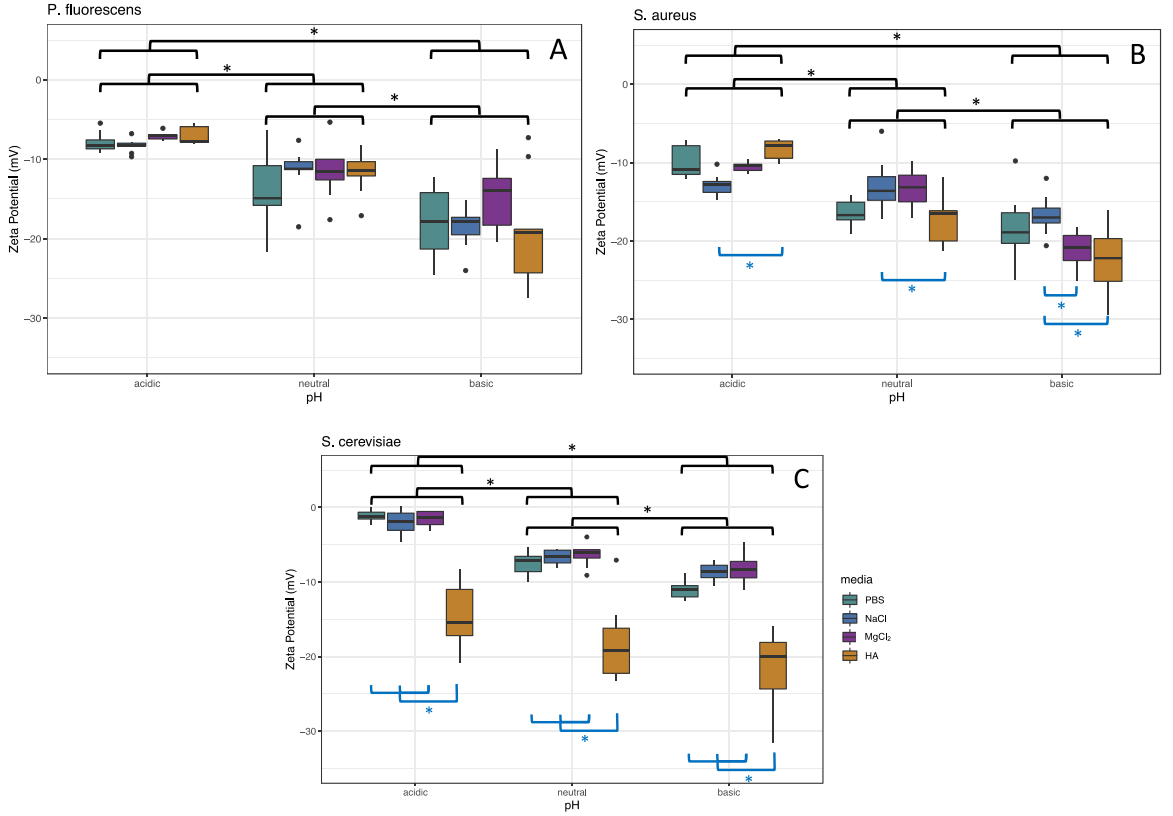

Fig. 2. Zeta potential (in mV) as a function of pH for each environmental condition for A) EVs from *Pseudomonas fluorescens*, B) EVs from *Staphylococcus aureus,* and C) EVs from *Saccharomyces cerevisiae*. The data represent nine measurements (three measurements each of three biological replicates). p-values < 0.05 are marked with an asterisk (*) indicating significant difference. "PBS" is 10 mM PBS; "NaCl" is 10 mM PBS supplemented with 10 mM additional NaCl; "$MgCl_2$" is 10 mM PBS supplemented with 1 mM $MgCl_2$; "HA" is 10 mM PBS supplemented with 10 mg/L HA. The acidic condition is pH = 4 ± 0.5; the neutral condition is pH = 7 ± 0.5; the basic conditions is pH = 10 ± 0.5. Additional points are outlier points. Black brackets denote statistically significant differences as a function of pH while blue brackets denote statistically significant differences as a function of environmental conditions.

HA. The specific testing conditions for the reported experiments were chosen to represent conditions typically found in the environment, similar to many other previous studies for engineered nanomaterials [33–35,52,53].

To compare the effects of environment on the stability of different EVs, we calculated zeta potential values from electrophoretic mobility measurements for each of the three parent cell organisms as a function of pH under four different environmental conditions (Fig. 2). The pHs were divided into acidic (pH = 4 ± 0.5), neutral (pH = 7 ± 0.5), and basic (pH = 10 ± 0.5) categories.

According to ANOVA, for *P. fluorescens*, changes in pH resulted in significant differences in zeta potential but not changes in other environmental conditions or the interaction between other environmental conditions and pH (Fig. 2 A). For *S. aureus*, ANOVA again indicated that pH was significant as well as the interaction between pH and environment, while the main effect of environment was not (Fig. 2B). Tukey's HSD showed that EVs in 10 mM PBS supplemented with 10 mM additional NaCl and EVs in 10 mM PBS supplemented with 10 mg/L humic acid were significantly different at acidic, neutral, and basic pHs. Additionally, EVs in 10 mM PBS supplemented with 10 mM additional NaCl and EVs in 10 mM PBS supplemented with 1 mM additional $MgCl_2$ were significantly different at basic pH. ANOVA indicated that both environment and pH had a significant effect on the zeta potential for *S. cerevisiae* EVs; however, the interaction between environment and pH was not significant (Fig. 2C). Tukey's HSD showed that the presence of HA significantly ($p < 0.05$) lowered the zeta potential compared to all other environment types.

Comparing the effect of different environmental conditions on the three different types of EVs, some trends emerge. Predictably, pH is inversely proportional to zeta potential for all three organisms (Fig. 2). While pH did alter their zeta potential, *P. fluorescens* EVs were the least affected by other differences in solution since none of the conditions caused a significant change in EV zeta potential. Similarly, EVs from *S. cerevisiae* were unaffected by all environmental conditions but the presence of HA.

The zeta potential for all EVs was negative throughout the pH range of the experiments. The source of this charge likely depends on the organism producing the EVs. For example, the surface of EVs from *P. fluorescens* is dominated by the essential, external leaflet lipid, lipopolysaccharide (LPS), which is negatively charged [44,54,55]. This compositional element is unique to Gram-negative organisms, and thus is not present in EVs from either *S. aureus* or *S. cerevisiae*. The exterior of Gram-positive bacteria such as *S. aureus* consists of a thick peptidoglycan wall with attached wall teichoic acids and membrane-anchored lipoteichoic acids on their surfaces, which are all negatively charged [56,57]. Lipoteichoic acids on the surface of *S. auerus* EVs [58] may be contributing to their observed negative surface charge. Wall teichoic acids may also contribute, but to-date they have not been shown to be present on EV surfaces.

S. cerevisiae EVs have a distinct composition from bacterial EVs. For yeast, evidence exists for yeast EVs being produced through at least two mechanisms: one from bulging out from the plasma membrane and the other from the fusion to the plasma membrane of a specialized intracellular compartment, the multivesicular body (MVB), and the subsequent release of the MVB-contained vesicles [59,60]. These different origins make characterizing the composition of yeast EVs challenging because yeast EVs could be a combination of MVB membrane and plasma membrane sources. One hypothesis regarding the negative charge for yeast EVs is the surface exposure of the various phospholipids known to be present in EVs [61–63]. The majority of pKa's (i.e., acid dissociation constants) of these phospholipids are in the acidic pH range [64,65], implying that for the experimental conditions here, phospholipids would impart at least a slightly negative charge across the evaluated pH range. However, some phospholipids are zwitterionic, which likely diminishes the capacity to impart strong negative charge to the EVs. These highlighted compositional differences may explain the trends we see in the zeta potential vs pH curves.

While embedded proteins, surface-associated proteins, or other adsorbed small molecules and nucleic acids could also contribute to the overall charge of the EV membrane, connecting trends in surface charge with surface composition is much more difficult due to the wide variety of possible molecules present, the difficulty of distinguishing which molecules are surface exposed, and the heterogeneous composition of the EVs. Especially for this study, where different media conditions were used for the different cell types, we acknowledge the possibility of the presence of impurities despite the multiple washing steps of the EVs.

Beyond differences due to pH, in the presence of HA, *S. cerevisiae* vesicles had a significantly more negative zeta potential relative to the same EVs in diluted PBS. In phosphate buffer, the *S. cerevisiae* EVs have a less negative surface charge, which results in less electrostatic repulsions between vesicles and other negatively charged particles like HA. This could lead to increased association of HA with the vesicles. Even if these organic molecules were not chemically attracted to the EVs, HA would not be repelled as strongly and thus, by nature of their tendency to adsorb to colloidal particles [35,66], could entangle the vesicles. Hence, the steric and bridging effects of HA overcome the weaker electrostatic forces of the yeast EVs to allow the HA and the EVs to bind, inducing a collective surface charge more like those of HA.

In addition to differences between EVs from different microbes, each parent cell may exhibit different surface properties from their respective EV products. From examining this relationship, we can relate different surface properties to differences in function, especially relating to hypothetical transport and colloidal stability. In particular, zeta potential trends provide information relating surface charge of colloidal particles. When comparing the zeta potentials of the cells and EVs for each species, distinct trends emerged (Fig. 3). *P. fluorescens* produces EVs that are more negatively charged than their parent cells (Fig. 3A), while *S. aureus* produces EVs that are less negatively charged than their parent cells (Fig. 3B). While it would be expected that the EVs from bacteria would be similar in charge to that of their parent cells, as these are produced from their outermost membrane [2], both of these EVs were significantly different in charge compared with their respective parent cell (Fig. 3A and B). For Gram-negative bacteria, the data indicate that some negatively-charged component (e.g. lipid and/or protein) is being overrepresented in vesicles relative to their parent cells. While we know that different subtypes of LPS can be enriched in EVs [67–69] and hence could be responsible for the different overall charges, differences in protein could also be responsible [70,71]. Functionally, it can be hypothesized that more negatively charged surfaces would induce greater electrostatic stability, allowing EVs to travel further distances compared to their less negatively charged parent cells due to increased electrostatic repulsion.

Intriguingly, the opposite trend is true when comparing *S. aureus* cells and their derived EVs (Fig. 3B). In this case, the cells are much more negatively charged than their respective vesicles. Furthermore, the magnitude of the difference between the charge of the cells compared with the EVs for *S. aureus* is greater than that of *P. fluorescens*. This trend may be attributed to the surface exposure of peptidoglycan on the surface of Gram-positive bacterial cells, which is more negatively charged than LPS [57]. Nevertheless, it is interesting to note that the vesicles from these two distinct bacterial types exhibit similar surface charge trends across the studied pH range for all environmental conditions, implying that bacterial EVs would be transported similarly in porous media despite originating from very distinct cell envelopes.

By contrast and unexpectedly, the surface charge was not significantly different for *S. cerevisiae* cells and the EVs it produces (Fig. 3C). As *S. cerevisiae* EVs originate at least in part from intracellular membranes through MVBs [72–74], compared with bacterial EVs, which are produced from the shedding of their outermost membranes, we predicted that the difference in surface charge between parent cell and respective EV would be greatest for *S. cerevisiae*. Our results contradicted that expectation. One possible explanation for these results is that the

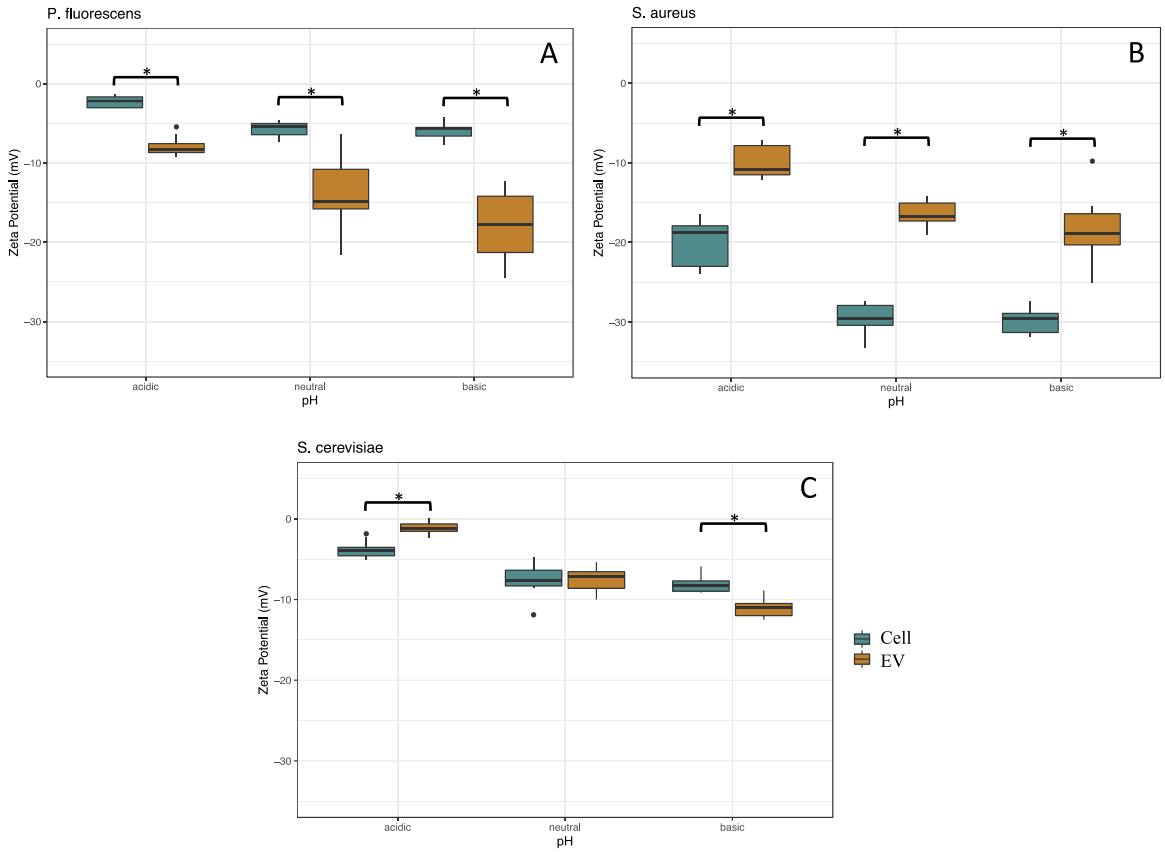

Fig. 3. Zeta potential (in mV) as a function of pH for parent cells and their corresponding EVs in 10 mM PBS. The data points represent nine replicate measurements (three measurements of three biological replicates). p-values < 0.05 are marked with an asterisk. "Cell" is the zeta potential of the whole cell; "vesicle" is the zeta potential of the corresponding EV. The acidic condition is pH $= 4 \pm 0.5$; the neutral condition is pH $= 7 \pm 0.5$; the basic conditions is pH $= 10 \pm 0.5$. Additional points are outlier points.

number of yeast EVs originating from the outer membrane dominate the number of those originating intracellularly. Another possibility is that the overall charge of the total heterogeneous vesicle suspension mirrors that of the cell surface charge, i.e., that the sum of the parts of the colloidal mixture has a net charge similar to that of the parent cells. By utilizing mutants that affect specific EV production routes, it will be possible to analyze the colloidal characteristics of sub-fractions of yeast EVs and test these theories.

4. Conclusions

Through this study, we sought to characterize the colloidal properties of EVs from three different microbial organisms regarding size and surface charge. We found many similarities between the three investigated EV populations, both regarding their size and their consistent robust surface charge with respect to changing environmental conditions. In sum, these results indicate that the stability of EVs is less likely to be affected by environmental changes in their surrounding media. If only considered with respect to electrostatic interactions, this stability would allow for longer range transport and delivery of vesicular content, compared with other colloidal particles that may be more likely to auto-aggregate or deposit in a natural environment.

This study also highlights the differences between surface characteristics of three species of microorganism parent cells and their corresponding vesicles. With *P. fluorescens*, cells can produce vesicles that are more electrostatically stable than the parent cell. Hence, in comparison to the parent cell, its EVs could be transported further distances if only considered under ideal electrostatic interactions (i.e., when not considering other forces such as steric interactions). In the other bacterial case, *S. aureus* EVs are less stable than the cells, suggesting they would interact with other surfaces more readily. This would likely result in greater EV aggregation or deposition, which would thus travel shorter distances than the parent cells. With regard to surface charge, *S. cerevisiae* EVs and their parent cells possess similar stabilities. This observation means that if the *S. cerevisiae* EVs are able to be transported longer distances compared to their parent cell, other factors must be stabilizing EVs, as in the presence of HA. Otherwise, we expect that *S. cerevisiae* EVs and cells would aggregate or deposit to a similar extent compared to other more negatively charged biocolloids.

This evaluation of physical-chemical properties provides a foundation for future research relating to EV transport in the environment and has implications for their stability in applications ranging from nano-agriculture to bioremediation. To generalize the findings beyond the three microbial EVs in this study, studies using a wide range of environmental conditions (e.g. ion composition, concentration, different organics, extreme pH) and organisms will be critical. Moreover, determining a quantitative metric of the physical transport capacity, such as attachment efficiency (α), would provide a complementary evaluation of EV electrostatic trends shown here to predict their range of fate outcomes. Current research has demonstrated the efficacy of methods in measuring an empirical α for a variety of environmental nanomaterials to predict their transport tendencies [35,75,76]. Finally, by either physically modifying surface elements or genetically altering the surface composition, the practical implications and applications of EVs could be exploited to engineer changes in the stability and transport of vesicles [77]. Either of these proposed future directions would validate or quantitate the qualitative predictions based on surface charge that were made in this report.

Synopsis

The surface chemistry of extracellular vesicles plays a potentially important role in mediating their environmental and physiological interactions. Our study shows species-specific and environmental condition-specific effects on surface charge of extracellular vesicles, and that the surface properties of cells and vesicles can be distinct.

CRediT authorship contribution statement

Nicholas M.K. Rogers: Methodology, Investigation, Writing – original draft. **Alexander W. McCumber:** Methodology, Formal analysis, Writing – review & editing. **Hannah M. McMillan:** Methodology, Writing – review & editing. **Meta J. Kuehn:** Conceptualization, Supervision, Writing – review & editing. **Ryan P. McNamara:** Methodology, Writing – review & editing. **Dirk P. Dittmer:** Resources, Writing – review & editing. **Christine Ogilvie Hendren:** Conceptualization, Supervision, Writing – review & editing. **Mark R. Wiesner:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2023.113249.

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