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1 **Lipopolysaccharide structure impacts the entry kinetics of bacterial outer**
2 **membrane vesicles into host cells**

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15

16 **Keywords:** outer membrane vesicles, lipopolysaccharide, serotype, FRET kinetics, extracellular
17 vesicles, cargo uptake, vesicle trafficking, endocytosis;

18

19 AUTHOR SUMMARY

20 All Gram negative species of bacteria, including those that cause significant disease, release
21 small vesicles from their cell membrane. These vesicles deliver toxins and other virulence factors
22 to host cells during infection. Current methods for studying host cell entry are limited due to the
23 nanometer size and rapid uptake kinetics of vesicles. Here we developed a method to monitor the
24 rapid vesicle entry into host cells in real-time. This method highlighted differences in kinetics
25 and entry route of vesicles into host cells, which varied with the bacterial cell wall composition
26 and thus, the vesicle surface. Increased understanding of vesicular entry mechanisms could
27 identify targets which may allow us to combat infections by inhibiting delivery of vesicle-
28 associated toxins to host cells.

29

30 **ABSTRACT**

31 Outer membrane vesicles are nano-sized microvesicles shed from the outer membrane of Gram-
32 negative bacteria and play important roles in immune priming and disease pathogenesis.
33 However, our current mechanistic understanding of vesicle - host cell interactions is limited by a
34 lack of methods to study the rapid kinetics of vesicle entry and cargo delivery to host cells. Here,
35 we describe a highly sensitive method to study the kinetics of vesicle entry into host cells in real-
36 time using a genetically encoded, vesicle-targeted probe. We found that the route of vesicular
37 uptake, and thus entry kinetics and efficiency, are shaped by bacterial cell wall composition. The
38 presence of lipopolysaccharide O antigen enables vesicles to bypass clathrin-mediated
39 endocytosis, which enhances both their entry rate and efficiency into host cells. Collectively, our
40 findings highlight the composition of the bacterial cell wall as a major determinant of secretion-
41 independent delivery of virulence factors during Gram-negative infections.

42

43 INTRODUCTION

44 Outer membrane vesicles (OMVs) are nano-sized proteoliposomes released from the bacterial
45 cell envelope [1]. OMV release is a highly conserved process, occurring in all growth phases and
46 environmental conditions [2]. OMVs contain and deliver a broad range of cargos, from large
47 hydrophobic molecules to DNA, making them a versatile and generalised form of secretion that
48 enhances bacterial fitness in hostile environments [3-6]. They also contribute significantly to
49 pathogenesis, via the delivery of virulence factors such as toxins, adhesins and
50 immunomodulatory compounds directly into the host cell [7-9]. In a mouse model, purified
51 OMVs from *Escherichia coli* were sufficient to cause lethal sepsis in the absence of intact
52 bacterial cells, indicating their potency in enhancing infection and inflammatory processes [10].
53 The immunogenicity and ubiquitous production of OMVs has also led to their clinical use in
54 vaccine preparations [11], representing an application for OMVs in generating immunity against
55 bacterial infections without the risks associated with live cell vaccines. Whilst many virulence
56 factors are known to be OMV cargos, the processes underlying their delivery to host cells during
57 infection are not well characterized. Understanding these mechanisms could help to identify
58 targets for inhibition of OMV-associated toxin delivery and lead to attenuation of bacterial
59 infections, as well as helping to achieve their therapeutic potential in medicine, via vaccines and
60 engineered delivery vehicles [12-14].

61 Release of OMVs occurs during infection, and has advantages over other secretion
62 systems. They can carry a broad range of cargos, from protein toxins to hydrophobic small
63 molecules such as the *Pseudomonas aeruginosa* quorum sensing molecule quinolone signal

64 (PQS), and vesicular cargos are protected from environmental insults [15, 16]. In addition,
65 OMV-mediated delivery of virulence factors can function over longer distances than contact-
66 dependent secretory pathways [17]. While much is known about the cargos contained within
67 OMVs, the small size of OMVs (20-200 nm) and rapid kinetics of entry (cargo-specific effects
68 can often be detected within minutes) have made studying their interactions with host cells
69 difficult. Previous work has often relied on OMVs labelled with dyes, non-discriminate probes
70 that modify vesicular contents during labeling. While such probes allow real-time analysis of
71 OMV entry and cargo delivery, their potential to modify vesicle components may interfere with
72 the vesicle's physicochemical characteristics, and alter the mechanism of OMV recognition,
73 entry and cargo release [18-20]. Other approaches rely on immunolabelling of OMV-associated
74 epitopes, but this often requires fixation of cells at pre-determined time points, and makes
75 assumptions about OMV cargo, which may ignore natural sub-populations of OMVs [21]. Some
76 experiments have used specific changes in host cell phenotypes in response to OMV contained
77 toxins as an indicator of OMV uptake [4]. However, such changes in host cell responses have
78 distinct dynamics from the OMV entry event, and allow only indirect conclusions about entry
79 kinetics [22]. These challenges have often lead to discrepancies in observations of OMV entry
80 and cargo delivery [14], demonstrating the need for an assay that can detect OMV entry
81 processes in a consistent and repeatable manner. In this paper we describe a novel assay to
82 continuously measure OMV entry and cargo release to host cells with high sensitivity, and in a
83 format that is adaptable for high throughput screening. Using this assay to study entry of OMVs
84 from different *E. coli* serotypes and pathovars into host cells, we identified key bacterial and host

85 factors that determine the route of entry, and thereby kinetics and efficiency of vesicular cargo
86 delivery and trafficking.

87 **RESULTS**

88 **A highly sensitive, kinetic assay for monitoring OMV entry into host cells.** We set out to
89 develop a highly sensitive and dynamic assay that would allow us to monitor the kinetics of
90 OMV entry into host cells. We used a genetically encoded hybrid reporter probe that is
91 incorporated into the bacterial outer membrane and subsequently targeted to the OMV surface.
92 ClyA, a cytolysin that is sorted into OMVs produced by pathogenic *E. coli*, acts as the targeting
93 component, and is fused to the TEM domain of β -lactamase (Bla), which acts as an
94 enzymatically active probe (Figure 1A), and prevents assembly of the toxin into its biologically
95 active oligomeric conformation [12]. Host cells were incubated with CCF2-AM, a dye composed
96 of a covalently linked coumarin and fluorescein molecule, resulting in FRET and green
97 fluorescence emission, specifically in the eukaryotic cytoplasm where it is processed by
98 esterases. Esterification decreases the hydrophobicity of the FRET probe, thus decreasing its
99 membrane permeability and trapping the probe in the host cell cytoplasm. When OMVs isolated
100 from the producing bacterial strain enter host cells, their Bla cargo is able to cleave CCF2-AM,
101 abolishing FRET and resulting in a shift in emission from green (530 nm) to blue (460 nm)
102 fluorescence (Figure 1A). We monitored the FRET kinetics upon incubation of OMVs with host
103 cells, and analyzed efficiency of OMV uptake by host cells ($[\text{Em}460/\text{Em}530]_{t=0\text{hrs}}/$
104 $[\text{Em}460/\text{Em}530]_{t=3\text{hrs}}$). We further analyzed data by fitting to a cubic spline function and
105 estimating gradients to extract maximal rate of entry (r_{max}) and rate over time (see SI Materials

106 and Methods). Experimental traces were limited to three hours, since beyond this time point the
107 FRET signal decayed, likely due to degradation of the substrate within the host cell cytoplasm.

108 **Figure 1. Genetically encoded Bla probes are enriched in *E. coli* OMVs and retain their enzymatic activity.**

109 (A) Expression of genetically encoded Bla probes is induced in bacteria and secreted OMVs are isolated for all
110 subsequent experiments. Entry of OMVs containing Bla probes into host cells can be detected using a continuous
111 FRET assay. (B) Whole cell lysate (WCL), supernatant (sup) and outer membrane vesicles (OMV) fractions isolated
112 from EHEC expressing ClyA-Bla, carrying empty vector, or no vector were separated by SDS-PAGE and
113 expression of ClyA-Bla was detected by Western Blotting and probing with α -Bla antibody. (C) Specific enzyme
114 activity in whole cell lysate, supernatant, OMV or solubilized OMV fractions isolated from EHEC expressing ClyA-
115 Bla, Bla-ClyA, or carrying empty vector (data shown are means \pm stdev, n=3).

116

117 **Genetically encoded Bla probes are targeted to *E. coli* OMVs and retain their enzymatic**

118 **activity.** First, we set out to verify whether ClyA-Bla fusion constructs retained ClyA's ability to
119 partition into vesicles, and were indeed targeted to *E. coli* OMVs. Following induction of probe
120 production, OMVs were isolated from enterohemorrhagic *E. coli* (EHEC) containing empty
121 vector, or expressing either ClyA-Bla (C-terminal fusion, enzyme exposed on the OMV surface)
122 or Bla-ClyA (N-terminal fusion, enzyme facing the OMV lumen) enzymatic probes. Probe
123 expression did not significantly change cross OMV morphology (Figure S1A) or charge (mean
124 ζ -potential -6.7 ± 3.6 mV, Figure S1C), but did cause a slight but significant increase in OMV
125 size distribution ($\sim 20\%$ increase in median diameter; Figure S1B). Probe expression did not
126 appear to result in cell envelope stress, as the amount of OMVs released per cell did not change
127 significantly compared to the untransformed strains (approximately 41 vs 39 vesicles/cell).
128 Sizing data (mean diameter 134 nm, range 10-400 nm across all OMV preparations) were in

129 accordance with previously published data for *E. coli* OMVs [12]. Intact ClyA-Bla fusion protein
130 was detected in samples from EHEC whole cell lysate, supernatant and OMV fractions (Figure
131 1B), suggesting that the fusion protein was targeted to and enriched in OMVs, as previously
132 reported for non-pathogenic *E. coli* [12]. The ClyA-Bla probe was oriented with Bla facing the
133 exterior of the OMV, as the protein was gradually degraded during treatment of ClyA-Bla OMVs
134 with papain protease, while the probe remained intact in OMVs containing Bla-ClyA, where Bla
135 faces the vesicle lumen (Figure S1D). The specific enzymatic activity was ~ 3-fold higher for
136 ClyA-Bla OMVs than for Bla-ClyA OMVs with similar activities in whole cell lysates, and both
137 activities were equalized by lysis of vesicles and probe solubilization, suggesting efficient
138 expression of active β -lactamase with the anticipated orientation (inward facing for Bla-ClyA,
139 outward facing for ClyA-Bla) in isolated OMVs (Figure 1C). Average OMV concentration was 5
140 $\times 10^{12}$ particles per ml, and particle concentrations of all samples were normalized to give a
141 consistent OMV concentration for subsequent experiments.

142

143 **OMV-targeted Bla probes report on rapid vesicle uptake and dismantling by host cells.**

144 Having verified the correct targeting, orientation and enzymatic activities of the Bla probes, we
145 used them to dissect OMV entry (i.e., exposure of ClyA-Bla to cytoplasmic dye) and release of
146 OMV luminal contents (i.e., exposure of Bla-ClyA to cytoplasmic dye) into epithelial cells. We
147 used both HeLa (cervical epithelial) and RKO (intestinal epithelial) cells loaded with CCF2-AM
148 dye and exposed to OMVs at an MOI of 1000 OMVs/cell. OMV yield was approximately $27 \pm$
149 13 OMVs/bacterial cell for the different pathovars used, so this corresponds to a bacterial MOI
150 of approximately 37 bacteria/cell, a dose commonly used in infection assays, or approximately

151 10 $\mu\text{g/ml}$ OMV protein (published assays use between 5-200 $\mu\text{g/ml}$ OMV protein). EHEC ClyA-
152 Bla OMVs caused a rapid increase in blue/green fluorescence over the course of a 3 hour
153 experiment. OMVs lacking probe did not cause a significant change in FRET signal. (Figure 2A-
154 C). While the rate of cargo release remains stable throughout the experiment (Figure S2B), the
155 rate of entry is initially high but gradually decreases and approaches the rate of cargo release
156 (Figure S2A). OMV entry kinetics are similar in intestinal epithelial (RKO) cells (Figure S3).
157 Results of these kinetic analyses were visually confirmed by capturing FRET of samples at the
158 onset and endpoint of the experiment (Figure 2D). The rapid kinetics inferred from the FRET
159 traces also correlated with rapid internalization and re-distribution of OMV lipid inside host
160 cells, with a significant portion of OMV material localized to an intracellular, tubular structure
161 surrounding the nucleus, likely the ER, even after 10 minutes, the fastest we could feasibly
162 prepare samples for imaging (Figure 2E). These results suggest that our approach is capable of
163 capturing the rapid internalization and dismantling of OMVs, which proceeds too fast to
164 adequately capture by imaging. As the rate limiting step for cargo release appears to be OMV
165 entry, we further focused on analyzing potential determinants of the entry process.

166

167 **Figure 2. Reporter OMVs capture rapid kinetics of vesicle uptake by host cells in real time.** (A) CCF2-AM
168 loaded HeLa cells were exposed to OMVs from EHEC carrying ClyA-Bla (red), or vector control (grey) at an MOI
169 of 1000 for 3 hours. Ratio of blue:green fluorescence) over time was plotted as mean \pm stdev (n=3). (B) R_{max} was
170 determined from data in S2A to visualize speed of uptake and is shown as means \pm stdev (n=3). Significance was
171 determined by analysis of variance, with a Brown Forsythe test to determine equal variance. (**) $p \leq 0.01$. (C)
172 Absolute FRET changes after 3 h were determined from data in (A) and plotted as efficiency of OMV uptake. Data
173 shown are means \pm stdev (n=3). Significance was determined by ANOVA, with a Brown Forsythe test to determine

174 equal variance. (**) $p \leq 0.01$. (D) CCF2-AM loaded HeLa cells were imaged by confocal microscopy and merged
175 blue/green images representative of 15 images ($n=3$) are shown. Scale bars, 20 μm . (E) HeLa cells incubated with
176 cellmask orange-labelled OMVs (red) for 10 and 60 min and slice views of z-stacks were acquired by confocal
177 microscopy. Scale bars, 10 μm ;

178

179 **EHEC OMVs enter host cells more rapidly and efficiently than OMVs from non-**
180 **pathogenic *E. coli*.** Next, we compared the uptake kinetics of OMVs isolated from EHEC and
181 non-pathogenic *E. coli* K12. Uptake of EHEC OMVs was faster (Figure 3A), and approximately
182 30% more efficient (Figure 3C), compared to K12 OMVs; the maximal rate was higher (Figure
183 3B), and a high rate of uptake was sustained for longer for EHEC than for the K12 strain (Figure
184 S2C). Both r_{max} (Figure S2D) and uptake efficiency (Figure S2E) increased with increasing
185 OMV concentration for both EHEC and K12, but for K12 vesicles r_{max} saturated at a lower OMV
186 concentration and a lower uptake efficiency was achieved. Taken together, these results suggest
187 EHEC OMVs contain cargos absent from K12 OMVs that accelerate and sustain the rate and
188 thus increase the efficiency of vesicle uptake by host cells.

189

190 **Figure 3. EHEC OMVs enter host cells more rapidly and efficiently than *E. coli* K12 OMVs.** (A) CCF2-AM
191 loaded HeLa cells were exposed to OMVs from EHEC (red) or *E. coli* K12 (blue) carrying ClyA-Bla, at an MOI of
192 1000 for 3 hours. Ratios of blue:green fluorescence over time were plotted as means \pm stdev ($n=3$). Maximum rates
193 (B) were determined from data in Figure S2 and absolute FRET signal changes after 3 hrs (C) were determined from
194 data in (A) and plotted to visualize overall efficiency of uptake for EHEC (red) and K12 (blue) OMVs. Data shown
195 are means \pm stdev ($n=3$). Significance was determined by ANOVA, with a Brown Forsythe test to determine equal
196 variance. (***) $p \leq 0.001$, (**) $p \leq 0.01$.

197

198 **Lipopolysaccharide structure shapes kinetics of OMV uptake by host cells.** Since OMVs are
199 derived from the outer membrane of Gram-negative bacteria, they contain lipopolysaccharides
200 (LPS), [23]. Whilst lipid A and the core oligosaccharide regions are well conserved, many
201 species including EHEC contain a highly variable polysaccharide domain known as O antigen
202 [24]. The O antigen constitutes the outermost structural region of LPS, and due to its length of up
203 to 40 nm [24], likely the first component in contact with host cells. These characteristics led us to
204 hypothesize that the O antigen present on EHEC OMVs may be a structural determinant of OMV
205 recognition and uptake by host cells.

206 To test this hypothesis, we carried out FRET assays with HeLa cells exposed to ClyA-Bla
207 reporter OMVs harvested from three pairs of strains, reflecting different *E. coli* serotypes and
208 pathovars and O antigen deficient isogenic mutants, to determine how the presence or absence of
209 O antigen would impact OMV uptake kinetics in each case. OMVs were derived from two
210 different pathovars of *E. coli*, EHEC (serotype O157) and enteroaggregative *E. coli* (EAEC,
211 serotype O42), and from the non-pathogenic lab strain K12 (serotype O16). For EHEC, OMVs
212 from O157 wild type cells and an isogenic strain lacking the O157 O antigen (*gne::IS629*, [25])
213 were compared (Figure 4). The O antigen deficient mutant *gne::IS629* carries a 1310 bp insertion
214 in *gne*, disrupting the epimerase required for synthesis of the oligosaccharide repeating unit in
215 the O antigen [25, 26], leading to a ~ 10 nm decrease in median OMV diameter (Figure S1B).
216 The r_{\max} for ClyA-Bla reporter OMVs derived from this O antigen deficient EHEC strain and the
217 isogenic wild type O157 strain were not significantly different (Figure 4B). However, OMVs
218 derived from wild type EHEC with intact O antigen sustained a higher entry rate over a longer

219 period (Figure S4A), and thus entered host cells ~ 43% more efficiently than those derived from
220 O antigen deficient EHEC (Figure 4D).

221 OMVs from wild type EAEC (serotype O42, intact O antigen) were compared with an
222 isogenic O antigen deficient mutant ($\Delta wbaC$, lacking a glycosyltransferase necessary for O
223 antigen synthesis; [27]). EAEC OMVs with intact O antigen were around 20 nm larger in
224 median diameter than EHEC OMVs, suggesting they carry a longer O antigen, and the diameter
225 dropped in the O antigen deficient mutant, to the same size as EHEC O antigen deficient OMVs
226 (Figure S1B). EAEC OMVs with intact O antigen entered host cells ~ 66% more efficiently than
227 OMVs without O antigen, due to a 77% higher r_{\max} (Figure 4D-F) and a higher sustained rate
228 over time (Figure S4B).

229 The non-pathogenic *E. coli* K12 strain MG1655 has lost its ability to produce O antigen
230 due to a disruption in *wbbL* encoding the rhamnosyltransferase required for O antigen synthesis
231 [28]. We compared entry of OMVs from this O antigen deficient strain (median OMV diameter
232 decreased by ~ 10 nm, compared to O16 positive strain), to those from an isogenic strain (DFB
233 1655 L9), where wild type *wbbL* has been restored, allowing for expression of the strain's
234 original O16 O antigen [27]. Similar to O157, the presence or absence of O antigen did not alter
235 r_{\max} , but the presence of O antigen allowed for a higher rate to be sustained for longer (Figure
236 S4C), leading to a ~ 22% higher efficiency overall (Figure 4G-I). A similar effect of O antigen
237 on uptake kinetics was observed in intestinal epithelial cells (Figure S3). Taken together, these
238 results suggest that the presence of the LPS O antigen increases the entry efficiency of OMVs
239 into host cells, independent of the specific mutation leading to O antigen deficiency. Depending
240 on the serotype used, this is caused by enhancing r_{\max} and/or by sustaining a higher uptake rate

241 over a longer period, compared to OMVs lacking O antigen. These variations may be due to
242 differences in physicochemical features and/or other vesicle cargos between the different
243 serotypes.

244

245 **Figure 4. LPS structure affects rate and efficiency of OMV uptake by host cells.** CCF2-AM loaded HeLa cells
246 were exposed to ClyA-Bla OMVs isolated from EHEC (serotype O157, A-C), EAEC (serotype O42, B-F) or K12
247 (serotype O16, G-I) containing O antigen (red), or lacking O antigen (blue), at an MOI of 1000 for 3 hours. Ratios of
248 blue:green fluorescence over time (A, D, G) were plotted as means \pm stdev (n=3). Maximum rates (B, E, H) were
249 extracted from data in Figure S4 and absolute FRET changes after 3 hrs (C, F, I) were determined from data shown
250 in A, D and G. Data shown are means \pm stdev (n=3); Significance was determined using ANOVA, with a Brown
251 Forsythe test to determine equal variance. (***) $p \leq 0.001$, (**) $p \leq 0.01$, (*) $p \leq 0.05$, (ns) not significant.

252

253 **LPS structure determines the preferred entry route of OMVs into host cells.** Next, we
254 evaluated the relative contribution of cellular trafficking pathways to OMV uptake and
255 determined if this was affected by LPS structure. Inhibition of macropinocytosis following
256 treatment of host cells with 20 μ M blebbistatin enhanced both the rate and efficiency of uptake in
257 the strains with shorter O antigen (EHEC and K12) and left it unaltered for EAEC (Figure S5).
258 These data suggest that only a small fraction of OMVs usually enters cells by micropinocytosis,
259 and inhibition of this relatively slow uptake route either does not affect or accelerates uptake.
260 Next, we tested if OMV uptake required dynamin, using the dynamin GTPase inhibitor dynasore.
261 Treatment of host cells with dynasore completely abolished uptake of OMVs, independent of
262 serotype and the presence of O antigen (Figure S5). Next, we determined whether OMV uptake
263 was via clathrin-coated pits, or via lipid raft-mediated endocytosis, both of which require
264 dynamin [29-31]. We inhibited clathrin-mediated endocytosis, either by proteolytic removal of

265 all protein receptors from host cells with papain prior to OMV incubation, or by blocking pit
266 assembly using chlorpromazine [32]. Removal of protein receptors from the host cell surface
267 increased uptake rate (Figure S6) and efficiency (Figures 5 and S6) for OMVs with O antigen,
268 but decreased or abolished uptake rate and efficiency of O antigen deficient OMVs. In general,
269 both papain and chlorpromazine treatment decreased the uptake of O antigen negative OMVs
270 but, although they had variable effects, they did not reduce uptake of O antigen positive OMVs
271 (Figures 5 and S6). This suggests that OMVs lacking O antigen require protein receptors for
272 uptake and use clathrin-mediated endocytosis as a main route of entry. In contrast, OMVs with
273 intact O antigen do not rely on protein receptors for entry, and inhibition of clathrin-mediated
274 endocytosis does not prevent their uptake into host cells.

275

276 **O antigen containing OMVs enter host cells faster because they can access raft-mediated**
277 **endocytosis more efficiently.** Since OMVs displaying O antigen on their surface accessed host
278 cells faster in the absence of clathrin-dependent endocytosis, we investigated whether this was
279 mediated by raft-dependent pathways. Disruption of raft-mediated endocytosis, either by
280 sequestration of membrane cholesterol from membrane microdomains via methyl- β -cyclodextrin
281 or by disrupting raft dynamics with filipin [33], led to a reduced r_{\max} (Figure 5) and uptake
282 efficiency (Figure S6). These data show that, while OMVs are able to access different uptake
283 routes including macropinocytosis, clathrin-dependent and raft-dependent endocytosis, OMVs
284 displaying O antigen on their surface are able to access raft-dependent endocytosis more
285 efficiently, while OMVs lacking O antigen are more reliant on clathrin-mediated uptake (Figure
286 6). Shifting a larger fraction of O antigen-positive OMVs to raft-mediated endocytosis further

287 accelerates their uptake, and we conclude the differences in uptake routes driven by LPS
288 structure account for differences in uptake rate and efficiency we observe.

289

290 **Figure 5. OMVs lacking O antigen are biased towards clathrin-mediated endocytosis, while OMVs with O**
291 **antigen can efficiently access host cells via lipid rafts.** HeLa cells were either left untreated (control, red), or pre-
292 treated with 5 µg/ml papain (lilac), 1 µg/ml chlorpromazine (pink), 5 mM methyl-β-cyclodextrin (light green) or 1
293 µg/ml filipin (turquoise), and exposed to ClyA-Bla OMVs isolated from EHEC (A), EAEC (B) or K12 (C) with or
294 without O antigen at an MOI of 1000 for 3 hours. Total FRET changes after 3 hrs were determined from data in
295 Figure S6 and data shown are means ± stdev (n=3). Significance compared to the control group was determined
296 using ANOVA, with a Brown Forsythe test to determine equal variance. (***) indicates $p \leq 0.001$, (**) $p \leq 0.01$, (*)
297 $p \leq 0.05$, (ns) not significant.

298

299 **Figure 6. LPS composition determines major route and kinetics of OMV entry into host cells.** Whilst it is well
300 established that pathogenic species utilize OMVs during infection, the specific adaptations which allow OMVs to
301 contribute to pathogenesis require further exploration. This work has developed a new approach to overcome current
302 methodological limitations and provide consistent data for future studies and allow new insights into the interactions
303 of OMVs with host cells during infection. This method has shown the relevance of LPS composition, in particular
304 the presence of O antigen, in determining the entry route and kinetics of OMVs. Further work in this area may
305 reveal targets for inhibition of these processes, and enable attenuation of infections by preventing the OMV-
306 associated delivery of virulence factors.

307

308

309 **DISCUSSION**

310 Interactions between bacterial outer membrane vesicles and epithelial cells are now recognized
311 as an important driver of bacterial pathogenesis. Yet, our ability to study vesicle-host cell
312 interactions has been limited by a lack of methods to capture the rapid kinetics of vesicle entry
313 and dismantling in real-time, and without altering the physicochemical properties of the vesicle.
314 Here we describe a novel assay that fulfils these requirements and allowed us to study the
315 kinetics of OMV uptake with enough temporal resolution to reveal critical differences in rate and

316 uptake efficiency of vesicles derived from different *E. coli* serotypes and pathovars. The method
317 uses a genetically encoded, OMV targeted probe and a cell-permeable dye, resulting in a change
318 in FRET upon reporter uptake and dye cleavage. Advantages of this system include its high
319 sensitivity (5 µg/ml OMVs, the lowest concentration reported in the literature, produced a
320 reproducible trace with good signal/noise ratio) and rapid response (signal was detected within
321 seconds). A potential drawback is, that it is not known if the ClyA-Bla probe is expressed
322 equally across the entire OMV population, but this is equally true for other markers and assays
323 currently in use. The system's use can be extended to a high-throughput format, allowing further
324 study of bacterial and host factors determining OMV uptake and trafficking. Using a transwell
325 format, the method can be applied to cell-based assays consisting of bacteria releasing OMVs,
326 and host cells without the need for OMV isolation. Although the specific probes used here were
327 functional across a range of *E. coli* isolates and different host cell types, their use in other
328 bacterial species will require further characterization to determine if they are targeted to OMVs
329 and retain correct orientation and enzymatic activity.

330 We selected EHEC and EAEC OMVs for this study, since OMVs have been shown to
331 play a crucial role in toxin stabilization and delivery for both pathovars [34, 35], and have been
332 considered as a means to vaccinate and protect against hemolytic uremic syndrome, a severe
333 complication of EHEC infection [36]. It is clear that LPS, and specifically O antigen, contributes
334 to bacterial within-host fitness and pathogenicity, by enhancing resistance to complement,
335 modulating phagocytosis and phage infection [37, 38]. The O antigen of most *E. coli* strains has
336 10-18 repeats, but can exceed 80 repeats [39, 40]. The length of the O antigen is equally variable

337 (~5-50 nm), and is positively correlated with the ability of the bacterial cell to adhere to host
338 cells and tissues, while loss of O antigen results in defects in colonisation, biofilm formation, and
339 increased pathogen clearance [24, 41-43]. Recent work showed that EHEC OMVs allow
340 efficient delivery of LPS into the host cell cytoplasm, resulting in inflammatory responses,
341 caspase-11 activation and cell death, but did not explore the role of LPS in uptake [44]. Our data
342 suggest that O antigen has an additional, previously unrecognized role during bacteria-host
343 interactions, which is to steer OMVs towards raft-mediated endocytosis, accelerating uptake and
344 delivery of vesicle associated virulence factors such as hemolysins and Shiga-like toxins [45] to
345 host cells and enhancing pathogenicity.

346 It is well known that OMVs contain different cargos, depending on pathovar and serotype
347 [46]. This means the comparison of O antigen deficient mutants with wild type OMVs as well as
348 comparison of different pathovars has the pitfall that other vesicle cargos may be modulated and
349 alter uptake kinetics. To dissect the effect of O antigen independent of other cargos, we
350 attempted to deplete O antigen of wild type OMVs by treatment with a glycoside hydrolase, but
351 found enzymatic activity was not limited to O antigen cleavage but modified the core LPS as
352 well. However, we observed a strong correlation between O antigen and uptake kinetics across
353 three different serotypes and pathovars, suggesting that O antigen is, if not the only factor, at
354 least a key determinant of uptake kinetics. Since EAEC OMVs showed the most distinct change
355 in entry kinetics upon O antigen deletion, with r_{\max} impacted as well as rate sustenance and
356 efficiency (Figure 4) and O42 antigen seemed to be much longer than EHEC O157 or K12 O16

357 antigens, which seemed similar in size and displayed similar changes upon O antigen deletion
358 (Figure S1B), we speculate that O antigen length may impact maximal entry rate.

359 We used our newly-devised assay to identify the relative contribution of cellular uptake
360 pathways to OMV entry into host cells. Clathrin- and raft-dependent endocytosis,
361 macropinocytosis and membrane fusion have all previously been reported as uptake pathways for
362 bacterial OMVs, and it is likely that discrepancies between studies result, at least in part, from
363 differences in species, strains and methodology used to study uptake [48]. Uptake of OMV cargo
364 by fusion of vesicles with the host cell membrane can be ruled out as a major route of uptake for
365 OMVs used in our study, since in this case ClyA-Bla would be exposed on the outer leaflet of
366 the host cell membrane and would not account for the rapid cleavage of the cytoplasmic FRET
367 dye. Assays using pharmacological inhibitors to block specific endocytic pathways, showed that
368 while all OMVs use multiple uptake routes, their surface structure biases them towards different
369 pathways. For example, O antigen deficient OMVs had a stringent requirement for surface
370 protein receptors for their uptake, while O antigen containing OMVs were able to access protein-
371 receptor independent pathways. Depletion of such receptors actually allowed them to access
372 protein-receptor independent pathways more efficiently and utilize raft-mediated endocytosis, a
373 more rapid mode of uptake, as main route of entry. While raft-mediated endocytic routes are not
374 as well characterized as clathrin-mediated endocytosis, it is clear there are multiple pathways,
375 including caveolin and non-caveolin dependent raft-mediated endocytosis. Our experiments
376 suggest that the entry of O antigen containing OMVs is raft- and dynamin dependent, but
377 protein-receptor independent, and no co-localization between OMVs and caveolin was detected.

378 The requirement of dynamin is likely, based on complete inhibition of uptake following
379 treatment with dynasore, however this is confounded by the dual inhibitory effect of dynasore
380 both on dynamin as well as cholesterol containing micro domains [49]. A recent study focusing
381 on vesicular cargo delivery of EHEC OMVs to host cells over longer time frames also concluded
382 that OMVs enter host cells via dynamin-dependent endocytosis [45]. We therefore conclude they
383 use a raft-mediated, and likely dynamin dependent, but protein-receptor and caveolin-
384 independent route of uptake, and the detailed requirements regarding their uptake are subject to
385 current studies.

386 **MATERIALS AND METHODS**

387 **Strains and growth conditions**

388 The strains used in this study were the *E. coli* serotype O157:H7 strain Sakai 813, a derivative of
389 enterohaemorrhagic *E. coli* (EHEC) RIMD 0509952, and its O antigen deficient derivative, MA6
390 (Δgne , [25]; the *E. coli* serotype O42 wild type strain (an enteroaggregative *E. coli* isolate, [47],
391 and its isogenic, O antigen deficient derivative strain ($\Delta wbaC$, [27]; the *E. coli* serotype O16
392 strain DFB 1655 L9 (a K12 strain containing a restored *wbbL* gene), and its isogenic, O antigen
393 deficient derivative, MG1655 [27]. All strains were transformed with plasmids pBAD ClyA-Bla,
394 Bla-ClyA, or empty vector (a kan^R derivative of the pBAD amp^R vector provided by Matthew
395 DeLisa, Cornell University), [12]. Strains were grown in LB containing 50 µg/ml kanamycin, at
396 37 °C with shaking at 200 rpm.

397

398 **Isolation of outer membrane vesicles by ultracentrifugation**

399 100 ml cultures were grown in LB at 37 °C, with agitation at 200 rpm. Once the OD₆₀₀ reached
400 0.5-0.6, expression of ClyA-Bla was induced with 0.2% L-arabinose and grown for a further 16
401 h. Cells were then pelleted at 6000xg, and the supernatants were removed and filtered with a
402 0.45um syringe filter. Aliquots of filtered supernatants were spread on LB agar and grown
403 overnight at 37 °C to check that all viable cells had been removed by filtration. 25 ml of filtered
404 supernatants were centrifuged in a Beckman XL90 ultracentrifuge using a 70Ti rotor at
405 100,000xg (30,000 rpm) for 2 h at 4 °C. After centrifugation, supernatants were removed, and the
406 OMV pellets were resuspended in 1 ml colorless DMEM or sterile water (for TEM) and stored at
407 -20 °C.

408 **Detection of Bla probes in cellular fractions**

409 12 µl of samples normalized for their protein content from EHEC ClyA-Bla and Bla-ClyA whole
410 cell lysate, supernatant and OMV fractions were added to 3µl 5X SDS loading dye and boiled for
411 10 min. Samples were loaded onto a 15 well BioRad pre-cast stain-free SDS-PAGE gel and run
412 at 120V, 200mA for 45 min. The gel was then transferred onto a PVDF membrane in transfer
413 buffer containing 20% methanol for 80 minutes at 100V. After transfer, the membrane was
414 blocked at room temperature in TBS 0.1% Tween-20 and 5% skim milk for 1h with agitation.
415 The membrane was washed 3 times with TBS 0.1% Tween-20 (5 min per wash). After blocking,
416 the membrane was incubated with a 1:2000 dilution of mouse anti-Bla primary antibody in TBS
417 0.1% Tween-20 and 5% skim milk overnight at 4 °C with agitation. The following day, the
418 membrane was washed 3 times as before, and incubated with a 1:5000 dilution of sheep anti-
419 mouse secondary antibody in TBS 0.1% Tween-20, 5% skim milk for 1h at room temperature

420 with agitation. The membrane was washed again 3 times, and 2 ml BioRad ECL reagents were
421 added to the membrane and incubated for 5 min, before visualization with a BioRad ChemiDoc
422 imager.

423

424 **Nitrocefin assay to determine β -lactamase activity**

425 50 μ l of samples were added in triplicate to a 96-well plate. Nitrocefin was diluted to 0.5 mg/ml
426 in PBS and 50 μ l was added to each sample. The absorbance at 486 nm was measured in the
427 FluoStar Omega plate reader for 2 h, and the change in absorbance over time was used to
428 determine the specific activity in samples, using the protein concentration determined by the
429 CBQCA kit.

430

431 **Protein Quantitation**

432 To quantify levels of protein in cell fractions, the ThermoFisher CBQCA Protein Quantitation kit
433 was used according to the manufacturer's instructions.

434

435 **Papain and detergent treatment of OMVs**

436 Triton X-100 and SDS were added at a concentration of 1% to 20 μ l OMVs for 45 min at 37 $^{\circ}$ C.
437 5ug/ml papain was then added for 30 or 60 min at 37 $^{\circ}$ C. The papain reaction was inactivated
438 using 1 mM PMSF at room temperature for 30 min. 5 μ l SDS-PAGE loading dye was added to
439 the samples, which were then boiled for 10 min. Samples were run on a 15-well pre-cast stain

440 free gel for 45 min at 120V, and then subjected to Western blotting with anti- β -lactamase
441 primary antibody (Pierce) as described above.

442

443 **Plate reader FRET experiments**

444 HeLa cells (passage 1-7) were seeded in triplicate in a black-walled, clear bottom 96-well plate
445 at a concentration of 1×10^5 cells per ml in Dulbecco's modified Eagle medium (DMEM)
446 supplemented with 1% L-glutamine, 1% Penicillin/Streptomycin and 10% heat inactivated fetal
447 bovine serum. The plate was incubated at 37 °C, 5% CO₂ for 24 h prior to experiments. The
448 following day, cells were loaded with 20 μ l 6X CCF2-AM with 100 μ l colourless
449 unsupplemented DMEM (cDMEM) and incubated at room temperature for 1 h in the dark to
450 allow dye loading. The dye was removed by washing 2x in PBS and 1x in cDMEM. Cells were
451 treated with 5 mM methyl- β -cyclodextrin or 1 μ g/ml filipin to inhibit cholesterol mediated
452 endocytosis, 80 μ M Dynasore for dynamin inhibition, or 20 μ M blebbistatin for
453 macropinocytosis inhibition for 1h at 37 °C. Cells were treated with 1 μ g/ml chlorpromazine for
454 1h at 37 °C to inhibit formation of clathrin-coated pits, or with 5 μ g/ml papain for 15 min at 37
455 °C to remove surface proteins, before inactivation of papain with 5 mM PMSF for 20 min.

456 Reporter OMVs were diluted in cDMEM and added to the cells for a final concentration of 10
457 μ g/ml, or 1×10^8 vesicles, corresponding to an MOI of 1000. The plate was immediately placed in
458 the PheraStar plate reader, with excitation at 405 nm and simultaneous dual emission at 530 nm
459 and 460 nm. The wells were scanned (bottom optic) with orbital averaging for a total of 150
460 cycles, equating to a measurement every 90 seconds for 3 hours. The ratio of blue to green

461 fluorescence intensity detected in the cells at each cycle was calculated using GraphPad Prism,
462 and ratios for uninfected, dye-loaded cells were used as the baseline value for each cycle. All
463 traces were normalized to 0 for their first ratio value. All experiments were performed with a
464 minimum of three technical replicates and three independent repeats.

465

466 **Efficiency of uptake and statistical analysis**

467 Efficiency of uptake was calculated as the absolute change in blue:green fluorescence intensity
468 ratio between 0 and 3 hours ($[Em460/Em530]_{t=0hrs} / [Em460/Em530]_{t=3hrs}$). Analysis of variance
469 (ANOVA) was used to determine statistical significance, with a Brown Forsythe test to
470 determine equal variance (GraphPad Prism software). A p-value of <0.05 was considered
471 statistically significant.

472

473

474 **Rate estimation and statistical analysis**

475 To estimate the gradients of the data, polynomials were fitted to each data set using the cubic
476 spline function *csaps* in Matlab. Numerical estimates of the gradients of the resulting
477 polynomials were determined using the *gradient* function. To ensure that the gradient estimates
478 were as smooth as possible whilst also retaining the overall shape and trend of the data, a small
479 smoothing parameter was used. Analysis of variance (ANOVA) was used to determine statistical
480 significance, with a Brown Forsythe test to determine equal variance (GraphPad Prism software).
481 A p-value of <0.05 was considered statistically significant.

482

483 Confocal Microscopy

484 HeLa cells (P3-7) were seeded on 13mm coverslips in a 12-well plate at a concentration of 1×10^5
485 cells per ml in complete DMEM, 24 h prior to experiments. The following day, cells were
486 washed and loaded with 100 μ l 6X CCF2-AM dye with 500 μ l colourless unsupplemented
487 DMEM, and incubated in the dye solution for 1 h at room temperature in the dark. Cells were
488 then incubated with ClyA-Bla reporter OMVs for 0-4 h. The cells were washed with PBS and
489 then fixed with 0.5 ml 4% PFA. The next day, coverslips were mounted onto slides with a drop
490 of Gold Anti-Fade mounting solution and then imaged using a Nikon A1R confocal microscope
491 (Birmingham Advanced Light Microscopy Facility), and fluorescence was observed from
492 excitation at 409 nm and dual emissions at 450 nm and 520 nm. Z stacks were produced with
493 gain, slice thickness, exposure and laser intensity kept the same for all slides, and images were
494 taken for 3 representative fields of view per slide and n=3 independent samples. The Z stacks
495 were converted to maximum intensity projection images. For OMV localization experiments,
496 OMVs were stained using cell mask orange (1:500) for 1 h at 22 °C and gentle agitation.
497 Following staining, samples were washed with 28 volumes of PBS and labelled OMVs pelleted
498 by ultracentrifugation (100,000xg, 2h). HeLa cells were exposed to labelled OMVs for 10 of 60
499 minutes prior to fixation in 3.2% formaldehyde. Slides were imaged using an Olympus IX83
500 inverted microscope fitted with a FV3000 confocal system and 100x Super Apochromat oil
501 objective. Images were captured using Olympus Fluoview software and processed using the
502 CellSens extension package.

503

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509

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- 646

647 **SUPPORTING INFORMATION LEGENDS**

648 **Figure S1. Morphology, size, charge and probe orientation of reporter OMVs.** (A) Electron
649 micrographs of negative stained OMV fractions from EHEC wt (left image) or EHEC ClyA-Bla
650 (centre and right images). Scale bars, 0.5 μm . (B) Isolated OMVs were diluted 1×10^{-6} fold and
651 nanoparticle tracking analysis was used to determine the size distribution. Black lines represents
652 median size from at least 200 tracks acquired per sample. Statistical significance was determined
653 by ANOVA, with a Brown Forsythe test to determine equal variance. (***) $p \leq 0.005$, (ns) not
654 significant. (C) ζ -potentials of isolated OMVs. Values represent means from 30 readings per
655 sample. (D) OMV fractions from EHEC expressing Cly-Bla, Bla-ClyA or carrying empty vector
656 were treated with papain for 30 or 60 minutes, and used for Western Blotting with α -Bla
657 antibody.

658

659 **Figure S2. Rates of uptake/dismantling and concentration dependency of uptake kinetics**
660 **for OMVs.** (A) CCF2-AM loaded HeLa cells exposed to EHEC OMVs carrying ClyA-Bla (red),
661 or empty vector (grey) at an MOI of 1000 for 3 h. Rate of uptake over time was extracted from
662 data in Figure 2A and data shown are means \pm stdev (n=3). (B) FRET change upon exposure of
663 HeLa cells to EHEC OMVs carrying ClyA-Bla (reporting on exposure to OMV surface to
664 cytoplasm) or Bla-ClyA (reporting on exposure of luminal cargo to cytoplasm). (C) HeLa cells
665 were exposed to EHEC or K12 ClyA-Bla OMVs at an MOI of 1000 for 3 hours. Rates of uptake
666 over time were extracted from data in Figure 3A and are means \pm stdev (n=3). (D) Experiments
667 were repeated as above but using different OMV concentrations (0-20 $\mu\text{g/ml}$ of protein,
668 corresponding to an MOI of 0- 2000), and maximum rates (D) and efficiency of uptake (E)
669 determined as described above. Data are means \pm stdev (n=3).

670

671 **Figure S3. Uptake for OMVs from serotypes O157, O42 and O16 with or without O**
672 **antigen.** CCF2-AM loaded RKO intestinal epithelial cells were exposed to OMVs from EHEC

673 O157 (A), EAEC O42 (B), and K12 O16 (C), with O antigen (red) and without O antigen (blue),
674 at an MOI of 1000 for 3 hours. FRET changes (blue/green fluorescence, A-C) and efficiency of
675 uptake (total change over three hours, D) are shown as means \pm stdev (n=3).

676

677 **Figure S4. Rates of uptake for OMVs from serotypes O157, O42 and O16 with or without**
678 **O antigen.** CCF2-AM loaded HeLa cells were exposed to OMVs from EHEC O157 (A), EAEC
679 O42 (B), and K12 O16 (C), with O antigen (red) and without O antigen (blue), at an MOI of
680 1000 for 3 hours. Polynomials were fitted to each data set using the cubic spline function csaps
681 in Matlab. Numerical estimates of the gradients of the resulting polynomials were determined
682 using the gradient function. Data shown are means \pm stdev (n=3).

683

684 **Figure S5. Effect of blebbistatin and dynasore on uptake of OMVs.** HeLa cells were either
685 left untreated or pre-treated 80 μ M Dynasore for dynamin inhibition (grey), or 20 μ M
686 blebbistatin for macropinocytosis inhibition (orange) for 1h at 37 °C and exposed to ClyA-Bla
687 OMVs isolated from EHEC (A, B), EAEC (C, D), or K12 (E, F) at an MOI of 1000 for 3 hours.
688 The FRET signal (ratio of blue:green fluorescence) over time was plotted as mean \pm stdev (n=3).

689

690 **Figure S6. Effect of pharmacological treatments on OMV uptake.** HeLa cells were either left
691 untreated or pre-treated with 5 μ g/ml papain (lilac), 1 μ g/ml chlorpromazine (pink), 5mM
692 methyl- β -cyclodextrin (light green) or 1 μ g/ml filipin (turquoise) and exposed to ClyA-Bla
693 OMVs isolated from EHEC (A, B), EAEC (C, D), or K12 (E, F) at an MOI of 1000 for 3 hours.
694 The FRET signal (ratio of blue:green fluorescence) over time was plotted as means \pm stdev
695 (n=3).

696

697

698