LIPOPOLYSACCHARIDE COMPOSITION DETERMINES THE ENTRY KINETICS OF BACTERIAL OUTER MEMBRANE VESICLES INTO HOST CELLS

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Abstract

Outer membrane vesicles (OMVs) are nano-sized proteoliposomes ubiquitously released from the outer membrane of Gram-negative bacteria, and are known to contribute to immune priming and disease pathogenesis. However, the current understanding of their interactions with host cells is limited by a lack of methods to study the rapid kinetics of vesicle entry and cargo delivery. This work has developed a highly sensitive method to study vesicle entry into host cells in real-time using a genetically encoded, vesicle-targeted probe. Using this approach, it was found that the route of vesicular uptake, and thus entry kinetics and efficiency, are shaped by bacterial cell wall composition. The presence of O polysaccharide in lipopolysaccharide creates a bias towards non-receptor mediated endocytosis, which enhances both the rate and efficiency of entry into host cells. This work indicates that the composition of the bacterial cell wall influences the behaviour of OMVs, and is therefore implicated in secretion-system independent delivery of bacterial virulence factors during Gram negative infection.

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To Elliot. Thank you for having me.

Table of Contents

List of Figures and Tables

DECLARATION OF AUTHORSHIP

This is to confirm that Eloise Jasmin O'Donoghue was the author of the review article entitled 'Mechanisms of outer membrane vesicle entry into host cells' published in the journal 'Cellular Microbiology', September 2016, with Anne-Marie Krachler as the corresponding author.

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1. Introduction

Communication is a vital aspect of bacterial life, allowing bacteria to interact with and react to their environment. Secretion of cellular material represents a versatile means of communication, and there are numerous mechanisms employed by bacteria in order to export a variety of cargo, for a wide range of purposes. One such method is via the secretion of Outer Membrane Vesicles.

1.1 Discovery of OMVs

The discovery of the immunogenic properties and detection of LPS in cell free supernatants from *Vibrio cholerae* and *Escherichia coli* over 50 years ago led to the observation of small membranous spherical structures via electron microscopy that later became known as Outer Membrane Vesicles (OMVs) (De, 1959; Bishop and Work, 1965). OMVs are nanosized (20-200 nm) spherical membranous structures that are observed to pinch off from all areas of the outer membrane of the bacterial cell, and contain LPS, and some inner membrane and periplasmic components (Devoe and Glichrist, 1973; Beveridge et al, 1999). Their ubiquitous production by all species of Gram negative bacteria studied to date, and in all growth phases and environmental conditions caused them to be dismissed as a by-product of cell wall turnover or lysis. Later evidence demonstrated the production of OMVs independently of cell lysis, and showed that they contained newly synthesised cellular material (Mug-Opstelten and Witholt, 1978; McBroom et al, 2006). Subsequent proteomic studies also revealed

enrichment or exclusion of certain molecules within the vesicles, suggesting a selective and active process of OMV release from the cell (Haurat et al, 2011; Elhenawy et al, 2014). The constant production, selectivity of cargo, and energy expenditure all indicate an important role for OMV release in bacterial survival (Kulp and Kuehn, 2010; Bonnington and Kuehn, 2014).

1.2 Roles of OMVs in stress

OMV release is now recognised as a form of generalised secretion system, with several advantages over other secretory mechanisms in Gram negative species. OMVs can export considerably more material than other types of secretion, with up to 5% of the outer membrane material of *E. coli* being shed in the form of OMVs, and up to 12% of total protein content for *Neisseria meningitidis* (Devoe and Gilchrist, 1973; Gankema et al, 1980). OMV release also allows protection of the cargo molecules within the enclosed lipid membrane, avoiding degradation in the extracellular environment, and also maintaining high concentrations of the substrates upon arrival at their destination (Kolling and Matthews, 1999). Molecules can be secreted within vesicles without requiring particular signal sequences, and can also be secreted alongside other molecules to sites distant to the cell of origin (Dorward et al, 1991; Wai et al, 2003; Bomberger et al, 2009).

The continuous production and versatility of OMVs allows them to play diverse roles in bacterial survival. OMVs are implicated in bacterial stress responses via the export of misfolded proteins from the cell, and *E. coli* with mutations in the heat shock response pathway σ^E showed increased vesiculation, suggesting a compensatory stress response is provided by OMVs (McBroom and Kuehn, 2007). OMV production increases following envelope stress and this increased production is also correlated with increased bacterial survival (McBroom and Kuuehn, 2007; MacDonald and Kuehn, 2013).

Although non-vesiculating mutants have so far been non-viable, hypervesiculating strains have been used to demonstrate the contributions of OMVs to stress responses. A hypervesiculating *yieM* mutant strain showed increased survival compared to the wild-type strains after exposure to membrane-acting stressors polymyxin B and colistin, and to T4 phage challenge (Manning and Kuehn, 2011). Treatment with alcohols, metal chelators, heat and osmotic shocks all resulted in increased vesiculation of *Pseudomonas putida*, leading to a more hydrophobic cell membrane, facilitating cell-cell attachment and therefore enhanced biofilm formation (Baumgarten et al, 2012).

1.3 Roles of OMVs in bacterial communities

OMV production can also help to adsorb toxic molecules such as antibiotics; gentamicin sensitive clinical isolates of extra-intestinal pathogenic *E. coli* (ExPEC) showed a 13.1 fold increase in OMV production compared with gentamicin resistant strains (Chan et al,

2017). Similarly, isolated OMVs from *E. coli* MG1655 were able to protect *P. aeruginosa* and *Acinetobacter radioresistens* against membrane active antibiotics, but not against those with different mechanisms (Kulkarni et al, 2015). The OMVs were shown to contain proteases which degraded the antibiotic melittin, whilst the membrane sequestered colistin, suggesting OMVs are well adapted to protecting a mixed community of bacteria against antibiotic treatment (Kulkarni et al, 2015).

Other contributions to bacterial communities have also been observed. OMVs from *P. aeruginosa* have been found to contain β-lactamase, an enzyme able to degrade penicillin antibiotics, and thus benefit not only the cells encoding the β-lactamase but also non-resistant strains occupying the same niche (Ciofu et al, 2000). This effect was observed directly for *Moraxella catarrhalis*, which often co-infects the upper respiratory tract with *Haemophilus influenzae* and *Streptococcus pneumoniae*. The *M. catarrhalis* OMVs were found to contain β-lactamase and were able to rescue the amoxicillin-sensitive strains of *H. influenzae* and *S. pneumoniae*, indicating a clinical implication for OMVs in facilitating antibiotic resistance (Schaar et al, 2011).

OMVs also contribute to group behaviour via the release of quorum sensing molecules. In *P. aeruginosa*, OMVs were found to contain the hydrophobic pseudomonas quinolone signal, PQS. Removing OMVs from the cultures resulted in a reduction in pyocyanin production, and it was also demonstrated that PQS mutants produced fewer OMVs, suggesting that the OMV cargo initiates OMV production (Mashburn and Whiteley,

2005). This was later shown to be due to its ability to induce membrane curvature, and promote OMV release from the cell envelope (Mashburn-Warren et al, 2008). PQS is able to bind iron, an essential and often scarce nutrient, and so release of OMV-associated PQS may enable the Pseudomonas population access to a higher concentration of iron in the extracellular environment (Kulp and Kuehn, 2010). It was recently demonstrated that OMVs enabled iron uptake and compensated for loss of other iron acquisition systems in mutant strains of *P. aeruginosa* (Lin et al, 2017).

OMVs from *Bacteroides succinogenes* were observed to bind and degrade cellulose in the rumen, and therefore aid in digestion of the large polymer and provide a source of sugars for species which lacked their own cellulolytic enzymes, and resulted in a stable microbial population (Forsberg et al, 1981).

Not all OMVs are beneficial to communities. OMVs from the soil dwelling predatory bacterium *Myxococcus xanthus* were shown to contain unique cargo molecules including proteases and phosphatases and selectively target *E. coli* cells and cause cell death (Evans et al, 2012; Berleman et al, 2014). A study by Li et al (1998) isolated OMVs from 15 different strains of Gram negatives, and demonstrated their ability to lyse a range of target cells, both Gram negative and Gram positive. The OMVs were most effective at killing cells that had a similar peptidoglycan structure to their own, allowing them to remove other competing members of a community (Li et al, 1998).

1.4 OMVs and nucleic acid export

OMVs have also been shown to export nucleic acids, with the DNA protected from degradation and resistant to DNAse (Kahn et al, 1983). DNA was detected in vesicles produced by enterohaemorrhagic *E. coli* (EHEC) encoding several virulence factors and resistance genes. The genetic material was efficiently transferred to the non-virulent *E. coli* JM109 recipient strains, conveying antibiotic resistance and increasing their cytotoxicity 6-fold (Kolling et al, 1999; Yaron et al, 2000). Transfer of resistance genes by OMVs was also observed for *N. meningitidis* (Dorward et al, 1989) and *H. influenzae*, where production of OMVs was also correlated with the competence state of the cells (Kahn et al, 1983).

As well as containing and releasing the enzymes which confer antibiotic resistance, OMVs can also transfer the genes which encode them. New Delhi metallo-β-lactamase-1 (NDM-1) is an antibiotic resistance gene that confers resistance to almost all penicillins, carbapenems and cephalosporins (Chatterjee et al, 2017). Isolation of OMVs from a clinically resistant strain of *A. baumannii* revealed the presence of the NDM-1 gene within the vesicles. Transfer of these vesicles to a non-resistant strain of *A. baumannii* and a strain of *E. coli* resulted in transfer of resistance, and was determined to be due to the plasmid encoded NDM-1 transferred by the OMVs (Chatterjee et al, 2017). OMVs therefore represent a successful means of spreading antibiotic resistance, both within and between species.

OMVs can transfer nucleic acids to mammalian cells. sRNA from *P. aeruginosa* OMVs was detected in human airway epithelial cells by RNAseq, and resulted in reduced LPS-induced IL8 secretion, and therefore a reduction in the inflammatory response, indicating an immunomodulatory function of OMVs (Koeppen et al, 2016).

1.5 Roles of OMVs in infection

1.5.1 In-vivo production of OMVs

The contribution of OMVs to bacterial stress responses is well established, and colonisation of a host can be considered a stressful environment (MacDonald and Kuehn, 2012). Pathogenic species generally release more OMVs than their non-pathogenic counterparts, with Enterotoxigenic *E. coli* secreting up to 10 fold more OMVs than the lab strain HB101 (Horstmann and Kuehn, 2000). Evidence of OMV production in vivo has been documented for many species, and observed via electron microscopy from human tissue biopsies (Fiocca et al, 1999; Heczko, 2000). It is likely that the role of OMVs in host colonisation and pathogenesis has been underestimated clinically, due to their size and their ability to disseminate deep into tissues and to to sites in the body distant to the initial infection (Dorward et al, 1991; Ellis and Kuehn, 2010). Intracellular pathogens such as *Legionella pneumophila* have also been shown to produce OMVs within intracellular compartments such as phagosomes (Fernandez-Moreira et al, 2006) whilst a recombinant *Salmonella enterica* Typhimurium strain engineered to express

Typhoid toxin were found to produce OMVs within the *Salmonella*-containing vacuole (SCV) and these OMVs were able to exit the host cell and deliver the toxin to neighbouring epithelial cells (Guidi et al, 2013). OMVs have been detected in sites distinct from the regions of bacterial colonisation, such as the cerebrospinal fluid, blood, urine and other organs (Wispelwey et al, 1989; Dorward et al, 1991).

1.5.2 Delivery of virulence factors

One of the most effective ways that pathogens appear to have adapted the use of OMVs is for the delivery of a wide range of toxins. OMVs from Enterotoxigenic *E. coli* (ETEC) were found to contain heat-labile enterotoxin (LT), which was delivered into intestinal epithelial cells, resulting in cytotoxicity (Kesty and Kuehn, 2004). The major virulence factor of *V. cholerae*, the cholera toxin (CT), can be delivered via OMVs into host cells where it causes increased cellular cyclic AMP, resulting in cytotoxicity in human intestinal epithelial cells (Chatterjee and Chaudhuri, 2011). The invasion plasmid antigens IpaB, C and D of *Shigella flexneri* are implicated in invasion of epithelial cells and have all been detected in OMVs (Menard, Sansonetti and Parsot, 1993; Kadurugamuwa and Beveridge, 1998). The periodontal pathogen *T. denticola* secretes OMVs containing the protease dentilisin, and facilitate disruption and penetration of epithelial layers (Rosen et al, 1995; Chi, Qi and Kuramitsu, 2003). OMVs from the intestinal pathogen *Campylobacter jejuni* have been observed to cause cytotoxicity in cultured intestinal cells and in the wax worm larvae *Galleria mellonella*. *C. jejuni* OMVs have been shown to export cytolethal distending toxin (CDT) and also a number of

proteases, including HtrA, which is able to bind and cleave the host trans-membrane protein E-cadherin (Elmi et al, 2012; Elmi et al, 2015). Co-culture of HtrA positive OMVs with the live *C. jejuni* resulted in increased bacterial adhesion and intracellular invasion of intestinal epithelial cell monolayers, indicating a role for OMVs in promoting bacterial colonisation and pathogenesis (Elmi et al, 2015).

Many toxins released by bacteria can be exported by OMVs or in their free form; Shiga toxin was detected in OMVs produced by EHEC O157:H7 and *Shigella dysenteriae*, with more Shiga toxin detected in the OMVs than in the supernatant or cell pellet (Kolling and Matthews, 1999; Dutta et al, 2004). Caspase mediated apoptosis was observed after delivery of shiga toxin into intestinal epithelial cells from the virulent outbreak strain *E. coli* O104:H4 (Kunsmann et al, 2015).

EHEC Hly, a hemolysin, can also be exported in its soluble form or in association with OMVs, and this has different consequences for the target cell. Free EHEC-Hly causes cell lysis, whilst Hly entering cells via OMVs targets the mitochondria, activates caspase-3 and caspase-9, triggering apoptosis (Bielaszewska et al, 2013).

Export via OMVs is the preferred route for some bacterial cargo. ClyA is an unusual pore-forming cytolysin expressed by various virulent *E. coli* and *S. enterica* strains which contains no typical signal sequence for secretion, but its inactive monomers accumulate in the periplasmic space (Wai et al, 2003; Ludwig et al 2004; von Rhein et al, 2009).

OMVs from ClyA-expressing strains were found to contain oligomerised ClyA in its active form, and this was dependent on the redox status of the OMVs, thus the activity of the toxin is specific to the vesicle environment (Wai et al, 2003). *A. actinomycetemcomitans* released OMVs containing 4-5 fold higher levels of biologically active leukotoxin, LtxA, than detected in the bacterial membrane (Kato et al, 2002).

OMV cargo can also restore function to parent cells. In *F. tularensis*, mutations in a lipase, FtlA, reduced its virulence *in vivo* and ability to infect host cells. It was subsequently found that FtlA is present in OMVs released from *F. tularensis*, and co-incubation of the mutant strain with the FtlA OMVs from the parent strain was able to complement the loss of the gene, and restored its virulence and invasion of A549 cells (Chen et al, 2017).

The opportunistic human pathogen *Pseudomonas aeruginosa* has been shown to produce cytotoxic OMVs containing a variety of known virulence factors including the hemolytic toxin Phospholipase C, and Cif, which inhibits the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) protein and enhances colonisation of *P. aeruginosa* in the cystic fibrosis lung (MacEachran et al 2007; Bomberger et al, 2009). Cif delivered via OMVs was 17,000 fold more effective at reducing CFTR levels than purified Cif protein, indicating that OMVs not only secrete biologically active virulence factors, but they are also delivered highly efficiently, representing an effective means of facilitating bacterial colonisation and pathogenesis (Bomberger et al, 2009). The secreted

and OMV-associated proteomes of *L. pneumophila* were analysed, and of 25 known virulence factors, 18 were found associated with OMVs, including 8 which were only detected in the vesicle fractions (Galka et al, 2008).

In contrast, the vacuolating toxin, VacA, in *H. pylori*, can also be secreted as free toxin or OMV-associated, but in this case only 25% of toxin was exported via OMVs, and was found to have reduced vacuolating activity in host cells compared with the freely secreted form (Ricci et al, 2005). However, OMVs from *H. pylori* are associated with other cytotoxic effects, including increased genetic mutations, which may suggest a role for OMVs in carcinogenesis associated with *H. pylori* (Chitcholtan et al, 2008). The causative agent of whooping cough, *Bordetella pertussis*, expresses an adenylate cyclase toxin, ACT. Only around 1% of the total secreted ACT is OMV associated, but it was found to enter host cells via a different mechanism to freely secreted ACT, using actin microfilaments instead of the CD11b/CD18 receptors (Donato et al, 2012). OMVs are hypothesised to act as a reservoir of virulence factors that help to promote further bacterial colonisation.

1.5.3 Biofilm formation

OMVs also contribute to the formation and maintenance of biofilms. Biofilms are aggregations of bacteria, which often form under stressful growth conditions, such as in the presence of antibiotics, and are implicated in chronic and persistent infections that are more resistant to treatment (Wang et al, 2015). Extracellular DNA is a known structural

component of *H. pylori* biofilms, but is not susceptible to DNAse treatment, due to its association with OMVs (Grande et al, 2015). A correlation between OMV release and biofilm production was observed in a strain of *H. pylori* that produced strong biofilms, and transfer of these OMVs to other strains resulted in their enhanced biofilm formation (Yonezawa et al, 2009). OMVs from *P. aeruginosa* were found to have different size distributions and protein composition when isolated from biofilms compared to those produced during planktonic growth, suggesting that OMVs play different roles depending on the lifestyle of the bacterial cells (Schooling and Beveridge, 2006).

OMVs may contribute to the resistance of biofilms to treatment. OMVs isolated from a *P. aeruginosa* biofilm contained 34 different antibiotic resistance proteins, compared with 10 identified in OMVs from planktonic cultures (Park, Surette and Khursigara, 2014). This is not just a reflection of the protein content of the cells of origin; one protein, penicillin binding protein 1B (mrcB) was not detected in the *P. aeruginosa* cells isolated from the biofilm, but was found at high levels in the OMVs. Another *P. aeruginosa* virulence factor, alkaline phosphatase, is involved in biofilm formation, and has also been detected in OMVs (Bomberger et al, 2009).

In *Porphyromonas gingivalis*, a causative agent of dental plaques and chronic periodontitis, a unique protein, HmuY, has been implicated in the secretion of the extracellular matrix necessary for biofilm formation (Olczak et al, 2010). This protein is also abundant in their OMVs, suggesting a role in the maintenance and accumulation of

biofilms. Dental plaques contain various bacterial species, and presence of OMVs has been observed within these polymicrobial biofilms, containing Gram negative species such as *P. gingivalis*, *Treponema denticola* and *Tannerella forsythia*, but additionally, the isolated OMVs from *P. gingivalis* have also been shown to cause aggregation of Gram positive species, such as *Staphylococcus aureus*, and fungal species such as *Candida albicans*, suggesting that the aggregative potential of OMVs is not limited to closely related Gram negative species of bacteria (Kamaguchi et al, 2003; Zhu et al, 2013).

1.6 Immunomodulation

1.6.1 Pro-inflammatory Roles

OMVs can also interact with the host immune system. Since they are derived from the outer membrane, they contain lipopolysaccharide (LPS), which elicits potent immune responses and release of pro-inflammatory cytokines via the binding of the Lipid A portion of the LPS to the host cell TLR4 receptor (Kuehn and Kesty, 2005; Park et al, 2010). A strain of *N. meningitidis* which caused fatal septicemia was shown to be highly vesiculating, and contributed to the fatally high levels of endotoxin in the patient (Namork and Brandtzaeg, 2002). Inoculation with OMVs from *E. coli* was sufficient to cause lethal systemic inflammatory response syndrome (SIRS) in a mouse model, in the absence of the bacterial cells (Park et al, 2010). Recognition of LPS by TLR4 requires hexa-acylated Lipid A, and mutations in the *msbB* gene prevents addition of the final acyl chain, resulting in penta-acylated Lipid A. *msbB* mutant OMVs showed a reduction in

endotoxicity and inflammatory responses compared with wildtype OMVs (Kim et al, 2009; Park et al, 2009; Rossi et al, 2014).

Many OMV-associated toxins have been observed to have increased activity compared with their freely secreted form. Similarly, in some cases OMVs were able to stimulate inflammatory responses more effectively than the purified components alone. NadA, an outer membrane adhesin of *N. meningitidis* was found to bind and activate macrophages and monocytes and cause cytokine release at 10 fold lower concentrations than required for the free form of NadA (Tavano et al, 2009). In the study by Park et al (2010), OMVs induced lethal sepsis but when purified LPS was added at the same concentration as that present in the vesicles, no sepsis was observed, even when the concentration of LPS added was doubled (Park et al, 2010). These findings indicate that OMVs can contribute to the stimulation immune responses, but this is not necessarily via LPS; LPS from *B. abortus* does not induce inflammation, and is considerably less endotoxic than LPS from *E. coli* (Kianmehr et al, 2015).

Other components of the cell wall present in OMVs can also contribute to immunogenicity. OMVs from *Aggregatibacter actinomycetemcomitans* entered host cells and delivered peptidoglycan, detected via intracellular NOD1 and NOD2 sensors and induced activation of NFKB (Thay et al, 2014), an effect also observed for OMVs from *Helicobacter pylori, P. aeruginosa* and *N. gonorrhoea*, independently of TLR signalling (Kaparakis et al, 2010).

Cytokine release in response to OMVs has been observed for a number of different species, and both *in vitro* and *in vivo*. OMVs from *N. meningitidis* triggered release of TNFα, IL1β, IL8 and MIP1a and MIP1b by neutrophils, and also in a whole blood model (Lapinet et al, 2000; Mirlashari et al, 2001), similarly to those of *N. gonorrhoeae* which induced secretion of IL6 and TNFα in macrophages (Makepeace et al, 2001). Proinflammatory cytokine release has also been detected in non-immune cells, with increased IL8 release observed for human intestinal and gastric cells when incubated with *V. cholerae, C. jejuni* or *H. pylori* OMVs (Ismail et al, 2003; Elmi et al, 2012; Mondal et al, 2016).

1.6.2 Anti-Inflammatory Roles

OMVs can also have anti-inflammatory effects. Monocytes incubated with OMVs from *B. abortus* showed reduced responses to LPS and flagellin, and subsequent infection with live *B. abortus* cells resulted in increased adhesion to and invasion of monocytes, implying that OMVs can help to promote infection by interfering with the innate immune response (Pollak et al, 2012).

Ulcerative colitis and Crohn's disease are chronic inflammatory diseases of the intestine. *E. coli* Nissle 1917 is a probiotic strain that is considered to be anti-inflammatory by inhibiting the release of inflammatory cytokines and as a result, reduce the symptoms and severity of ulcerative colitis (Losurdo et al, 2015; Fabrega et al, 2017). In a mouse model

of colitis, pre-treatment with OMVs from *E. coli* Nissle showed less weight loss, lower production of inflammatory markers, and less damage to the colon than observed in the control mice (Fabrega et al, 2017). Other studies have found *E. coli* Nissle to be as effective in inducing remission from colitis as the current recommended treatment mesalazine, and this work suggests that it may be the OMVs produced by this strain that have an important role as anti-inflammatory mediators (Losurdo et al, 2015; Fabrega et al, 2017).

The commensal species, *Bacteroides fragilis*, secretes OMVs carrying capsular polysaccharide polysialic acid (PSA). PSA from the OMVs was able to bind dendritic cells via TLR2, and resulted in increased numbers of T regulatory cells and a suppression of pro-inflammatory cytokine release, indicating a mechanism for non-pathogenic species to avoid triggering immune responses (Shen et al, 2012).

The immunomodulatory effects of OMVs can also benefit other bacterial species. OMVs from *M. catarrhalis* containing ubiquitous surface proteins UspA1/A2 were able to bind C3 of the complement system and prevented the complement cascade directed against *H. influenzae*, and promoted its survival (Tan et al, 2007).

OMVs from *P. gingivalis* carrying the cysteine proteases known as gingipains were shown to cleave the LPS co-receptor CD14 from the cell surface of macrophages, which

resulted in a suppressed TNF-a response to subsequent addition of purified *E. coli* LPS, and may represent a means of immune evasion (Duncan et al, 2004).

OMVs can also affect the adaptive immune system. OMVs from *H. pylori* induced apoptosis of T cells, independently of the presence of the VacA toxin, and thus suppressed T-cell mediated immune responses to infection (Winter et al, 2014). Interferon-ɣ stimulated endothelial cells present antigens to CD4+ T cells via the surface display of major histocompatibility complex (MHC Class II). After infection with *P. gingivalis* OMVs, IFN-ɣ signalling was inhibited which prevented the expression of the MHC II genes and the display of antigens, and therefore the T-cell response was not initiated (Srisatjalik et al, 2002). The Opa surface proteins of *N. meningitidis* are known virulence factors and are found in OMVs. Opa from *N. meningitidis* OMVs was able to bind human carcinoembryonic antigen-related cell adhesion molecule (CEACAM1) and inhibit the activation and proliferation of CD4+ T cells (Lee et al, 2007). These studies all suggest a means for OMVs to suppress adaptive immune responses and enhance infection.

1.7 Vaccine Applications

The ability of OMVs to elicit immune responses can also be advantageous for the host. OMVs from *S. enterica* Typhimurium were able to induce maturation of dendritic cells and activation of macrophages, and OMV-vaccinated mice were resistant to disease when

inoculated with live *Salmonella* cells due to the production of antibodies and CD4+ T cell responses (Alaniz et al, 2007). This indicated that the OMVs were able to generate both innate and adaptive immune responses and protected the host from subsequent infection. OMVs from the fish pathogen *Francisella noatunensis* protected zebrafish from a high dose inoculation of the live bacteria (Brudal et al, 2015).

OMVs have been investigated as vaccine candidates due to their immunogenicity and their relative safety as non-replicating antigens (Price et al, 2016). Their small size facilitates access to tissues and lymphatic vessels, and they are stable when stored for long periods of time (van der Pol, Stork and van der Ley, 2015). However, their use also presents significant challenges; the toxicity of their LPS, and the heterogeneity of their cargo which may not elicit a sufficiently strong and specific immune reaction (van der Pol, Stork and van der Ley, 2015). Several OMV-based vaccines have been developed against *N. meningitidis*, which typically use detergent extraction of OMVs to detoxify their LPS, and these vaccines have shown high efficacy against the specific strain used in the vaccine preparation but are often not cross-protective. A newer OMV vaccine, Bexsero, has been developed to also incorporate recombinant antigens and provide more immune coverage against other *N. meningitidis* serogroups (Acevedo et al, 2014).

B. parapertussis is a close relative of *B. pertussis*, and also causes whooping cough although its prevalence has been underestimated. Current vaccines against *B. pertussis* are not effective against *B. parapertussis*; in contrast, OMVs from *B. parapertussis* were

protective against both species in a mouse model, and were also effective when administered in the typical combination with tetanus and diphtheria antigens (Bottero et al, 2013). *Burkholderia pseudomallei* is the causative agent of melioidosis, a significant public health problem in South East Asia and Northern Australia, where it is endemic, and associated with high levels of morbidity and an estimated 42% mortality rate (Nieves et al, 2014). Several virulent strains are co-endemic, and there is currently no vaccine and little antibiotic susceptibility. OMVs from a clinical isolate of *B. pseudomallei* were protective against sepsis after challenge with live bacteria from the same strain, but also against other clinical isolates (Nieves et al, 2014). OMVs may therefore be more broadly antigenic and protective than currently used vaccines, and this could be exploited further against infectious diseases for which there is no vaccine available.

Engineering of OMVs to reduce their endotoxicity but enhance their expression of specific relevant antigens has been explored. OMVs from EHEC O157:H7 with a mutation in the *msbB* gene to reduce the endotoxicity of the LPS have been used to protect mice against hemolytic uremic syndrome (HUS) and also against a lethal dose of WT O157:H7 OMVs (Choi et al, 2014). This mutation was also used in the non-pathogenic *E. coli* W3110, and the LPS-modified OMVs showed reduced endotoxicity and stimulation of cytokine production, but caused comparable T-cell priming and upregulation of antigen presenting molecules, suggesting their potential use as safe adjuvants in vaccine preparations (Lee et al, 2011).

The protective immunity generated by OMVs can be expanded by the use of engineered vesicles. *V. cholerae* OMVs expressing PhoA from *E. coli* were able to generate specific PhoA antibody production in mice (Schild et al, 2009).

OMVs can be used to deliver antigens from unrelated species. *S. enterica* Typhimurium OMVs were generated to incorporate the surface protein PspA from the Gram positive pathogen *S. pneumoniae*, and vaccination of mice with these modified OMVs protected them from *S. pneumoniae* infection, whilst no protection was conferred after inoculation with purified PspA or empty vesicles (Muralinath et al, 2011). *Salmonella* OMVs were also used to display antigens from the intracellular pathogen *Mycobacterium tuberculosis*, and the antigens were then presented via MHC II on dendritic cells to result in activation of CD4+ T cells and initiate immune priming (Daleke-Schermerhorn et al, 2014). Similarly, *E. coli* OMVs were engineered to display the HtrA antigen from *Chlamydia muridarum*, resulting in antibody production and protection against Chlamydial infection (Bartolini et al, 2013). Non-pathogenic *E. coli* OMVs have been engineered to display antigens from *C. jejuni* and *S. pneumoniae* to elicit production of antibodies in vivo (Price et al, 2016).

As well as forming the basis of the vaccine preparations, OMVs have also been demonstrated to be useful adjuvants. Addition of OMVs to Protein D vaccination against non-typeable *H. influenzae* showed increased IgG production compared to Protein D alone, and was comparable to the commonly used adjuvant alum (Behrouzi et al, 2016).

Mice vaccinated against *Salmonella* showed a 6-40 times higher antibody titre when OMVs were subsequently administered, suggesting that the OMVs improved the immune response to the specific antigen (Schroeder and Aebischer, 2009).

1.8 Engineering of OMVs

Engineering of the cargo and surface of OMVs has been explored to broaden their potential applications in immunotherapy and as anti-cancer treatments. An early study by Kesty and Kuehn showed that fusion of GFP to the TAT signal sequence to allow its accumulation in the periplasm enabled incorporation of GFP in the OMVs subsequently isolated from non-pathogenic and pathogenic strains of *E. coli* (Kesty and Kuehn, 2004). The green fluorescent vesicles were also observed internalised in eukaryotic cells. However, there is not a linear relationship between periplasmic protein concentration and its concentration in OMVs (Alves et al, 2015). More recently, efforts have focused on ways to develop a more homogenous population of engineered OMVs, and to increase their overall yield. OMVs are cheap and quick to produce compared to the cost and labour involved in expression and purification of other antigens required for vaccines (Chen et al, 2010). Several genes have been identified which affect the amount of OMV production, including *tolR,* a gene of the Tol-Pal system which is required by Gram negative species for maintaining the integrity of the bacterial surface (Baker et al, 2014). Mutations in *tolR* result in a hypervesiculating phenotype, which has since been used for scaling up OMV production for vaccine preparations against *S. sonnei* and *F. tularensis* (McBroom et al, 2006; Chen et al, 2010; Berlanda Scorza et al, 2012).

ClyA is a cytolysin encoded by many strains of *E. coli*, with homologs in *Shigella* and *Salmonella* (Fahie et al, 2013). Despite containing no typical signal sequence for secretion, ClyA is exported from the bacterial cell and causes cytotoxicity upon contact with the mammalian cell membrane (Wai et al, 2003). Monomers of the 34kDa ClyA protein accumulate in the periplasm, in an inactive oxidised state (Wai et al, 2003). ClyA is exported into OMVs, where it becomes reduced, and forms an active oligomeric complex that is embedded in the membrane (Roderer et al, 2016). This suggests the redox conditions within the OMVs allow for the assembly and activity of ClyA, representing a form of protein export that is independent of other secretion systems present in Gram negative bacteria (Wai et al, 2003).

Since ClyA is targeted into OMVs, its fusion with other molecules allows their export in vesicles (Kim et al, 2008). ClyA-GFP fusions carried by OMVs elicited strong anti-GFP antibodies, compared with inoculation with the fusion protein alone, indicating that engineered OMVs can be used to generate immune responses to molecules that are usually not strongly immunogenic (Chen et al, 2010).

The ability of ClyA to localise in OMVs has also been investigated for treatment of cancers. Fusion of ClyA to an affibody against Human Epidermal Growth Factor 2 (HER2), a membrane receptor that is overexpressed in 18-25% of breast and ovarian cancers, allowed OMVs to target only cells expressing HER2 on the surface (Gujrati et

al, 2014). Furthermore, the ClyA-HER2 OMVs were loaded with siRNA against the kinesin spindle protein, which is upregulated in cells undergoing rapid proliferation. The engineered OMVs were able to target the HER2 expressing cells, and deliver siRNA, resulting in apoptosis and the reduction of tumour growth in a mouse model (Gujrati et al, 2014).

The glycan synthesis pathway has also been exploited for engineering of OMVs. Insertion of plasmids containing the locus for synthesis of a complex carbohydrate from pathogenic species such as *S. pneumoniae* or *C. jejuni* resulted in display of the glycan on the surface of the OMVs, and induced specific antibody production in mice and chickens respectively (Price et al, 2016). Glycan display is not just associated with infectious disease; tumour cells often have altered expression of surface glycans. Expression of Thomsen-Friedenrich antigen (T-antigen) on tumour cells is a hallmark of malignancy in many common cancers such as breast and prostate (Valentine et al, 2016). Mice immunised with OMVs engineered to display the T-antigen yielded high levels of anti-T-antigen antibodies, suggesting these engineered OMVs could enable the immune system to recognise its own cells (Valentine et al, 2016).

1.9 Biogenesis

The potential uses for OMVs could be expanded if more was understood about the biogenesis of OMVs. Whilst many genes have been implicated in their production, the highly conserved and ubiquitous nature of OMV shedding across all Gram negative

species suggests there may be a conserved mechanism but this is as yet unidentified (McBroom et al, 2006). Release of extracellular vesicles is not unique to Gram negatives, or indeed to bacteria, as it is also observed for some Gram positive species and archaea, and eukaryotic organisms (Lee et al, 2013; Gould et al, 2016).

Several mechanisms for vesicle biogenesis have been proposed, and there is ongoing debate surrounding these theories. There is evidence to support each of them, and whilst it is possible there is a single conserved mechanism, it is also likely that there are several different pathways that all contribute to OMV production.

Some theories have indicated a role of periplasmic peptidoglycan in enabling or directing OMV release. Peptidoglycan forms a rigid inner layer beneath the outer membrane and determines cell shape and holds its structure (Silhavy, Kahne and Walker, 2010). The outer membrane is attached to the peptidoglycan layer by Lpp, a highly abundant protein with around 500,000 molecules present in each cell (Silhavy, Kahne and Walker, 2010). One theory has suggested that a reduction in the cross-linking of peptidoglycan to the outer membrane allows the vesicles to pinch off from the cell surface, caused by the outer membrane expanding faster than the periplasm, allowing areas of the OM to become detached (Burdett and Murray, 1974; Hoekstra et al, 1976; Wensink and Witholt, 1981). Mutations in genes implicated in cross-linking, such as *lpp*, result in hypervesiculating phenotypes, supporting this theory (Bernadac et al, 1998). However, it does not account for the constitutive and ubiquitous release of OMVs.

Another theory suggests that it is not the reduced cross-linking of peptidoglycan that causes OMV release, but instead the accumulation of peptidoglycan fragments in the periplasm during cell growth exerts pressure on the cell wall, and release of OMVs allows this pressure to be alleviated (Zhou et al, 1998). A mutation in *yfgl*, a gene involved in accumulation and turnover of peptidoglycan, resulted in a reduction in OMV production, which was hypothesised to be due to reduced pressure on the cell wall (Rolhion et al, 2005). Vesiculation is highest during log phase, which supports the theory of OMVs being released as a consequence of cell growth and division (Hoekstra et al, 1976). However, it does not explain how cytosolic proteins are exported in the OMVs, nor for the exclusion of some OM proteins (Jan, 2017).

In contrast, it has been proposed that domains of the membrane that are enriched in certain molecules allow ionic interactions that induce curvature of the membrane and release of vesicles (Li et al, 1996; Nguyen et al, 2003). Specifically, the structure of the lipopolysaccharide molecules on the outer membrane have been implicated in inducing membrane curvature (Nguyen et al, 2003). *P. aeruginosa* producing more B-band LPS produced more OMVs, which is proposed to be due to the repulsion between the more negatively charged B-band LPS molecules, compared with A-band LPS, which could cause localised destabilisation of the membrane (Kadurugamuwa and Beveridge, 1995; Sabra et al, 2003). Although this accounts for enrichment and exclusion of certain molecules within OMVs, it does not explain production of OMVs in species with only

one form of LPS, or studies which show that OMV release is independent of membrane instability (McBroom et al, 2006).

The most recent mechanism that has been proposed has described a link between phospholipid trafficking and OMV production, and is the first theory to propose a general secretion mechanism that may be widely conserved and explain the ubiquity of OMV production (Roier et al, 2016). The conserved VacJ/Yrb system is responsible for maintaining the asymmetry of the OM by transporting phospholipids from the external surface of the membrane to the inner leaflet (Malinverni and Silhavy, 2009). Disruption within genes in this system resulted in increased vesiculation in the distantly related species *H. influenzae*, *V. cholerae* and *E. coli*, whilst overexpression had the reverse effect (Roier et al, 2016). The authors proposed that loss of retrograde phospholipid transport caused accumulation of phospholipids in the outer leaflet of the OM, causing membrane bulging and subsequent release of vesicles. Iron limitation is a common stressor for bacteria, particularly within a host, and it was also shown that this transport system was downregulated in response to iron starvation, and therefore increased phospholipid accumulation and OMV release (Roier et al, 2016). Iron limitation may trigger the production of OMVs during infection, helping the bacteria to exploit the broad uses of OMVs in pathogenesis, and may help to explain why pathogenic species produce more OMVs (Horstmann and Kuehn, 2000; Roier et al, 2016).

These conflicting theories all help to explain certain aspects of OMV release, but it remains to be identified whether there is a single universal process, or whether there are several contributing mechanisms which vary depending on species, growth phase or environmental conditions (Schwechheimer and Kuehn, 2015).

Whilst much has been learnt about the biogenesis, secretion and cargo of vesicles in recent years, there are still many areas left to explore. Their existence and function was ignored for several decades, despite their ubiquity across Gram negative species and beyond: it is likely that the scope of their role in bacterial interactions with the environment is still underestimated. Understanding their true contributions to bacterial survival requires new approaches, to overcome the problems caused by the heterogeneity of their production, cargo and size.

1.10 Entry of OMVs into host cells

(The following is adapted from O'Donoghue and Krachler, 2016.)

Whilst many studies have identified the cargo delivered into host cells by OMVs, there is still little agreement over how this delivery process occurs.

Endocytosis is a process by which small molecules can cross the membrane bilayer of a cell (Doherty and McMahon, 2009). In non-phagocytic cells, there are four main pathways for the entry of small solutes: Macropinocytosis, clathrin mediated endocytosis, caveolin mediated endocytosis, or non-caveolin, non clathrin mediated endocytosis

(Rewatkar et al, 2015). These pathways have all been implicated in mediating OMV entry into host cells (Figure 1.1).

OMV-mediated delivery of virulence factors occurs without requiring close proximity between the bacterial cell and the host cell (Bomberger et al, 2009). The small size of OMVs (20-200 nm) has made studying their interactions with host cells in real time difficult. Previous work has often relied on OMVs labelled with dyes such as fluorescein isothiocyanate (FITC) or dioctadecyloxacarbocyanine perchlorate (DiO). FITC labelling of OMVs from Enterotoxigenic *E. coli* (ETEC) revealed delivery of heat labile enterotoxin (LT) into host cells via lipid raft mediated endocytosis (Kesty and Kuehn, 2004). While such dyes allow real time study of OMV entry and cargo delivery processes, the use of membrane labelling of the vesicles may interfere with their physiological characteristics, and obscure the natural mechanisms of OMV entry and cargo release (Bauman and Kuehn, 2009; Parker et al, 2010). Current methods used for studying OMV entry are outlined in Table 1.

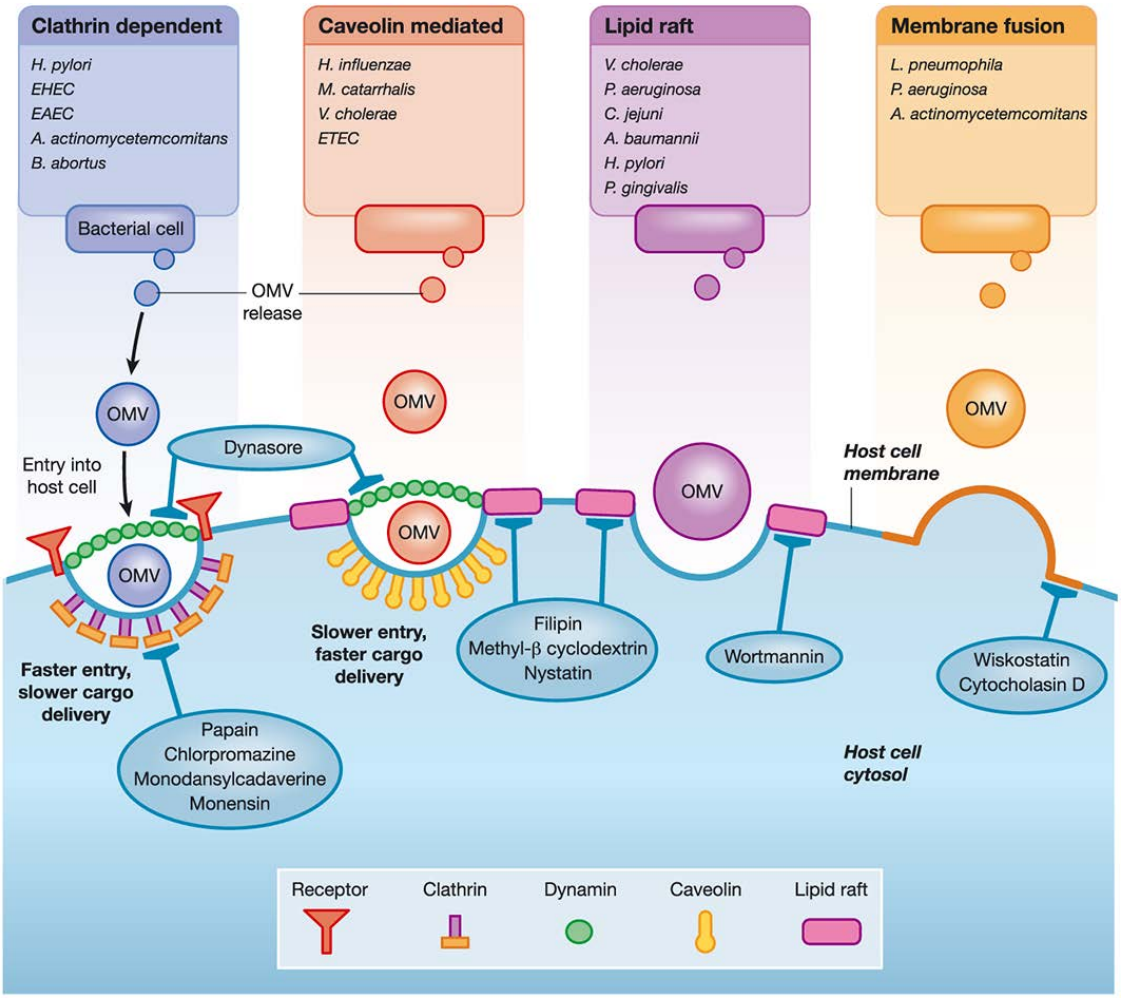

Figure 1.1. (From O'Donoghue and Krachler, 2016). Routes of OMV entry into host cells.

Several different pathways allowing OMVs from a variety of Gram negative species of bacteria to enter host cells have been described. These routes can require clathrin coated pits, formation of caveolae, and use of lipid rafts or direct membrane fusion. OMV entry can be impaired by the use of inhibitors against components of these pathways: chlorpromazine–inhibits clathrin coated pit formation; papain–proteolytically degrades surface protein receptors; monensin–ionophore, dissipates proton gradient; monodansylcadaverine–inhibits receptor internalization; dynasore–inhibits dynamin GTPase activity; methyl-β cyclodextrin–extracts cholesterol from membrane; filipin and nystatin–intercalate and disrupt cholesterol-rich membrane domains; wortmannin–inhibits phosphatidylinositol kinases; wiskostatin–inhibits N-WASP, which regulates actin polymerization; cytocholasin D–depolymerises actin.

Table 1. Overview of methods to determine OMV uptake by host cells.

Method of detecting OMV uptake by host cell	External	Bound	Internal	Advantages of method	Disadvantages	References
Antibody staining	✔	✔	✔	Shows delivery of OMV cargo Allows study of contributions of cargo to interactions with host cell for binding/entry processes or downstream cellular effects Enables visualisation of colocalisation with particular cellular compartments Detection via flow cytometry or microscopy	May obscure OMV epitopes that facilitate uptake Requires prior knowledge of OMV cargo and so may ignore subpopulations that are not detected with the antibody No data on kinetics of uptake due to requirement of fixation at pre-determined time points Need high concentrations of OMVs and epitopes in order to visualise with immunofluorescence microscopy	Furuta et al, 2009; Guidi et al, 2013; Jin et al, 2011; Kaparakis et al, 2010; Kim et al, 2010; Kunsmann et al, 2015; Mondal et al, 2016; Parker et al, 2010; Rompikontal et al, 2012; Thay et al, 2014; Vanaja et al, 2016
Lipophilic dyes for membrane labelling eg. DiO, PKH26	✔	✔	✔	Allows labelling of the whole OMV population Can determine interaction between OMV membrane and host receptors or lipid raft regions Can be used on live cells to resolve kinetics of uptake	Requires controls to prevent labelling of host cell membrane with excess dye Washing steps to remove extracellular vesicles Membrane labelling may affect normal behaviour of the OMV and its interactions with the host cell membrane Often required in combination with antibody labelling to prove the labelled membrane is OMV derived	Guidi et al, 2013; Kunsmann et al, 2015; Parker et al, 2010; Thay et al, 2014; Waller et al, 2016
Rhodamine R18		✔	✔	Allows labelling of the whole OMV population Can determine interaction between OMV membrane and host receptors or lipid raft regions Can be used on live cells to resolve kinetics of uptake	Requires controls to prevent labelling of host cell membrane with excess dye Washing steps to remove extracellular vesicles Membrane labelling may affect normal behaviour of the OMV and its interactions with the host cell membrane Often required in combination with antibody labelling to prove the labelled membrane is OMV derived	Bomberger et al, 2009; Rompikontal et al, 2012;
FITC labelling	✔	✔	✔	Allows non-specific labelling of OMV proteins Can be used in live cells Allows detection of OMVs outside and inside host cells	Unknown effects on natural OMV behaviours or uptake processes Non-specific so often required in combination with antibody labelled components	Chatterjee and Chaudhuri, 2011; Kesty and Kuehn, 2004; Pollak et al, 2012; Schaar et al, 2011; Sharpe et al, 2011
OMV targeted GFP	✔	✔	✔	No processing required Can be used in live cells Specific OMV fluorescence No observable effects on OMVs or host cells	Targeting sequence specific for *E. coli*, not tested for other species Need to engineer and verify strain prior to use	Kim et al, 2008.

1.10.1 Macropinocytosis

Macropinocytosis, or 'cell drinking' is characterized by the formation of large (over 200 nm in diameter), actin-driven, ruffled protrusions from the cell membrane, which allow the sampling and internalization of extracellular medium (Weiner et al, 2016). Its role in infection has been observed for *S. flexneri*, which invades host cells via macropinosomes (Weiner et al, 2016). The pathway is also utilized by viruses, which are comparable in size to OMVs (Mercer and Helenius, 2008). It has therefore been suggested that OMVs can enter host cells via macropinocytosis (Kaparakis-Liaskos and Ferrero, 2015). Inhibition of actin polymerization by cytochalasin D or wiskostatin has been observed to reduce the entry of OMVs from *P. aeruginosa* into airway epithelial cells (Bomberger et al, 2009). However, macropinocytosis is generally not a cargo induced process, and it is likely that entry via this route is not a deliberate OMV-driven event (Lim and Gleeson, 2011). Treatment with actin inhibitors is not entirely specific for macropinocytosis; movement of endosomes also requires actin remodeling, and so reduced cargo delivery after these treatments may also be due to the inadvertent effect on other endocytic routes (Soldati and Schliwa, 2006).

Macropinocytosis allows internalization of endocytic vesicles up to 1 um in diameter, whereas clathrin dependent and caveolin or lipid raft mediated endocytosis generally allows internalization of considerably smaller cargo (120 nm, 60 nm, and 90 nm respectively; Amano et al, 2010). The size of OMVs ranges from 20 to 500 nm, and this

heterogeneity may influence their preferred route of uptake (Amano et al, 2010; Kaparakis-Liaskos and Ferrero, 2015).

1.10.2 Clathrin dependent endocytosis

Clathrin mediated endocytosis occurs via the formation of clathrin coated pits up to 200 nm in diameter (Vercauteren et al, 2010). Unlike macropinocytosis, internalisation can be triggered by ligand binding to cell surface receptors (Rewatkar et al, 2015). Budding off of the vesicle requires dynamin, and the internalised vesicle enters the endosomal trafficking routes, from where its cargo can be returned to the cell surface or targeted to lysosomes for degradation (Ritter et al, 1995). Many bacterial virulence factors, such as shiga toxin, cholera toxin and the arg-gingipain adhesin of *P. gingivalis* have been shown to utilise clathrin mediated endocytosis to gain entry into host cells during infection (Sandvig and van Deurs, 2002; Boisvert and Duncan, 2008). Since OMVs are known to transport various virulence factors during infection, it is reasonable to infer that they can utilise toxin-receptor interactions to facilitate their cargo delivery via clathrin dependent endocytosis. Clathrin mediated endocytosis is typically inhibited using drugs such as chlorpromazine to prevent formation of clathrin coated pits, or dynamin inhibitors to prevent scission of the endosome from the membrane (Vercauteren et al, 2010).

Several studies have identified clathrin mediated endocytosis as a route for OMV entry. Vacuolating toxin VacA in *H. pylori* is an important cytotoxic virulence factor that is found in OMVs during infection (Parker et al, 2010). VacA containing OMVs entered

host cells more efficiently than their VacA deficient counterparts, in a cholesterol independent fashion, but inhibition of clathrin mediated endocytosis by chlorpromazine had a stronger inhibition on VacA deficient OMVs, suggesting that VacA is not a receptor ligand but may enable the OMVs to adapt to use alternative pathways in the absence of the clathrin mediated pathway (Parker et al, 2010). The OMVs were labelled with the lipophilic dye DiO, and intracellular fluorescence was measured using flow cytometry. It is not clear whether membrane labelling of OMVs affects their function or interaction with the membranes of host cells, and the affinity of lipophilic dyes for plasma membranes necessitates stringent controls and washing steps to ensure the dye does not label the cell membrane in addition to the vesicle (Mulcahy et al, 2014). Lipophilic dye molecules have been extensively used due to their efficient incorporation into membranes. However, the dye molecules can also form aggregates and enriched domains resulting in changes to the mobility and stiffness of the lipid bilayer, and these physical alterations may in turn affect the behavior of the labeled membrane (Lulevich et al, 2009).

Contradictory findings to work from Parker et al. were presented by Kaparakis et al (2010), who observed that entry of *H. pylori* OMVs was dependent on lipid rafts, and entry was significantly reduced after sequestration of cholesterol from the host cell membrane. A similar finding was also observed by Olofsson et al, which demonstrated a role for lipid-raft associated cholesterol in entry of *H. pylori* OMVs, which was inhibited by treatment with methyl-β cyclodextrin or filipin (Olofsson et al, 2014). OMV release is

a conserved phenomenon, but there are considerable differences in composition and activity of OMVs between species, between strains, and even between the same strain under different external pressures (McBroom and Kuehn, 2007). This may explain some of the discrepancies in the data regarding the uptake routes of OMVs from the same strains. The study by Kaparakis et al used Alexa Fluor labeled OMVs, with antibody labeling used to determine internalization and lipid raft stains to observe colocalization. However, using light microscopy to observe OMVs can be problematic due to their small size (often less than ~100 nm) and there is a need for a more reliable and high resolution method of quantifying and identifying internalization of OMVs, particularly when attempting to assess colocalization of OMVs with particular compartments of the cell (Mulcahy et al, 2014). Furthermore, antibody labeling may obscure OMV epitopes important in determining association with host receptors and thus, entry mechanism. Uptake of OMVs has been shown to be a rapid process, with internalization detected as little as 15 min following infection (Wai et al, 2003). Many methods involving use of immunofluorescence microscopy require fixation at pre-determined time points, and a live cell imaging method would be beneficial to define the kinetics of OMV interactions with host cells.

Methods used to isolate OMVs can also vary, with most using ultracentrifugation but others using sucrose gradients or commercially available isolation columns (Chutkan et al, 2013). The size of the OMV population is relevant when studying endocytic routes; clathrin mediated endocytosis generally allows internalisation of larger cargo than

clathrin-independent routes (El-Sayed and Harashima, 2010). Different isolation methods can introduce a bias towards particular sizes of OMVs, for example with the use of filters to exclude particles over 200 nm in diameter, or columns which allow retention of smaller particles and the lack of standardized isolation procedures may also explain some of the differences in findings in studies of OMVs from the same species (Kulp and Kuehn, 2010).

Other evidence for the entry of OMVs via receptor mediated endocytosis was recently described by Vanaja et al, (2016) who showed that in cells with an siRNA knockdown of AP2, an adaptor protein required for internalization of clathrin coated pits, there was a reduced response to the LPS delivered by EHEC OMVs. This indicated a reduction in the ability of the OMVs to enter the cell, which was also observed when the LPS of the OMVs was neutralized with polymyxin B, suggesting a functional link between LPS, clathrin and the induction of inflammatory responses (Vanaja et al, 2016). The fate of the LPS was to escape the endosomal compartments and induce caspase-11 activity, causing cytokine production and cell death. LPS can be a highly immunogenic component of OMVs, (Vanaja et al, 2016) and modification of LPS has been used as a way to reduce immunogenicity and enhance the suitability of OMVs as an adjuvant in vaccine preparations (Kim et al, 2009). The role of LPS during OMV host cell interactions is thus an attractive and important area for further investigation.

Caspase induction was also observed after incubation with enteroaggregative *E. coli* O104:H4 OMVs (Kunsmann et al, 2015). Labeled OMVs were found to contain several antigens, including shiga toxin, flagellin and enterotoxin, and caused cell death by the induction of caspase-9 mediated apoptosis, and inflammation through increased IL-8 release (Kunsmann et al, 2015). Treatment with dynasore and chlorpromazine significantly reduced the uptake of OMVs, suggesting entry of OMVs and their cargo occurs via the receptor mediated endocytic pathway (Kunsmann et al, 2015). Neutralization of OMV LPS with polymyxin B reduced the secretion of IL-8, in agreement with other studies indicating a role of LPS in driving pro-inflammatory responses (Kunsmann et al, 2015; Vanaja et al, 2016).

OMVs from EHEC containing the hemolysin HlyA were shown to enter host cells, with HlyA released from lysosomes into the cytoplasm where it was then trafficked to mitochondria, resulting in caspase-3 and caspase-9 activation and subsequent death of epithelial and endothelial cells (Bielaszewska et al, 2013). Treatment with dynasore and chlorpromazine significantly reduced OMV entry, suggesting EHEC-HlyA OMVs enter via clathrin mediated endocytosis. Fluorescence microscopy confirmed the colocalization of HlyA and clathrin, while there was no colocalization observed between HlyA and caveolin (Bielaszewska et al, 2013). When free HlyA was added to the cells, it remained at the cell surface and was not internalized, suggesting that the association with OMVs is necessary to allow efficient delivery into the cell (Bielaszewska et al, 2013).

Many studies have demonstrated a role for clathrin in the internalization of OMVs, but with the caveat that OMVs are able to compensate well in the absence of this entry route. Similarly to the finding by Parker et al that OMVs can utilize more than one route of entry, OMVs from *A. actinomycetemcomitans* showed a 25% reduction in uptake when clathrin mediated endocytosis was inhibited by monensin, and an equivalent reduction when cholesterol was bound by filipin (Parker et al, 2010; Thay et al, 2014). OMVs from *B. abortus* were also shown to enter monocytes primarily via a clathrin dependent route, with monodansylcadaverine treatment resulting in a 33% inhibition of OMV entry, and no effect seen after filipin treatment (Pollak et al, 2012). However, the partial level of inhibition implies that the OMVs are able to use alternative pathways. Interestingly, the study also showed that pre-incubation with OMVs prior to infection with whole cells inhibited the TNF responses, and increased the numbers of internalized *B. abortus*, demonstrating a role for OMVs in immunomodulation during or prior to subsequent infection. The ability of toll-like receptors to activate upon addition of their agonists was also reduced after pre-treatment with OMVs (Pollak et al, 2012). This study was conducted with monocytes rather than epithelial cells and there may be differences in entry of OMVs into phagocytic cells compared with non-phagocytic cell lines used in many studies (Pollak et al, 2012).

Incomplete levels of inhibition were also seen in studies with *H. pylori* OMVs, with a method termed 'Quantification of internalised substances' which labeled the *H. pylori* OMVs with a dye containing a cleavable disulphide bond, allowing quenching of

extracellular OMV-associated fluorescence by the addition of a reducing agent (Olofsson et al, 2014). Fluorescence inside the epithelial cells was then assessed with microscopy (Olofsson et al, 2014). This work demonstrated involvement of dynamin, with dynamin inhibition causing an 80% reduction in internalization, but chlorpromazine only reducing internalization by 40% (Olofsson et al, 2014). Dynamin is involved in both clathrin mediated and caveolin mediated endocytosis, and so it appears that there is a contribution of both clathrin mediated and caveolin mediated endocytosis towards OMV entry (Vercauteren et al, 2010).

Entry into a cell via the clathrin mediated endocytic pathway typically utilizes receptor-ligand binding to drive internalization (El-Sayed and Harashima, 2013). While this route has been implicated in many studies of OMV entry, the possible ligands have remained elusive. If internalization of OMVs requires these interactions, then identifying the components involved could allow the design of inhibitors to attenuate infections by preventing the delivery of OMV-associated virulence factors.

1.10.3 Non clathrin mediated endocytosis

Many studies have indicated a role for lipid rafts in enabling OMV entry (Furuta et al, 2009; Kaparakis et al, 2010; Jin et al, 2011; Schaar et al, 2011; Sharpe, Kuehn, and Mason, 2011; Elmi et al, 2012; Kim et al, 2010; Thay et al, 2014; Mondal et al, 2016) . Lipid rafts are domains of the plasma membrane that are enriched in sphingolipids and cholesterol (Mulcahy et al, 2014). The lipid composition of these domains causes them to

be more ordered and compact than neighboring regions (Simons and Ehehalt, 2002). Cholesterol-rich regions are abundant in the bilayer, and it is hypothesized that clustering of the regions allows curvature of the membrane, driving formation of invaginations in the host cell and entry of particles into the cell (Pelkmans, 2005). It is well-established that viruses exploit lipid rafts to enter host cells and the similarities between enveloped viruses and OMVs in terms of size and composition would suggest a potential affinity for this route of entry (Kulp and Kuehn, 2010).

Cholesterol is a principal component of lipid raft domains, and cholesterol dependency has been demonstrated for entry of OMVs from a variety of species (Bomberger et al, 2009; Furuta et al, 2009; Kim et al, 2010; Jin et al, 2011; Schaar et al, 2011; Sharpe et al, 2011; Elmi et al, 2012; Olofsson et al, 2014; Thay et al, 2014; Mondal et al, 2016). Cholesterol-rich microdomains are commonly disrupted by using chemicals such as methyl-β-cyclodextrin (mbcd, sequesters and depletes cholesterol from the cell membrane) or filipin (binds to cholesterol in the membrane and disrupts lipid packing, Danthi and Chow, 2004; Vercauteren et al, 2010; Maxfield and Wustner, 2012).

Many reports have used this approach to demonstrate the importance of membrane cholesterol for delivery of OMV cargo. OMVs from *Vibrio vulnificus* delivered cytolysins into epithelial cells to induce cell death, but this effect was diminished in the presence of filipin (Kim et al, 2010). Treatment with mbcd prevented delivery of OmpA from *A. baumannii* OMVs to host cells (Jin et al, 2011). OMVs commonly cause immune

activation via the induction of cytokines, and their production is measured using ELISAs to determine the level of inflammatory stimulation (Schaar et al, 2011; Sharpe et al, 2011; Elmi et al, 2012; Pollak et al, 2012; Kunsmann et al, 2015; Mondal et al, 2016; Waller et al, 2016). Treatment of host cells with mbcd prior to infection with OMVs from *Campylobacter jejuni* resulted in reduced production of IL-8, IL-6 and TNF-α (Elmi et al, 2012). The cargo of OMVs can also assist in allowing lipid-raft mediated entry processes. OMVs from a clinical isolate of *P. aeruginosa* displayed PaAP aminopeptidase on the surface and showed a 40% higher association with lung cells than the OMVs from a PaAP deletion strain, and this association was dependent on membrane cholesterol (Bauman and Kuehn, 2009).

1.10.4 Caveolin mediated endocytosis

Lipid raft domains can also be enriched in caveolin, and the oligomerization of caveolin allows formation of caveolae (Rewatkar et al, 2015). Caveolae are cave-shaped invaginations that are formed on the cell membrane, around 80 nm in diameter, and enriched in cholesterol, caveolins, and sphingolipids (Mulcahy et al, 2014). Similarly to clathrin mediated endocytosis, dynamin is also required for scission and internalization of caveolae (Rewatkar et al, 2015). Although the speed of caveolae internalization is around five times slower than that of clathrin mediated endocytosis, the efficiency of cargo delivery into the cytosol is much higher (Ritter et al, 1995).

Interactions between pathogens and caveolae have been reported, and caveolae have been suggested as a preferential invasion mechanism for many pathogens, including bacteria, viruses and fungi, as the internalised caveolae are thought to avoid fusion with lysosomal compartments and subsequent degradation, in contrast to clathrin coated pits (Anderson, Chen, and Norkin, 1996; Long et al, 2012; Lim et al, 2014). *E. coli* and *Leishmania chagasi* internalized via caveolae are able to persist within macrophages (Baorto et al, 1997; Rodriguez, Gaur, and Wilson, 2006). Chlamydial species are able to avoid detection during intracellular infection by using caveolins to disguise the internalized phagosome as a host-derived vesicle (Norkin, Wolfrom, and Stuart, 2001). Simian virus 40 (SV40) also enters host cells through caveolae, and uptake of exosomes produced from cells infected with Epstein Barr virus also requires caveolae (Anderson et al, 1996; Nanbo, Kawanishi, Yoshida, and Yoshiyama, 2013).

There are now numerous examples of OMVs utilizing caveolin mediated endocytosis to enter host cells. However, many studies often fail to distinguish between lipid raft dependency, which is inhibited by cholesterol depletion, and caveolin-specific lipid raft dependency, which is sensitive to both cholesterol and dynamin depletion. OMVs from non-typeable *Haemophilus influenzae* were shown to enter and colocalize with caveolin 1 (Cav-1), a marker of caveolae, by western blotting of epithelial cell lysates after infection (Sharpe et al, 2011). Treatment of cells with filipin to disrupt cholesterol rich microdomains in the membrane abolished this interaction. The same study showed that while binding of OMVs to the cell membrane could occur at 4 °C, internalization only

occurred after incubation at 37 °C. This is in agreement with work by Kesty et al. (2004) which showed reduced entry of ETEC OMVs into HT29 intestinal epithelial cells at 4 °C compared with 37 °C, and also by Jager et al. (2014) which demonstrated the temperature dependence of uptake for OMVs from *Legionella pneumophila*, suggesting that OMV entry is not a passive process.

Caveolin mediated endocytosis of OMVs has been found in many cases to utilize interactions between bacterial ligands and host cell receptors. OMVs from *M. catarrhalis* entered human epithelial cells via interactions between toll-like receptor 2 (TLR2) and lipid rafts (Schaar et al, 2011). Internalization of FITC-labeled OMVs was not observed after treatment with filipin, suggesting that the receptors were localized in cholesterol-rich regions of the membrane. Cholera toxin (CTx) is a virulence factor of *V. cholerae* known to bind to the ganglioside GM1 present in caveolin enriched lipid rafts on the host cell surface, and is secreted in both soluble and OMV-associated forms (Chatterjee and Chaudhuri, 2011). During infection of intestinal epithelial cells, OMV-associated CTx was shown to rapidly target GM1 after only 15 min and facilitate internalization of the OMVs (Chatterjee and Chaudhuri, 2011).

Similarly, entry of ETEC OMVs relied on the association of heat labile toxin (LT) contained within the OMV membrane with the toxin receptor, and immunofluorescence microscopy revealed colocalization of labeled caveolin and vesicles (Kesty et al, 2004). OMVs derived from an LT deficient strain showed a 60% lower association with host

cells, demonstrating the role of specific OMV cargo in driving uptake processes. Together, these reports suggest that OMVs from pathogens are adapted for delivery of virulence factors (Kesty et al, 2004).

1.10.5 Non caveolin mediated endocytosis

Alternatively, lipid raft mediated endocytosis can be independent of caveolin and dynamin and instead require small GTPases (Rewatkar et al, 2015). These GTPase dependent processes are the least well characterized of endocytic routes, but are generally defined as uptake into the cell via uncoated membrane invaginations (Mayor, Parton, and Donaldson, 2014).

OMV-associated proteases from *V. cholerae* reportedly were delivered into intestinal epithelial cells via this dynamin independent, lipid raft mediated endocytic route (Mondal et al, 2016). Induction of pro-inflammatory cytokines following infection with OMVs was measured using ELISA, and cytotoxicity determined using flow cytometry. Both responses were reduced after treatment with mbcd to deplete cholesterol, but no effect was observed after dynamin inhibition (Mondal et al, 2016).

The oral pathogen *P. gingivalis* secretes OMVs containing virulence factors such as gingipains and fimbriae, and these OMVs were shown to enter HeLa and gingival epithelial cells in a Rac1/lipid raft dependent manner, and independent of caveolin, clathrin, and dynamin (Furuta et al, 2009). Interestingly, these OMVs were rapidly

directed to lysosomes, but avoided degradation for over 24 hr following entry (Furuta et al, 2009). Despite being unable to deliver their contents into the cytosol, the strong and prolonged acidification of lysosomes induced by the OMVs caused cellular damage, even without delivery of specific virulence factors (Furuta et al, 2009).

OMVs can also influence host cellular responses without entering cells. When all endocytic routes were inhibited, OMVs from *P. gingivalis* were still able to cause suppression of immune signaling and increase tolerance to subsequent infection through the induction of TLR4 on the cell surface of monocytes (Waller et al, 2016). In some cases, OMVs can even cause effects in host cells distal from the initial site of infection and OMV production. OMVs from *S. enterica* were produced by whole bacteria in the *Salmonella* containing vacuole (SCV) and the vesicles were able to escape not just the SCV but the infected host cell, and enter uninfected neighboring cells to deliver the genotoxin cytolethal distending toxin (CDT), revealing the ability of OMVs to migrate from the cell of origin (Guidi et al, 2013).

1.10.6 Membrane fusion

Despite the different architecture of the membrane bilayer present in OMVs and that of host eukaryotic cells, membrane fusion has been described as a mechanism for OMV entry into host cells. The self-quenching fluorescent dye Rhodamine-R18 was used to label OMVs from *P. aeruginosa* (Bomberger et al, 2009). When added to host epithelial cells, an increase in fluorescence was observed due to lipid mixing between vesicle and

host membrane bilayer, leading to dilution and de-quenching of the dye. The increase in fluorescence was used as a quantitative determinant of membrane fusion between the OMV and the cell membrane (Bomberger et al, 2009). Lipid rafts were labeled with CTxB subunit, and there was colocalization between sites of membrane fusion and the labeled lipid rafts, and the fusion events were inhibited in the presence of filipin. This indicated that membrane fusion events preferentially occur at lipid raft domains (Bomberger et al, 2009). A similar technique was used to assess membrane fusion between OMVs from *A. actinomycetemcomitans* and HeLa cells, which utilized confocal microscopy to identify colocalization between the toxin component cytolethal distending toxin (CDT) and the labeled lipid rafts and sites of dequenched membrane labeling (Rompikuntal et al, 2012). A caveat of studies employing mbcd and filipin to deduce the involvement of lipid rafts in OMV uptake is that both agents, by disrupting a major constituent of the membrane, affect membrane organization on a large scale and may have effects on processes not limited to lipid rafts.

Model membranes have been utilized to confirm that membrane fusion can occur between OMVs and host cell membranes, despite their structural differences. Phospholipid liposomes mimicking host cell membranes were labeled with a pair of FRET dyes to demonstrate that OMV membrane material from *L. monocytogenes* can be included into the model host bilayer, by monitoring the change in FRET signal, which increased upon incorporation of OMV membrane due to an increase in surface area (Jager et al, 2014). The fusion events occurred just seconds after addition of OMVs,

highlighting the rapid and efficient kinetics behind OMV interactions with host cells (Jager et al, 2014). The interaction was also observed to be partially temperature dependent, with a lower level of incorporation when the experiment was conducted at 4 °C compared to 37 °C, but there was still a notable level of membrane fusion detected, suggesting that fusion does not entirely depend on active, energetic processes (Jager et al, 2014).

There are considerable discrepancies between findings determining OMV entry routes into host cells. Differences in uptake routes between OMVs from different species may well be explained by the fact that OMV composition is adapted to direct vesicles towards a specific uptake route, and thus allow them to undergo ideal processing within the host cell to facilitate infection. However, discrepancies also exist between studies analyzing entry of OMVs from the same species. This may be due to variation in methodologies, such as the isolation and quantification of OMVs, the labeling or imaging techniques, or the strains, host cell types and cell lines used. It is also apparent that OMVs can use multiple routes to enter host cells.

Different isolation techniques result in different levels of purity and/or size distributions of the OMV preparations. Quantification of OMVs used for uptake assays also varies between studies, and particularly the use of total protein contained within the OMV preparation as a means of normalization is problematic, as protein content and composition can vary widely between OMV preparations derived under different growth

conditions (McBroom and Kuehn, 2007). Despite these challenges, the existing literature clearly demonstrates that OMVs are well adapted to direct and deliver their cargo into host cells. In order to fully elucidate the mechanisms underpinning these processes, it is necessary to develop a consistent, quantifiable and dynamic approach to measure OMV association, entry and cargo delivery to host cells.

Deepening our understanding of how the structure of the vesicle surface can direct OMVs towards a specific uptake route, and thus, determine the fate of vesicular contents within the host cell is essential and will allow the future exploitation of OMVs for medical applications (Berleman et al, 2013). OMVs engineered to display a ClyA-HER2 targeting probe were able to target cells over-expressing HER2, a common biomarker of cancer cells, and induce cell death and tumor shrinkage via the delivery of siRNA targeting the expression of kinesin spindle protein (Gujrati et al, 2014). Insights on OMV entry would allow production of engineered OMVs with high affinity for specific cell types or tissue locations, and enhance their potential as novel therapeutic agents (Alves et al, 2015; Gao et al, 2015).

1.11 Composition of Outer Membrane Vesicles

1.11.1 The cell envelope of E. coli

Exploring the interactions between OMVs and host cells requires an appreciation of their origin from the bacterial cell envelope. Bacteria experience a wide range of challenging

and dynamic environmental conditions, and are able to survive and adapt in part due to their sophisticated cell envelope (Silhavy, Kahne and Walker, 2010). The unique and complex structure allows for compartmentalisation of the cytoplasm and confers protection and resistance to hostile environmental conditions, such as heat, acid and the presence of antibiotics (Ruiz, Kahne and Silhavy, 2006). Cell envelope synthesis and maintenance requires a significant energy contribution by the bacterial cell; a fifth of the *E. coli* genome is dedicated to the cell envelope, an indication of its vital role in determining cell structure, passage of molecules into and out of the cell, and as a site for many important metabolic processes (Goemans et al, 2014).

1.11.2 Inner membrane

The cell envelope of the Gram negative model organism *E. coli* consists of 3 layers. The inner membrane is a typical phospholipid bilayer that forms a fluid boundary between the cytoplasm and the cell wall. In *E. coli*, it is around 5.5 nm thick and is composed primarily of three phospholipid species: Phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Weiner and Rothery, 2007). It allows protection and separation of cytosolic contents. Due to the absence of organelles in bacteria, many cellular functions, such as lipid and peptidoglycan synthesis, active transport and cell division are performed at the inner membrane. It therefore contains a diverse range and high concentration of lipoproteins and integral membrane proteins necessary for carrying out these roles (Weiner and Rothery, 2007).

1.11.3 Periplasm

External to the inner membrane is a viscous section known as the periplasm (Goemans et al, 2014). It is enriched in proteins, and segregation of these proteins, such as nucleases and proteases, in the periplasmic layer allows their functions to be performed away from the cytoplasm and prevent cell damage, analogous to lysosomes in eukaryotic cells (Weiner and Li, 2008; Silhavy et al, 2010). The periplasm is estimated to comprise 20-30% of the total cell volume, occupying around 50 nm (van Wielink and Duine, 1990). Within the periplasm is a layer of peptidoglycan, a polymer consisting of repeating units of the disaccharide N-acetylglucosamine-N-acetyl muramic acid (Vollmer et al, 2008). Peptidoglycan helps to maintain cell rigidity and shape, and is involved in cell growth and division. Its essentiality to bacteria has been exploited through the use of β-lactam antibiotics which inhibit the formation of peptidoglycan cross linkages and lead to cell lysis (Kohanski et al, 2010). The thin layer of peptidoglycan in Gram negative species is around 10 fold thinner than in Gram positives, which have many layers of peptidoglycan, and a thickness of up to 100 nm (Vollmer et al, 2008).

1.11.4 Outer membrane

Anchored to the peptidoglycan in the periplasm is the second, outer-most membrane, that is absent in Gram positive bacteria. This membrane forms a considerable, selective barrier that is the interface between the bacterial cell and its environment. Its integrity is crucial for survival. Use of antibiotics against Gram negative species of bacteria require the ability to permeate the outer membrane (Delcour, 2009). Some antibiotics, such as

vancomycin, are ineffective against Gram negatives as they cannot traverse this barrier (Miller, 2010). Unlike the inner membrane, this is not a typical membrane bilayer. The outer membrane is asymmetrical, with phospholipids comprising the inner leaflet, but the outer leaflet is composed of lipopolysaccharide (LPS). It contains fewer proteins than the other layers of the envelope, and these proteins are typically either trans-membrane anti-parallel β-barrel structures, (known as OMPs) in contrast to the α-helices found in the inner membrane, or lipoproteins (Weiner and Li, 2007). Unlike in the inner membrane, there is no proton motive force available to drive substrate transport across the OM and instead these highly expressed β-barrels form channels known as porins which allow the free movement of small (<600 Da) hydrophilic molecules through the outer membrane (Bos et al, 2007; Miller, 2016). Cells which down regulate expression of porins are able to resist β-lactam antibiotics which enter via this route (Harder et al, 1981).

1.11.5 Lipopolysaccharide (LPS)

At the outer surface of the bilayer is lipopolysaccharide (LPS). This molecule consists of 3 main regions, from inside to out: Lipid A, core, and O polysaccharide. Forming the top of the outer membrane bilayer is Lipid A, a glucosamine disaccharide with 6 or 7 acyl chains, which is attached to the polysaccharide core region which, in turn, is linked to the highly variable polysaccharide termed the O polysaccharide. The variability of the O polysaccharide is used to classify and differentiate serotypes of *E. coli*.

1.11.6 Lipid A

Lipid A is synthesised by 9 enzymes, and its formation occurs in the cytoplasm and the inner membrane (Wang and Quinn, 2010). LpxA, C, and D convert UDP-GlcNAc into UDP-diacyl-GlcN by the addition of two fatty acid chains. This is then hydrolysed, condensed and phosphorylated by LpxH and LpxB, before phosphorylation by LpxK. The KdtA enzyme then adds Kdo (deoxy-D-manno-oct-2-ulosonic acid) residues. The final two steps result in the addition of a lauryl and myristoyl residue to the final glucosamine residue by LpxL and LpxM acyltransferases (Raetz et al, 2007). MsbA is an essential ABC transporter that is required to transfer newly synthesised Lipid A from the inner membrane to the outer membrane; in its absence, LPS accumulates at the inner membrane (Zhou et al, 1998).

Lipid A is a highly abundant molecule, and although the majority of species are not viable without Lipid A or LPS, its absence can confer advantages. Spontaneous loss of genes required for LPS and Lipid A biosynthesis in *A. baumannii* allow resistance to colistin, an antibiotic which targets Lipid A (Henry et al, 2012). Lipid A is a potent innate immune activator in mammalian cells, specifically macrophages (Raetz and Whitfield, 2002). Innate immunity is triggered by the presence of pathogen-associated molecular patterns (PAMPs) which are recognised by pattern recognition receptors (PRRs) (Lizundia et al, 2008). In the case of LPS, this occurs via the recognition of the acyl chains by Toll-like Receptor 4 on the cell surface (Akira et al, 2006). LPS is bound by the LPS binding protein, LBP, which converts LPS into monomers that can in turn

associate with CD14 on the cell membrane (Miller et al, 2005). Lipid A is then transferred to MD2-TLR4 at the extracellular surface and oligomerises to recruit signal transduction adaptor proteins via the TIR (Toll Interleukin-1 Receptor) domain of TLR4. Signal transduction is either MyD88-adaptor dependent or independent, resulting in release of pro-inflammatory cytokines, or maturation and activation of adaptive immune cells (Kawai et al, 2001; Akira et al, 2006; Lu et al, 2008; Needham and Trent, 2013).

Recognition of Lipid A by macrophages results in the release of pro-inflammatory modulators such as TNFα and IL1β, and in turn activates the adaptive immune response in order to control the infection (Rietschel et al, 1994). Regulation of these responses is linked to other diseases; severity of asthma is positively correlated with the concentration of LPS in house dust (Michel et al, 1996). A strong and uncontrolled over-induction of these responses can lead to septic shock, organ failure and mortality (van Deuren, 2000).

Sepsis is a severe complication of infection, and over half of cases occur in the context of a Gram negative bacterial infection (Wenzl, 1992). A single nucleotide polymorphism from aspartic acid to glycine at residue 299 of the TLR4 gene is a relatively common genotype, (~10-20% of the population) that results in changes to the extracellular domain of the receptor and is associated with a reduced response to LPS, increased number of infections, and a subsequent defect in clearing infection (Arbour et al, 2000; Agnese et al, 2002). Although this mutation resulted in a reduced localised response to LPS, surprisingly it was also found exclusively in patients with septic shock and was absent in

the healthy controls, suggesting a fine balance between the induction of the TLR4 response and the control of infection (Lorenz et al, 2002).

Lipid A is well conserved amongst Gram negatives, and combined with its essentiality it has long been a target for the development of new antibiotics (Jackman et al, 2000). In addition, engineering of strains with less immunogenic Lipid A have also been explored for use as vaccine adjuvants (Needham et al, 2013).

Variations in the number of acyl chains affects the ability of TLR4 to recognise Lipid A. Six acyl chains has been demonstrated to elicit the strongest binding between Lipid A and MD-2/TLR-4 (Park et al, 2009). LpxM/MsbB catalyses the final myristoylation step during the synthesis of Lipid A, and its mutation did not affect growth but resulted in penta-acylated Lipid A in *E. coli* (Clementz et al, 1997). Penta-acylated Lipid A in *E. coli* reduced immune activation and inflammatory responses and also reduced its virulence in a mouse model, compared with the wildtype hexa-acylated Lipid A (Kim et al, 2004). Similarly, the purified LPS from an *msbB* mutant caused a 10,000 fold reduction in TNFα production by monocytes (Somerville et al, 1996).

Other host defence mechanisms which target Lipid A include the production of cationic anti-microbial peptides (CAMPs). CAMPs are found in phagocytes, mucus layers and secretions (Needham and Trent, 2013). These positively charged molecules are able to bind and integrate into the negatively charged bacterial membrane, altering the membrane

potential and causing cell death (Kagan et al, 1990; Needham and Trent, 2013). In the human intestinal epithelia, one of the most commonly expressed CAMPs are the α-defensins which are formed of β-sheets with 3 disulphide bridges (Peschel and Sahl, 2006). They have anti-microbial activity against diverse pathogenic species, encompassing Gram negative and Gram positive bacteria, but also fungi and viruses (Gallo and Hooper, 2012). CAMPs represent a broad spectrum defence against infection, but bacteria have also found means of defending themselves against them. In *Salmonella* species, addition of positively charged groups to Lipid A via PhoP/PhoQ and PmrA/B reduces the affinity of the positively charged CAMPs for the bacterial membrane (Guo et al, 1998).

Platelet Factor 4 (PF4) is a chemokine released upon activation of platelets, and is another positively charged molecule that is able to bind to Lipid A. The PF4-Lipid A complex triggers the production of specific antibodies. The bacterium then becomes opsonised and targeted for destruction and clearance by the immune system (Krauel et al, 2012).

1.11.7 Core

Attached onto the synthesised Lipid A is the core oligosaccharide. The inner core is mostly well conserved, and consists of the relatively unusual sugars Kdo and Hep (L-glycero-D-manno heptopyranose), with other common sugar residues decorating the backbone (Heinrichs et al, 1998; Frirdich and Whitfield, 2005). The Kdo residue is

necessary for survival, and one unit of Kdo is the smallest possible core structure that retains viability (Helander et al, 1988).

KdsA is responsible for synthesising Kdo-8-phosphate from D-arabinose 5-phosphate and phosphoenol pyruvate (Schnaitman and Klena, 1993). The resulting CMP-Kdo is activated by KdsB, and attached to Lipid A by WaaA (Schnaitman and Klena, 1993). Heptose residues are required for membrane integrity, and are synthesised by WaaC, and transferred to the inner core by WaaF and WaaQ (Heinrichs et al, 1998). Phosphoryl groups are added to the inner core by WaaP (Heinrichs et al, 1998).

The outer core in *E. coli* differs in composition but the structure consists of three linked hexose sugars, usually of glucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine, that are attached by glycosyltransferases encoded by the *waa* gene cluster, with two side chain residues (Heinrichs et al, 1998; Erridge et al, 2002). For all *E. coli* and *Salmonella* species, the first residue is glucose (Heinrichs et al, 1998). The outer core consists of more typical sugars compared with the inner core but is less well conserved within a species, with 5 types found in *E. coli*; the K12 type and types R1-R4. Core R1 and R4 are the most similar to each other, with one alteration between the side branch (Amor et al, 2000). The R1 core type is the most common, while R3 predominates in verotoxinogenic strains, but is not necessarily linked to virulence *per se* and may have been acquired alongside more relevant virulence determinants (Amor et al, 2000; Frirdich and Whitfield, 2005).

1.11.8 O polysaccharide

The O polysaccharide (or O antigen) is attached to the final sugar of the core by WaaL (Han et al, 2012). In 'rough' strains such as *E. coli* K12, the outer core is the outer-most region of the membrane, and no O polysaccharide is attached (Amor et al, 2000). Strains with LPS containing O polysaccharide are termed 'smooth'. The O polysaccharide is a polysaccharide made up of up to 40 repeats of 2-6 sugars (Lerouge and Vanderleyden, 2002). Unlike the core region of LPS, there is considerably more variation in the O polysaccharide structure, with 186 different serotypes present in *E. coli* and *Shigella* (Samuel and Reeves, 2003). The O polysaccharide is variable in the sugar composition, linkages between units, and the number of repeats (Samuel and Reeves, 2003).

The genes for synthesis of the O polysaccharide are found in clusters, with three groups of genes generally required, for synthesis, formation of the O unit, and translocation and polymerisation into the full O polysaccharide (Figure 1.2, Samuel and Reeves, 2003). The first step occurs in the cytoplasm, where the sugar biosynthesis enzymes catalyse the production of the sugar precursors. Glycosyltransferases then transfer the precursors to the carrier lipid undecaprenyl pyrophosphate (UndPP) in the inner membrane, and then transfer the subsequent precursors to form the oligosaccharide. Next, processing and translocation occurs: wzx allows the UndPP linked O unit to transfer into the periplasm, where wzy polymerises the units into the completed O polysaccharide, the length of which is controlled by wzz (Samuel and Reeves, 2003). WaaL then catalyses the

attachment of the O polysaccharide to the core, by forming a glycosidic bond between the UndP-linked sugar and the final residue of the core region (Han et al, 2002).

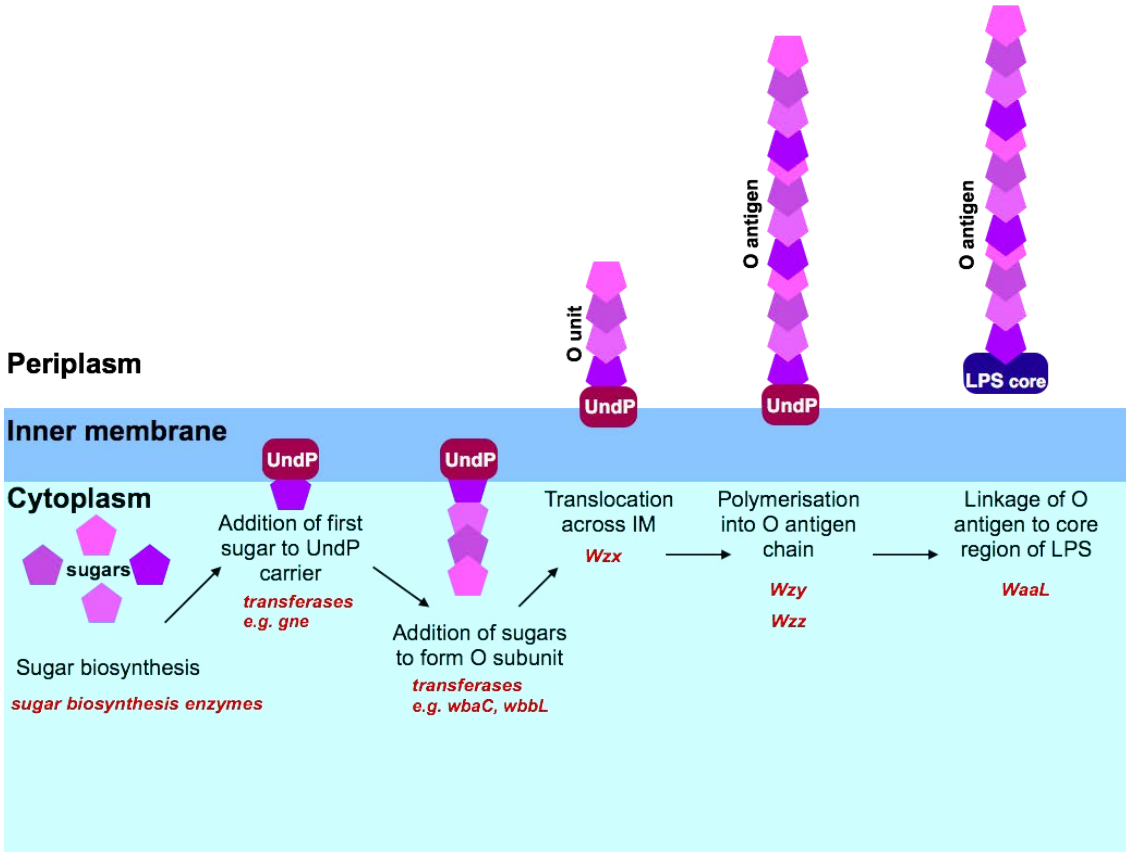

Figure 1.2. O polysaccharide synthesis in E. coli

The O polysaccharide is not essential for viability, but it is certainly associated with survival, particularly outside of a laboratory setting (Reeves, 1995). Presence of O polysaccharide can confer resistance to bacteriophages which use outer membrane proteins as receptors prior to invasion (van der Ley et al, 1986; Ho and Waldor, 2007). The O polysaccharide can also aid in responding to environmental conditions; In *M. xanthus*, the O polysaccharide is required for social motility and the formation of the multi-cellular fruiting body, which occurs when nutrients are limited (Bowden and Kaplan, 1998). At a pH of below 5, *H. pylori* was found to increase transcription of *wbcJ*, a gene involved in O polysaccharide biosynthesis (McGowan et al, 1998). *wbcJ* mutants were found to be acid sensitive, and since *H. pylori* is able to colonise the stomach which has a pH of around 2, it is possible that the O polysaccharide contributes to acid resistance. The protozoan *Naegleria gluberi* prey on *Salmonella* species and are able to select their choice of prey based on the expression of certain O polysaccharides, suggesting that the wide variation in O polysaccharide composition has developed as a means of avoiding predation (Wildschutte et al, 2004). The wide variation in the structure of O polysaccharides cannot be explained as a mechanism to avoid immune detection; O polysaccharides are highly immunogenic and many pathogenic strains express only one type of O polysaccharide, when it would be more beneficial to alter this structure if it would facilitate immune evasion.

In the host, the presence of the O polysaccharide can reduce phagocytosis, and the longer the O polysaccharide (up to 100 repeating units) the lower the level of ingestion of *S.*

enterica by macrophages (Murray et al, 2006). A *P. aeruginosa* strain that expressed an unusually long O polysaccharide was not cleared from the blood during infection, compared with the strain expressing shorter O polysaccharide (Ohno et al, 1995). Likewise, a rough strain of *S. enterica* was eliminated quickly from the bloodstream, whereas the smooth strain persisted (Ohno et al, 1995).

O polysaccharide can also increase resistance to complement and bile (Murray et al, 2006; Crawford et al, 2012). It is not just the length that can increase resistance to complement and phagocytosis, as it was also observed in Salmonella that the differences in the sugar composition affected the rate of uptake, and the virulent strains altered to express O polysaccharides from less virulent strains were phagocytosed faster (Liang-Takasaki et al, 1982). In addition, those expressing O polysaccharides from the most virulent strains were the most resistant to phagocytosis, implying that the carbohydrate composition of the O polysaccharide is able to alter the recognition by macrophages. The density of O polysaccharide affects serum resistance, as Grossman et al (1987) found that steric hindrance of the C5b-9 complex of complement requires a minimum concentration of 4-5 O polysaccharide units per LPS molecule.

Loss of O polysaccharide in the lung pathogen *Actinobacillus pleuropneumoniae* caused a defect in the ability to form biofilms (Hathroubi et al, 2016). In contrast, clinical isolates of *P. aeruginosa* showed no expression of O polysaccharide during biofilm growth, but this was restored when in planktonic culture (Ciornei et al, 2009). The

'rough' LPS isolated from the cells in the biofilm was also more immunogenic, and induced an enhanced cytokine response compared with the planktonic smooth LPS (Ciornei et al, 2009).

Presence of O polysaccharide does not always confer enhanced pathogenicity; the opposite effect is also observed. *Yersinia pestis* is the causative agent of plague, and a primary virulence factor is a surface expressed protease, Pla, which is able to activate plasminogen in the bloodstream and cause proteolysis, leading to invasive and systemic infections (Kukkonen et al, 2003). Activity of Pla is dependent on the absence of O polysaccharide, likely due to the fact that its long chains would obscure Pla from its target (Kukkonen et al, 2003). Studies in *S. flexneri* showed that rough mutants of virulent strains were still able to invade HeLa cells, and transfer of the O polysaccharide from a virulent smooth strain to an avirulent rough strain had no effect on its ability to penetrate epithelial cells (Okamura et al, 1983).

The gastrointestinal pathogen *Yersinia enterocolitica* contains a virulence plasmid, pYV, but presence of this plasmid alone is not sufficient for pathogenesis, unless in combination with the presence of O polysaccharide (Bengoechea et al, 2004). Strains without O polysaccharide had an LD50 1000 fold higher than the parent strain, whereas strains expressing just one O polysaccharide unit had an LD50 just 10 fold higher in a mouse model of infection, suggesting expression of a single O unit can contribute to virulence (Bengoechea et al, 2004). O polysaccharide has been linked with serum

resistance in other species. For *Y. enterocolitica*, presence of O polysaccharide does not confer serum resistance when grown at room temperature, but 'smooth' strains were more resistant than the 'rough' strains when grown at 37 °C. The expression of its O polysaccharide is temperature regulated, with highest expression at 25 °C, and lower expression at 37 °C, the temperature within a host. Since other virulence factors of *Y. enterocolitica* are also temperature regulated, the O polysaccharide status may act as a regulator or signal for expression of other virulence factors (Bengoechea et al, 2004; Perez-Gutierrez et al, 2007).

In this work, the role of O polysaccharide in OMV entry into host cells was studied in the context of OMVs from three different strains: two pathogenic strains of *E. coli*, EHEC and EAEC, and the lab strain *E. coli* K12.

1.12 Enterohemorrhagic *E. coli* (EHEC)

EHEC O157 is an important human pathogen, spread via contaminated food and water, and infects around 100,000 people per year in the USA (Rangel et al, 2005). Whilst the number of infections is low compared with other enteric bacteria, such as *Campylobacter jejuni* or *Vibrio cholerae*, the severity of disease and its complications, particularly in children and the elderly, leads to the hospitalisation of up to 50% of those infected (Eppinger and Cebula, 2015). All EHEC strains produce shiga-like toxins, but the O157 serotype is particularly virulent in humans and is responsible for the majority of cases of

acute hemorrhagic colitis, which can progress to hemolytic uremic syndrome (HUS) in around 10% of cases in children under 10 years old (Tarr, 1995). Due to the low infectious dose (less than 100 organisms) and the severity of symptoms, EHEC O157 is a reportable disease agent and is responsible for significant outbreaks of disease, typically originating from contaminated food, including meat, or vegetables such as cucumbers or bean sprouts (Tarr, 1995). It also has a considerably higher mortality rate than other serotypes of EHEC; an outbreak in Central Scotland in 1996, traced back to a butcher's shop, caused 279 confirmed cases and 21 deaths (Pennington, 2014).

EHEC strains have a considerable armory of virulence factors. Shiga-like toxins are potent virulence factors, but expression of toxins in non-pathogenic strains does not enable them to cause disease (Law, 2000). EHEC O157 produces Stx2 type shiga toxin, which is 1000 fold more toxic to endothelial cells than the Stx1 form (Louise and Obrig, 1995). The toxin is formed of 2 subunits: The 32 kDa A subunit, and a pentamer of 7 kDa units forms the B subunit ring. This binds to the host cell membrane via Globotriaosylceramide (Gb3) and enters the cell through receptor mediated endocytosis. Once inside the cell, if the A subunit has avoided lysosomal degradation, it is converted to its active form by cellular proteases, whereupon it cleaves 28S ribosomal RNA and inhibits protein synthesis, leading to cell death (Law, 2000). EHEC O157 is commonly isolated from cattle, yet is not usually associated with disease in the animal host, and this is proposed to be due to the lack of expression of the Gb3 receptor (Tree et al, 2009).

The EHEC O157 chromosome also encodes a pathogenicity island known as the locus of enterocyte effacement (LEE). The LEE genes encode proteins for the assembly of the Type 3 secretion system, which enables both intimate attachment to host cells, via intimin, and the direct secretion of proteins into the host cell, facilitating rearrangements in the actin cytoskeleton. This results in actin 'pedestals' on cultured cells, which promote colonisation of EHEC, and causes attaching and effacing (A/E) lesions in the gut (Tree et al, 2009).

EHEC O157 is far more commonly associated with human disease than other EHEC serotypes, despite expression of the same virulence factors. The O157 O polysaccharide has been suggested as a possible virulence factor (Ho and Waldor, 2007). Synthesis of the O157 antigen in EHEC requires a 14 kb gene cluster. These genes encode 5 enzymes for synthesis of the sugar units, 4 transferases to export to the inner membrane, 1 flippase to transfer across the outer membrane and 1 polymerase to add the subunits together to form the long O polysaccharide (Wang and Reeves, 1998). Sequence analysis of the genes for O polysaccharide biosynthesis revealed that the transferases, flippase and the polymerase are unique for the synthesis of O157 (Wang and Reeves, 1998). The O157 subunit is comprised of N-acetyl D-perosamine, L-fucose, D-glucose and N-acetyl D-galactose (Shimizu et al, 1999). Precursors of the O polysaccharide unit are assembled onto Und-P-P at the inner membrane by transferase enzymes, prior to their diffusion across the outer membrane and their polymerisation into the completed O polysaccharide (Samuel and Reeves, 2003). In EHEC, the *gne* gene encodes an epimerase enzyme required for the

synthesis of GalNAc-PP-Und lipid intermediate, the precursor for the first GalNAc unit (Figure 1.2, Rush et al, 2010).

Analysis of the O157 O polysaccharide determined an average length of 30 nm, and the length of the O polysaccharide was positively correlated with its adhesive strength (Strauss et al, 2009). Other studies have also linked O157 to adherence. EHEC mutants lacking the O157 antigen had a reduced ability to bind to lettuce compared with the wildtype, and this may be due to the increased hydrophobicity of the cells in the absence of the hydrophilic O polysaccharide (Boyer et al, 2011). The ability of EHEC to survive and contaminate food surfaces is key to its route into humans to facilitate disease. O polysaccharide deficient mutants showed impaired growth and survival in a silkworm model, due to their increased sensitivity to anti-microbial peptides and serum, and were also unable to colonise and kill the host compared with the wildtype (Miyashita et al, 2012). Similarly, O157 negative mutants were 100 fold more sensitive to an AMP produced by the epithelial cells of the intestine, which would significantly impair the ability to colonise the gut (Ho and Waldor, 2007).

Cattle are reservoirs of EHEC O157. Clearance of infection occurred faster for O polysaccharide negative EHEC, compared with the wildtype EHEC O157. After 32 days, 6 of the 7 cattle infected with the O polysaccharide deficient strain were culture negative, compared with only 1 of the 7 for EHEC O157, indicating that the O polysaccharide plays a role in colonising and persisting within the bovine intestine (Sheng et al, 2008).

The *wzz* gene in *E. coli* controls O polysaccharide chain length. Typically, O polysaccharides of *E. coli* contain 10-18 repeats (Osawa et al, 2013). Mutations or deletions in *wzz* lead to variations in the chain length, and in these experiments, three different phenotypes were generated: short chain (with less than 15 units), intermediate (with 15-20 units) and long (over 20 units). The strains with longer chain lengths had a higher survival in serum, particularly at higher concentrations, compared with intermediate or short chain lengths (Osawa et al, 2013).

1.13 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* (EAEC) are pathogenic strains which cause diarrhoea and are able to form characteristic aggregative biofilms in the intestinal tract, as well as a characteristic 'stacked brick' pattern of adhesion to epithelial cells *in vitro* (Wilson et al, 2001; Chaudhuri et al, 2010). Unlike for EHEC, animals are not considered to be a reservoir for disease, and instead EAEC is typically acquired from its ability to grow on food sources (Okhuysen and Dupont, 2010). EAEC infection is commonly associated with 'travellers diarrhoea' and is prevalent in Africa and South America, where it can be endemic in the population, especially in children (Harrington et al, 2006; Okhuysen and Dupont, 2010). EAEC is frequently detected in patients with diarrheal disease, and in one large cohort study in the UK, it accounted for almost 5% of 3506 cases, compared with just 0.1% for EHEC O157, and 4% for *Salmonella* (Wilson et al, 2001). It was also found in healthy people, and was detected in 1.7% of 2772 control samples, whereas only 0.4% of samples contained *Salmonella*, and EHEC O157 was not detected at all (Wilson et al,

2001). This indicates that, unlike O157, presence of EAEC is not always associated with disease, and as EAEC infection commonly affects the immunocompromised and HIV patients, it may be that the immune system can typically control infection to an extent (Garcia et al, 2010). The sub-clinical persistence of EAEC in the generally hostile environment of the gut, even in healthy subjects, may be due to the formation of its thick, aggregative biofilms that help it to evade immune responses, and confer protection from anti-microbial peptides and antibodies (Okhuysen et al, 2010). EAEC triggers pro-inflammatory responses through the interaction of flagellin with Toll-like receptor 5, causing the release of IL-8 (Khan et al, 2004).

Persistent diarrhoea is an illness that affects millions of children globally, particularly in the developing world, where malnutrition is both a risk factor and an outcome of the disease (Lima et al, 2000). A study in Brazil found that EAEC was responsible for 68% of cases of persistent diarrhoea in children under 3 years of age (Fang et al, 1995). In a neonatal mouse model, persistent EAEC infection caused up to 47% growth impairment, and malnutrition increased the burden of infection by up to 4 logs (Roche et al, 2010). Persistent EAEC infection can therefore lead to a continual cycle of malnutrition and diarrhoea, especially in impoverished communities with poor sanitation (Lima et al, 2000).

The aggregation and adherence of EAEC is conferred by the plasmid pAA. The genes of pAA are used clinically to distinguish EAEC from commensal *E. coli* (Harrington et al,

2006). Transfer of this plasmid to K12 also transferred its aggregative ability (Nataro et al, 1992). pAA encodes the genes for the highly positively charged aggregative-adherence fimbriae (AAF). The transcription of genes on pAA is controlled by AggR, upstream of which is a gene encoding a small, immunogenic 10.2 kDa protein known as dispersin (Sheikh et al, 2002). Dispersin mutants show hyperaggregation, indicating the role of this protein is to counteract the AAF and reduce aggregation, which may then allow the bacteria to penetrate the intestinal mucus layer (Sheikh et al, 2002). Dispersin is secreted to the outer membrane of the bacterial cell, where it non-covalently binds to LPS. Dispersin is hydrophilic, unlike AAF, and so its abundance on the membrane may act to reduce the auto-aggregation of the hydrophobic fimbriae by preventing their interactions (Sheikh et al, 2002).

EAEC also secretes a 116 kDa serine protease autotransporter known as Pic. This protein was demonstrated to have a nutritional role for EAEC; it allowed use of mucin as a carbon source, allowing the removal of the thick mucus layer that can inhibit colonisation of the intestinal epithelium, but also providing a nutritional benefit to the cells in an environment where sources of nutrients can be limited (Harrington et al, 2009).

O42 is a common serotype of EAEC (Chaudhuri et al, 2010). Although it was observed that EAEC can be carried asymptomatically, 3 out of 5 adults infected with EAEC O42 developed diarrhea, while the other three EAEC serotypes investigated did not cause disease in any of the subjects (Nataro et al, 1995). The O42 antigen subunit is comprised

of 4 sugars: Glycero a-D glucopyranose, 2-acetamido 2-deoxy b-D Glucopyranose, 2-acetyl b-D galactofuranose and a-D N-acetyl glucopyranose. *wbaC* encodes a mannosyl transferase, which catalyses the formation of mannosyl linkages between the residues, and disruption to this gene inhibits O polysaccharide synthesis (Figure 4.1, Browning et al, 2013).

1.14 *E. coli* K12

E. coli K12 has been used in laboratory settings as a model organism for almost a century (Browning et al, 2013). It was originally isolated from a fecal sample from a diphtheria patient (Bachmann, 1972). Since this isolation in 1922, it has undergone a series of mutations that have enabled it to adapt to its life in the laboratory (Stevenson et al, 1994). Culturing of *E. coli* in the lab is not representative of their natural habitats (Fux et al, 2005). Laboratory strains of *E. coli* appear to readily lose their O polysaccharide, suggesting that it is not advantageous in this context, in contrast to *E. coli* isolated in the 'wild' which almost invariably express O polysaccharide (Stevenson et al, 1994). A K12 strain, MG1655, is a 'rough' strain that has undergone an IS-5 insertion, called the rfb50 mutation, in the *wbbL* gene of the rfb cluster, causing loss of expression of its native O16 O polysaccharide (Liu and Reeves, 1994). *wbbL* was found to encode a rhamnosyl transferase, required for the addition of rhamnose as the second sugar in the O16 subunit (Figure 4.1, Stevenson et al, 1994). The O16 antigen subunit consists of N-acetyl glucosamine, a-L rhamnose, a-D glucose, and B-D galactofuranose (Stevenson et al, 1994).

When expression of the O16 antigen was restored to generate MG1655 L9, it was able to colonise the intestine of *Caenorhabditis elegans* and cause disease, suggesting that the O polysaccharide is a requirement for pathogenesis (Browning et al, 2013). It also casts doubts upon the use of K12 as a model organism, when it does not replicate the behaviour of its restored O polysaccharide counterpart, let alone naturally occurring strains of *E. coli* (Browning et al, 2013).

Sequence comparisons of K12 and O157 revealed that K12 has not lost the genes necessary for pathogenesis, but in fact O157 has acquired them (Hooper and Berg, 2002). Around 4.1 kb of chromosomal DNA is conserved in *E. coli* strains, but the EHEC O157 chromosome is 5.5 kb. However, it does not just gain genes, as it has also lost 0.53 kb that is present in K12, suggesting it has selected its genome during its evolution as a successful pathogen (Perna et al, 2001; Lim et al, 2010).

The use of OMVs as delivery vehicles for virulence factors by pathogenic strains has been well established. The vast majority of Gram negative bacteria in the human body are non-pathogenic. However, this does not mean that they are harmless. OMVs released by the avirulent DH5α were able to enter cells of the intestinal line Caco-2 and induce DNA damage and production of free radicals, similar to the effects caused by OMVs from a pathogenic invasive strain of *E. coli* (Tyrer et al, 2014). Since all species of Gram negative bacteria that have been studied have been found to release OMVs, it is likely that their role in both disease and health have been under explored.

1.15 Aims

Whilst understanding and appreciating the behaviour and contributions of OMVs to bacterial fitness is an area of increasing interest, there are still many methodological barriers to overcome. The primary aim of this work is to address the need for a new approach to studying the entry kinetics of OMVs into host cells, through the development of an assay that can sensitively and quantitatively monitor their uptake in real time.

This assay can then be used to explore whether the presence of O polysaccharide in OMVs influences the ability of OMVs to enter host cells.

2. Materials and Methods

2.1 Strains used

The strains used in this study were the *E. coli* serotype O157:H7 strain Sakai 813, a derivative of enterohaemorrhagic *E. coli* (EHEC) RIMD 0509952, and its O polysaccharide deficient derivative, MA6 (Δ*gne,* Rump et al, 2010); the *E. coli* serotype O42 wild type strain (an enteroaggregative *E. coli* isolate, Chaudhuri et al, 2010), and its isogenic, O polysaccharide deficient derivative strain *(*Δ*wbaC*, Browning et al 2013); the *E. coli* serotype O16 strain DFB 1655 L9 (a K12 strain containing a restored *wbbL* gene), and its isogenic, O polysaccharide deficient derivative, MG1655 (Browning et al, 2013).

2.2 Cloning of ClyA-Bla and Bla ClyA into pBad Kan

pBad24 ClyA-Bla and Bla-ClyA (provided by Matthew DeLisa, Cornell University) and pBad18 KanR were all digested using NcoI and HindIII restriction enzymes (NEB) at 37 °C for 2h. The digested products were run on a 0.8 % agarose gel at 100V for 1h. The new vector and insert bands were excised from the gel and purified using the Qiagen Gel Extraction kit. 1 µl of vector was added to 13 µl of insert, with QuickStick DNA ligase for 15 minutes at room temperature to ligate the insert and vector. The ligation mixture was then transformed into chemically competent *E. coli* 5-alpha (NEB) using the heat shock protocol and plated on Lysogeny Broth (LB) agar with 50 µg/ml kanamycin (Sigma) overnight at 37 °C (Froger and Hall, 2007). Colonies were then selected, and the plasmid was isolated using a Qiaprep plasmid miniprep kit (Qiagen). 5 µl of purified

plasmid was added to 100 µl of electrocompetent EHEC, EAEC and MG1655 strains in 0.1 cm electrocuvettes (BioRad) on ice, and transformed using electroporation with the BioRad Micropulser, before outgrowth in SOC media (NEB) for 1h at 37 °C. Cells were plated onto LB Kan overnight at 37 °C (Gonzales et al, 2013). To verify the presence of the ligated plasmid and insert, colonies were added to 5 ml LB and grown overnight at 37 °C, with shaking at 200 rpm, and the following day, plasmid was isolated from 1 ml of cells, using a Qiaprep plasmid miniprep kit (Qiagen) and 10 µl was digested again with NcoI and HindIII (NEB) for 1h at 37 °C. After digestion, 10 µl DNA loading dye (NEB) was added to the digestion mix, and run on a 0.8 % agarose gel stained with SybrSafe (Thermo Fisher) for 1h at 100V, and imaged under UV light in the BioRad ChemiDoc imaging system to verify presence of insert (2 kb) and vector (4 kb).

2.3 Preparation and analysis of OMVs

2.3.1 Isolation of outer membrane vesicles

Overnight cultures of EHEC, EAEC and MG1655 containing pBad ClyA Bla or Bla ClyA were set up in 5 ml LB with 50 µg/ml Kanamycin (Sigma). The following day, the cultures were diluted 1:100 in 100 ml LB and were grown at 37 °C, with agitation at 200 rpm until OD600nm reached 0.5-0.6. The cells were then induced with 0.2% L-arabinose (Sigma) and grown for a further 16h. Cells were pelleted for 20 minutes at 6000 xg, and the supernatants were removed and filtered with a 0.45 µm syringe filter (Merck). Aliquots of filtered supernatants were spread on LB agar and grown overnight at 37 °C to check that all viable cells had been removed by filtration. 25 ml of filtered supernatants

were centrifuged in a Beckman XL90 ultracentrifuge using a 70Ti rotor at 100,000 xg (32,000 rpm) for 2h, 4 °C. After centrifugation, supernatants were removed, and the OMV pellets were resuspended in 1 ml colourless DMEM or sterile water (for TEM) and stored at -20 °C.

2.3.2 Fluorescent labelling of OMVS

For fluorescently labelled vesicles, 1 µl of 1x Cellmask Orange plasma membrane stain (ThermoFisher) was added to 1 ml isolated OMV fractions for 1h, in the dark with gentle shaking, at room temperature. After 1h, the OMV samples were ultracentrifuged as before to remove excess dye. The stained OMVs were resuspended in 1 ml colourless unsupplemented Dulbecco's Modified Eagle Media (DMEM).

2.3.3 Nanoparticle tracking analysis

Nanoparticle tracking analysis allows for the measurement of size distribution and concentration of nanosized particles suspended in liquid by tracking their Brownian motion and the scattering of laser light (Filipe, Hawe and Jiskoot, 2010). After purification, 1 ml of OMV samples were diluted by adding 10 µl of the neat fraction to 1000 µl of filtered sterile PBS for a 1:100 dilution, and this was repeated twice more for 1:10,000 and 1:1,000,000 dilutions. Particle diameter and concentration was measured using the Nanosight LM10 (Malvern) nanoparticle tracking analysis, with a minimum of 100 tracks per sample, performed in triplicate. Camera shutter 1495 and gain of 450 were used, and the size frequency distribution was determined using GraphPad Prism.

2.3.4 Measurement of OMV charge

The Malvern Zeta sizer applies an electric charge to particles in a solution, and the rate of their movement is related to their zeta potential and surface charge (Clogston and Patri, 2010). 700 µl of OMVs were added to cuvettes and the surface charge of the sample was measured using the Malvern Zeta sizer. An average of 30 readings was used to determine the charge of particles in the samples. Values between +10 mV and -10 mV are considered to be uncharged (Clogston and Patri, 2010).

2.3.5 Visualisation of outer membrane vesicles by Transmission Electron Microscopy

10 µl of isolated outer membrane vesicles in sterile deionised distilled water were added to 400-mesh copper grids, and negatively stained with 4% uranyl acetate for two minutes. Samples were then observed using a Jeol 1200Ex transmission electron microscope with an acceleration of 75 kV (Birmingham Electron Microscopy Facility).

2.3.6 Protein Quantitation

To quantify levels of protein in cell fractions, the ThermoFisher CBQCA Protein Quantitation kit was used. 10 µl of protein was added to 125 µl 0.1M sodium borate, 5µl 20mM KCN, and 10 µl 5 mM ATTO-TAG in a 96 well plate, and incubated in the dark for 1-2h. The fluorescence in samples was measured with an excitation at 465 nm and an emission at 550 nm in a FluoStar OMEGA plate reader. The level of fluorescence in

samples was compared to known quantities of bovine serum albumin, and this was used to determine the protein concentration in the samples in μg/ml.

2.3.7 Nitrocefin assay to determine β-lactamase activity

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

2.3.8 Western blotting of EHEC ClyA-Bla fractions

12 μl samples from EHEC ClyA-Bla and whole cell lysate, supernatant and OMV fractions (induced, uninduced) were added to 3 μl 5X SDS loading dye and boiled for 10 minutes. Samples were loaded onto a 15 well BioRad pre cast stain-free SDS PAGE gel and run at 120 V, 200 mA for 45 minutes. The gel was then transferred onto a PVDF membrane in transfer buffer containing 20% methanol for 80 minutes at 100 V. After transfer, the membrane was blocked at room temperature in TBS 0.1% Tween-20 and 5% non fat milk for 1h with agitation (Liu et al, 2014). The membrane was washed 3X with TBS 0.1% Tween-20 (5 minutes per wash). After blocking, the membrane was incubated with a 1:2000 dilution of mouse anti-bla primary antibody (Abcam) in TBS 0.1% Tween-20 and 5% non fat milk overnight at 4 °C with agitation. The following day, the membrane was washed 3X as before, and incubated with a 1:5000 dilution of sheep

anti-mouse secondary antibody (Sigma) in TBS 0.1% Tween-20, 5% non fat milk for 1h at room temperature with agitation (Liu et al, 2014). The membrane was washed again 3x, and 2 ml BioRad ECL reagents were added to the dried membrane and incubated for 5 minutes, before visualisation with the BioRad ChemiDoc imager.

2.3.9 Papain and detergent treatment of OMVs

The detergents Triton X-100 and SDS were added at a concentration of 1% to 20 µl OMVs for 45 minutes at 37 ºC to disrupt OMV membranes. 5 µl of 0.01% cysteine protease papain (Sigma) was then added for 30, 45 or 60 minutes at 37 ºC to degrade proteins in the sample. The papain reaction was inactivated using 1 mM of the serine protease inhibitor PMSF at room temperature for 30 minutes. 5 µl SDS-PAGE loading dye was added to the samples, which were then boiled for 10 minutes. Samples were run on a 15 well pre-cast stain free gel (BioRad) for 45 minutes at 120V, and then proceeded to Western blotting with anti-bla primary antibody (Abcam) as described previously (Liu et al, 2014).

2.4 Cell culture

HeLa (Human cervical epithelial) cells (ATCC number CRM-CCL-2) were maintained in Dulbecco's Modified Eagle Medium, (DMEM, Sigma) with 1000 mg glucose per litre, and supplemented with 1% L-glutamine, 1% Penicillin/Streptomycin and 10% heat inactivated fetal bovine serum (FBS, Sigma). Cells were grown at 37 ºC, 5% carbon dioxide, and were split via trypsinisation upon reaching 60-80% confluency in T75 culture flasks.

RKO (human colonic epithelial) cells (ATCC number CRL-2577) (Ahmed et al, 2013) were maintained in DMEM with 4500 mg glucose per litre (Sigma), with 1% L-glutamine, 1% Penicillin/Streptomycin and 10% heat inactivated fetal bovine serum (FBS, Sigma). Cells were grown at 37 ºC, 5% carbon dioxide, and were split via trypsinisation upon reaching 60-80% confluency in T75 culture flasks.

Mycoplasma contamination was checked twice throughout the project using MycoAlert detection kit (Lonza).

2.5 Confocal Microscopy

2.5.1 CCF2-AM loaded HeLa cells

HeLa cells (P3-7) were seeded on 13 mm coverslips in a 12 well plate at a concentration of $1x10^5$ cells per ml in complete DMEM 24h prior to infection. For infection with reporter OMVs, cells were washed and loaded with 100 µl 6X CCF2-AM dye with 500 µl colourless unsupplemented DMEM, and incubated in the dye solution for 1h at room temperature in the dark. Cells were then infected with 15 µg/ml reporter OMVs for 0-4h at 37 ºC. The cells were washed with PBS and then fixed with 0.5 ml 4% PFA for 15 minutes. The next day, coverslips were mounted onto slides with a drop of Gold Anti-Fade mounting solution (ThermoFisher) and then imaged using the Nikon A1R confocal microscope (Birmingham Advanced Light Microscopy Facility), with fluorescence observed from excitation at 409 nm and two separate emissions at 460 nm and 530 nm, to visualise green and blue cells. Z stacks were produced from 20 slices, from the top to bottom of cells to show the distribution of fluorescence throughout the whole cell volume. Z stacks were converted to maximum intensity projection images to show the fluorescence through 20 layers within the cells.

2.5.2 Fluorescently labelled OMVS

HeLa cells (P3-7) were seeded on 13 mm coverslips in a 12 well plate at a concentration of $1x10^5$ cells per ml in complete DMEM 24h prior to infection. The following day, cell mask orange labelled OMVs were added to cells at a concentration of 10^8 vesicles per ml. Cells were incubated with OMVs at 37 ºC for 10 minutes, or 60 minutes, prior to fixation

with 0.5 ml 4% PFA for 15 minutes at room temperature. Coverslips were mounted as before, and slides were imaged using an Olympus IX83 inverted microscope fitted with a FV3000 confocal system and 100x Super Apochromat oil objective. Images were captured using Olympus Fluoview software and processed using the CellSens extension package, to visualise membrane labelled OMVs on the surface and within HeLa cells.

2.6 Plate reader FRET experiments

2.6.1 Loading of cells with CCF2 substrate prior to infection

HeLa cells or RKO cells (passage 1-7) were seeded in triplicate in a black-walled, clear bottom 96-well plate (Greiner) at a concentration of $1x10^5$ cells per ml in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 1% L-glutamine, 1% Penicillin/Streptomycin and 10% heat inactivated fetal bovine serum. The plate was incubated at 37 ºC, 5% CO_2 for 24h prior to experiments. The following day, cells were loaded with 20 µl 6X CCF2-AM with 100 µl colourless DMEM (cDMEM) with 10mM HEPES and incubated at room temperature for 1h in the dark to allow dye loading. The dye was removed by washing 2x in PBS and 1x in cDMEM.

2.6.2 Transwell assay for monitoring delivery from whole bacterial cultures

HeLa cells were seeded in 24 well plates at a concentration of $1x10^5$ cells per ml in complete DMEM as before. Overnight cultures of EHEC ClyA Bla and EHEC empty vector were set up in 5 ml LB, and grown at 37 ºC with shaking at 200 rpm. The next day, the bacterial cultures were diluted 1:100 in colourless unsupplemented DMEM, HeLa cells were washed and media was replaced with 700 µl colourless DMEM with 10 mM HEPES, and 200 µl of bacterial culture was added to 0.4 µm transwells above the cells. 0.2% L-arabinose was added to the bacterial cells to induce plasmid expression. The plate was added to the Phera Star Omega (BMG) plate reader at 37 ºC. The wells were scanned (bottom optic) with orbital averaging, with excitation at 405 nm and simultaneous dual

emission at 530 nm and 460 nm, and the fluorescence was detected every 2 minutes for a total of 15 hours.

2.6.3 Inhibition of uptake processes

For inhibition experiments, cells were treated with previously verified chemical inhibitors of endocytic pathways. HeLa cells were treated with 5 mM methyl-β cyclodextrin (mbcd) to remove cholesterol (Contreras et al, 2010) or 1 μg/ml filipin to bind cholesterol (Ilnytska et al, 2013), 80 μM Dynasore for dynamin inhibition (Girard et al, 2011), or 20 μM blebbistatin for macropinocytosis inhibition (Ramanathan et al, 2015) (all Sigma), for 1h prior to infection at 37 ºc. Cells were treated with 1 μg/ml chlorpromazine for 1h at 37 ºc to inhibit formation of clathrin coated pits (Papatheodorou et al, 2010). Wells were washed with 100 μl PBS prior to infection. To remove surface proteins, 5 μg/ml of the cystein protease papain (Sigma) was added for 15 minutes at 37 ºc, before inactivation of papain by 5 mM PMSF for 20 minutes. Due to detachment of cells from the well after papain treatment, these wells were not washed prior to infection.

2.6.4 Supplementation with LPS, and inhibition of TLR4

Rough LPS (from *E. coli* EH100 Ra mutant strain, Sigma) or smooth LPS (from *E. coli* O55:B5, Sigma) was added at a concentration of 1 μg/ml for 30 minutes prior to infection, at 37 ºC. The TLR4 inhibitor C34 (Sigma) was added at a concentration of 10 μM for 30min at 37 ºC prior to co-incubation of OMVs and cells. Wells were not washed prior to infection, to maintain both LPS and C34 in the media.

2.6.5 Infection with OMVs

The CBQCA protein quantitation kit was used to determine the protein concentration in isolated OMVs, which were then diluted in cDMEM and added to the cells for a final protein concentration of 10 μg/ml to allow for OMVs from the same isolation to be used for all the biological and technical replicates (Jager et al, 2015) This concentration was determined using the Nanoparticle tracking analysis to be equivalent to $1x10^8$ vesicles. HeLa cells were seeded at a concentration of $1x10^5$ cells per ml, corresponding to an MOI of 1000. This MOI (equivalent to 10 μg/ml) represents a relatively low number of OMVs, in comparison with other studies which have used up to 250 μg/ml, to test the sensitivity of the assay (Chitcholtan et al, 2008). After addition of OMVs, the plate was immediately placed in the PheraStar Omega (BMG) plate reader, with excitation at 405 nm and simultaneous dual emission at 530 nm and 460 nm. The wells were scanned (bottom optic) with orbital averaging for a total of 150 cycles, equating to a measurement every 90 seconds for 3 hours.

2.6.6 Efficiency of uptake and statistical analysis

The ratio of blue to green fluorescence intensity detected in the cells at each cycle was calculated using GraphPad Prism, as an indicator of OMV uptake. Ratios for uninfected, dye-loaded cells were used as the baseline value for each cycle. All traces were normalized to 0 for their first ratio value. All experiments were performed with three

technical replicates and three biological replicates. Means and standard deviation were plotted over time.

Efficiency of uptake was calculated as the total change in blue:green fluorescence intensity ratio after 3h. Three technical replicates and three biological replicates were performed for each plate reader experiment and these were treated as 9 independent replicates. An ANOVA with correction for multiple comparisons, with a Brown Forsythe test for equal variance, was used to determine the statistical significance of the total change between samples and between samples and the relevant controls, or a Student's t test where there were less than three samples, using GraphPad Prism software. A p value of < 0.05 was considered statistically significant.

2.6.7 Rate estimation and statistical analysis

To estimate the gradients of the data, polynomials were fitted to each data set generated from the fluorescence assay using the cubic spline function *csaps* in Matlab (by Dr. Sara Jabbari, School of Mathematics, University of Birmingham). Numerical estimates of the gradients of the resulting polynomials were determined using the *gradient* function. To ensure that the gradient estimates were as smooth as possible whilst also retaining the overall shape and trend of the data, a small smoothing parameter was used.

As before, an ANOVA with correction for multiple comparisons was used to analyse data sets, with a Brown Forsythe test to determine equal variance using GraphPad Prism

software, with multiple comparisons used to assess differences between experimental samples and the relevant control. A p-value of < 0.05 was considered statistically significant.

3. Developing methods for studying uptake of OMVs by host cells
(Adapted from O'Donoghue et al, 2017)

3.1 Introduction

The discrepancies in previous observations of OMV entry and cargo delivery may be attributable to the different methods used in these studies, demonstrating the need for an assay that can detect OMV entry processes in a consistent and repeatable manner. This chapter describes a novel assay to continuously measure OMV uptake and cargo delivery to host cells with high sensitivity, that is adaptable for high throughput screening, and explores the kinetics of OMV interactions with host cells.

This assay relies on the use of a genetically encoded hybrid reporter probe that is carried by OMVs. ClyA, a cytolysin that is preferentially targeted into OMVs by *E. coli*, acts as the targeting component, and is fused to β-lactamase at either the C- or N-terminus, which acts as the reporter component (Kim et al, 2008). This fusion disrupts the ability of ClyA to assemble into its toxic oligomeric conformation (Kim et al, 2008). Host cells are incubated with CCF2-AM, a dye composed of a covalently linked coumarin and fluorescein molecule. The dye readily enters the host cells, where it is modified by esterases, preventing its exit from the cell. Uncleaved CCF2-AM has an emission at 530 nm, detected as green fluorescence. When the reporter OMVs enter the host cells, the β-lactamase cargo is able to cleave the bond between the dye molecules, causing a disruption to FRET, and a detectable shift in emission from green (530 nm) to blue (460 nm) (Figure 3.2A). This shift in emission can be monitored in real time to determine the rate of uptake and delivery of OMV cargo to host cells, by comparing the ratio of blue to

green fluorescence intensity as an indicator of OMV-host cell interactions. This method uses a plate reader to measure the change in fluorescence in host cells during infection with the reporter OMVs.

3.2 Results

3.2.1 Cloning of ClyA Bla and Bla ClyA

ClyA-Bla and Bla-ClyA fusion genes were subcloned into an arabinose inducible Kan resistant pBad vector and used to transform EHEC, MG1655 and EAEC (Figure 3.1).

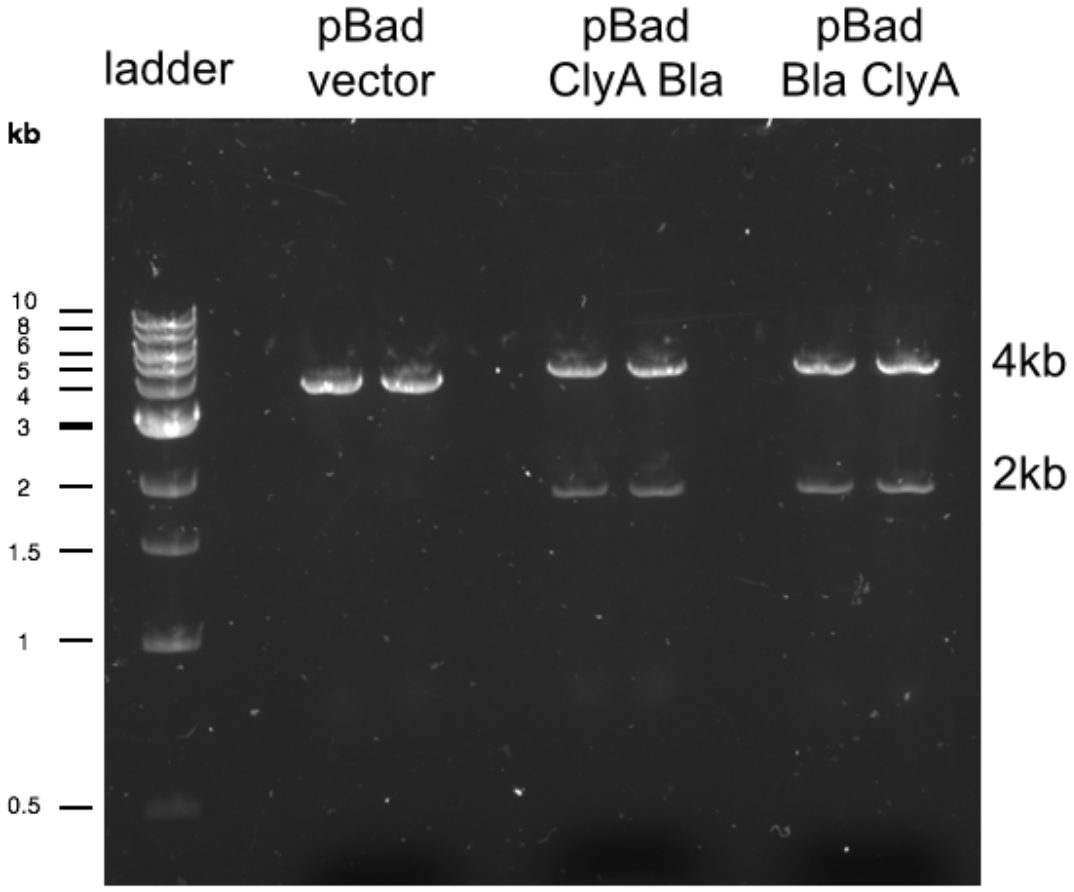

Figure 3.1. Restriction digest of pBad, pBad ClyA Bla and pBad Bla ClyA
pBad vector alone or containing ClyA-Bla or Bla-ClyA fusion genes were isolated from transformed EHEC strains, and digested with NcoI and HindIII. Digested samples were run on a 0.8% agarose gel, and 4 kb bands were detected, representing the vector backbone, and 2 kb fragments detected for the digested plasmids containing the inserts.

3.2.2 Characterisation of reporter OMVs

Outer membrane vesicles were isolated from EHEC ClyA-Bla (C-terminal fusion) and EHEC Bla-ClyA (N-terminal fusion). The OMVs containing β-lactamase enter host cells and cleave the cephalosporin ring connecting the fluorescein and coumarin molecules, causing a disruption to FRET, using a single excitation at 405 nm, and the separation of these fluorescent molecules will cause a detectable change in emission from green (530 nm) to blue (460 nm) (Figure 3.2A). Anti-Bla Western blotting showed presence of the intact 69 kDa ClyA-Bla fusion protein in samples from EHEC whole cell lysate, supernatant and OMVs, suggesting that the fusion protein is targeted to and retained in OMV fractions, as previously reported (Kim et al, 2008) (Figure 3.2B). The β-lactamase activity was detected by its ability to hydrolyse nitrocefin, indicated by an increased absorbance at 486 nm, and the specific β-lactamase activity was determined by the change in absorbance normalised to the total protein concentration measured in each sample using the CBQCA protein quantitation kit (Figure 3.2C). The specific activity was high in the OMV samples, suggesting efficient localisation and expression of active β-lactamase in the isolated OMV fractions. Triton treatment to lyse the vesicles resulted in increased β-lactamase activity in the N-terminal fusion Bla-ClyA OMVs, while there was no effect on the C-terminal ClyA-Bla OMVs, suggesting that the β-lactamase is on the interior of the Bla-ClyA OMVs.

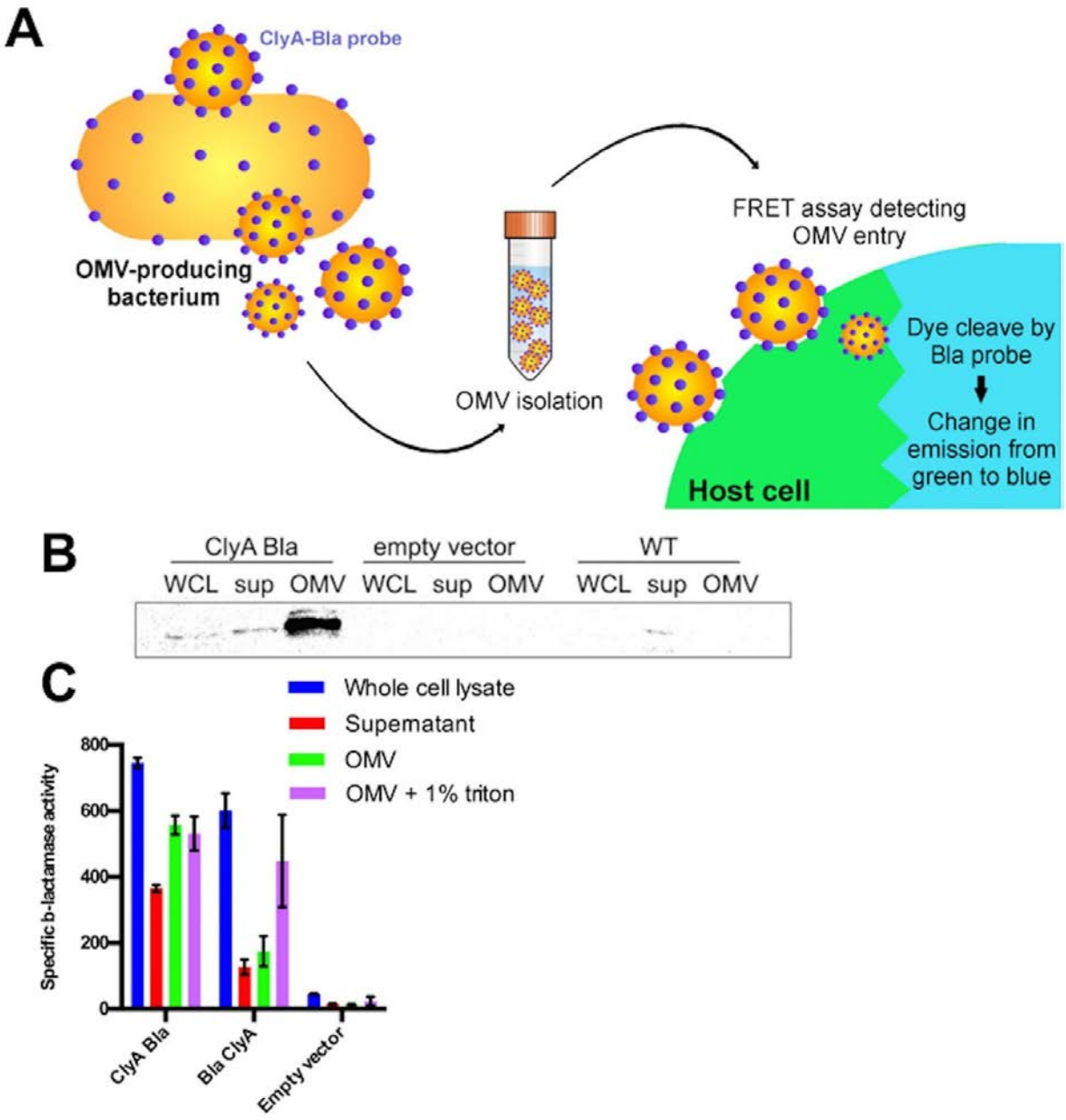

Figure 3.2. Reporter OMVs contain active β-lactamase
Using reporter probes, this assay can monitor uptake of OMVs into host cells in real time (A). Western blotting of cell fractions showed that the ClyA-Bla probe is exposed and incorporated into vesicles (WCL= whole cell lysate, sup= supernatant, OMV= outer membrane vesicle, WT= wildtype) (B). The β-lactamase within the reporter OMVs is active and able to hydrolyse nitrocefin (C) Data shown are the mean specific activities of 3 technical replicates. Error bars represent standard deviation.

Transmission electron microscopy (TEM) was used to observe their morphology which showed intact spherical membranous particles, confirming that the ClyA-Bla fusion protein has no observable effect on OMV morphology (Figure 3.3A). The size and concentration of OMVs was determined using Nanoparticle Tracking Analysis (NTA), which allows for the measurement of size distribution and concentration of nanosized particles suspended in liquid by tracking their Brownian motion and the scattering of laser light (Filipe, Hawe and Jiskoot, 2010), with a minimum of 200 tracks per sample (Figure 3.3B). Average OMV concentration was 5 x 10^{12} particles per ml, with size ranging from 10-400 nm in diameter, with a mean of 134 nm. The size ranges were in accordance with data obtained previously for OMVs from *E. coli* (Kim et al, 2008).

Expression of the probe did not affect the number of OMVs produced, determined by comparing the OD of cultures, prior to isolation, with the number of OMVs detected by Nanosight (Figure 3.3 B, C). There was an average of 38 OMVs isolated per cell by wildtype EHEC, and 41 OMVs per cell for EHEC ClyA Bla strains (Figure 3.3 C).

The Malvern Zeta sizer applies an electric charge to particles in a solution, and the rate of their movement is related to their zeta potential and surface charge (Clogston and Patri, 2010). Probe expression did not change OMV charge (Figure 3.3D). Values between +10 mV and -10 mV are considered to be uncharged (Clogston and Patri, 2010). In EHEC ClyA-Bla, protease protection experiments revealed that the probe is oriented with Bla

facing the exterior of the OMV, as β-lactamase detection by Western blot was abolished after treatment of ClyA-Bla OMVs with papain for 60 minutes (Figure 3.3E).

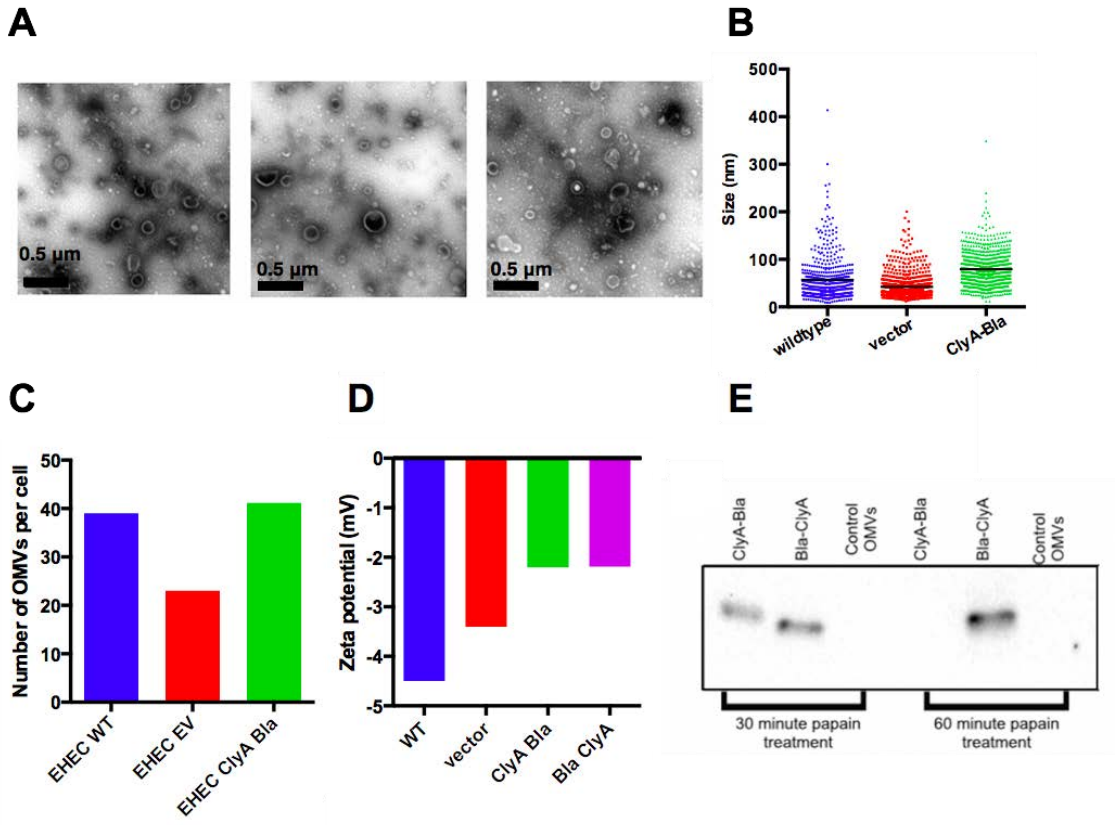

Figure 3.3. ClyA-Bla does not affect morphology, size or charge of reporter OMVs

Expression of the probe does not change vesicle morphology, as observed with Transmission Electron microscopy (A) or size, using Nanosight particle tracking analysis (B). Data shown represent 3 technical replicates, with a minimum of 100 tracks per sample. Black line represents median size value. Production of OMVs was not affected by probe expression (C). Data represents one biological replicate. The probe has no effect on charge, as determined using Zeta potential analysis (D). Data represents mean charge from 30 readings of one biological replicate. OMVs were treated with papain prior to anti-bla Western blotting, which revealed the orientation of Bla is internal for Bla-ClyA OMVs, and external for ClyA-Bla OMVs (E).

3.2.3 β-lactamase cargo probe from reporter OMVs enters host cells

Lipid membranes of OMVs were fluorescently labelled with the lipophilic dye Cellmask Orange prior to infection of host cells. HeLa cells were incubated with labelled OMVs for 10 minutes or 60 minutes prior to fixation, and were then imaged using confocal microscopy (Figure 3.4A). This revealed rapid entry and trafficking of vesicles into host cells, and highlighted the requirement of a real-time approach to study OMV uptake. HeLa cells were then infected with the reporter OMVs from EHEC, followed by fixation at 0h or 4h, to represent early and late timepoints of infection, and imaged using confocal microscopy to visualise blue and green fluorescence. There was an observable colour change from green to blue in HeLa cells infected with reporter OMVs compared with uninfected cells or those infected with the vector only control OMVs (Figure 3.4B). This indicated that active β-lactamase was delivered from the reported OMVs into the HeLa cells, resulting in disrupted FRET.

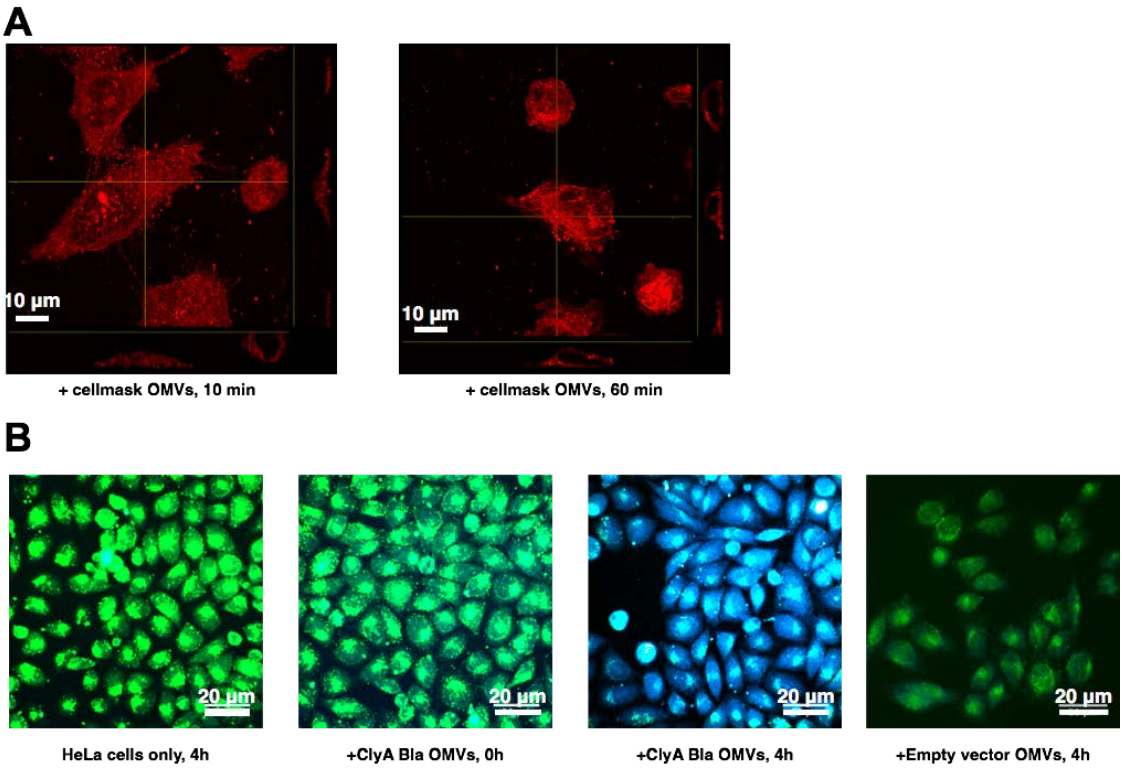

Figure 3.4. OMV entry can be detected using confocal microscopy

Fluorescently labelled OMVs were incubated with HeLa cells for 10 or 60 minutes, and were visible inside the cells at both time points, observed using confocal microscopy (top and side view of Z stack) (A). Scale bar = 10 µm. Reporter OMVs caused a colour change from green to blue in host cells after 4h, suggesting entry of active β-lactamase (B). Scale bar = 20 µm.

3.2.4 Kinetics of vesicle entry into host cells

Next, the kinetics of vesicle entry into host cells were investigated. The confocal microscopy experiments revealed that reporter OMVs had caused almost complete colour change in host cells by 4h, so the plate reader experiment was run for 0-3h to capture the kinetics of uptake. In the plate reader experiments, the fluorescence in dye loaded HeLa cells at 530 nm (green, uncleaved) and 460 nm (blue, cleaved) was measured simultaneously every 90 seconds to determine the speed and efficiency of β-lactamase entry and dye cleavage within the cells. The rate and maximum rate was calculated from estimating the change in gradient over time, whilst the efficiency of uptake was calculated from the total change in blue:green fluorescence ratio over the 3h experiment.

There was an increase in the blue:green fluorescence intensity ratio over time for HeLa cells infected with reporter OMVs for 3h, compared with cells infected with empty vector control OMVs from EHEC (Figure 3.5A). The gradient over time showed a faster rate of change for cells infected with ClyA Bla OMVs compared with Bla ClyA or empty vector (Figure 3.5B). There was an increase in the maximum rate of change from green to blue in cells infected with reporter OMVs (Figure 3.5D) and a significant difference in the total change after 3h (Figure 3.5C). These results showed that the β-lactamase from the reporter OMVs is able to enter host cells and cause dye cleavage and a change in emission from green to blue. The slower rate of change for EHEC Bla ClyA OMVs is probably due to the N-terminal fusion of ClyA to β-lactamase, resulting in Bla facing the interior of the OMV and not becoming exposed immediately upon cell entry (Kim et al,

2008). Entry of OMVs via fusion of lipid membranes may prevent internalisation of the β-lactamase probe from the OMV surface to the cell cytoplasm, and so fusion could be difficult to detect using this assay. These experiments indicate that this approach can be used to detect and quantify OMV entry into host cells.

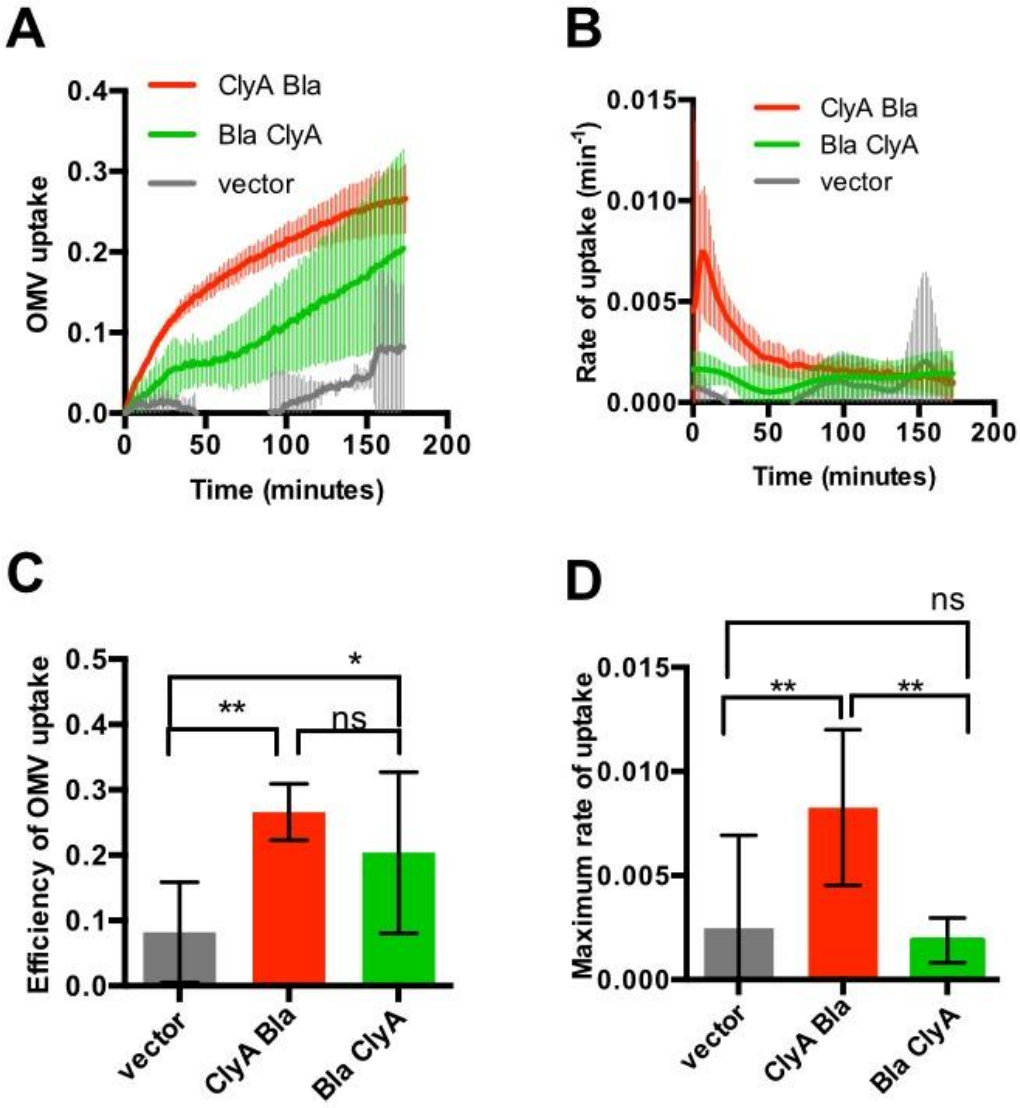

Figure 3.5. Probe OMVs can be detected upon entry into host cells, and capture dynamics of entry in real time. *OMV uptake (Y axis) is defined as the ratio of blue:green fluorescence measured over 3h, as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred (A). Data shown are means with SD, with three technical replicates for each of three separate experiments. Efficiency of uptake was defined as the total change in blue:green ratio over 3h, and was highest in cells infected with ClyA-Bla OMVs (C). The gradient of the colour change was calculated (rate, Y axis) (B) and the maximum rate of change was determined as the highest change in gradient over the 3h (D). ANOVA was used to analyse results in C and D, with a Brown Forsythe test for equal variance. Replicates were treated as 9 independent replicates, and corrected for multiple comparisons*

between samples. A p (Figure 3.5 continued) ...value < 0.05 was considered significant (*), p < 0.01 (**), while p > 0.05 was not considered statistically significant (ns).

3.2.5 Reporter OMVs can enter intestinal epithelial cells

E. coli is primarily found in the intestine of mammalian hosts (Tenaillon et al, 2010). The previous experiments were conducted in HeLa cells, where the CCF2 reporter system has been frequently used and verified (Lineberger et al, 2002; Charpentier and Oswald, 2004). To determine if the reporter system could be used in a more biologically relevant cell line, RKO cells were selected. RKO cells are derived from a human colorectal carcinoma and therefore represent a more typical environment for *E. coli,* and their derived OMVs (Savageau, 1983; Ahmed et al, 2013). The experiment was conducted as before, and it was found that the cells were able to take up the dye, and also that the ClyA Bla reporter OMVs caused a significant change from green to blue fluorescence over 3h, compared with the control OMVs and with uninfected cells (Figure 3.6). Although the change in fluorescence for the cells infected with the empty vector OMVs was higher than that observed in HeLa cells, which may be due to reduced dye stability, there was still a significant difference between the cells infected with the reporter OMVs and those with the control.

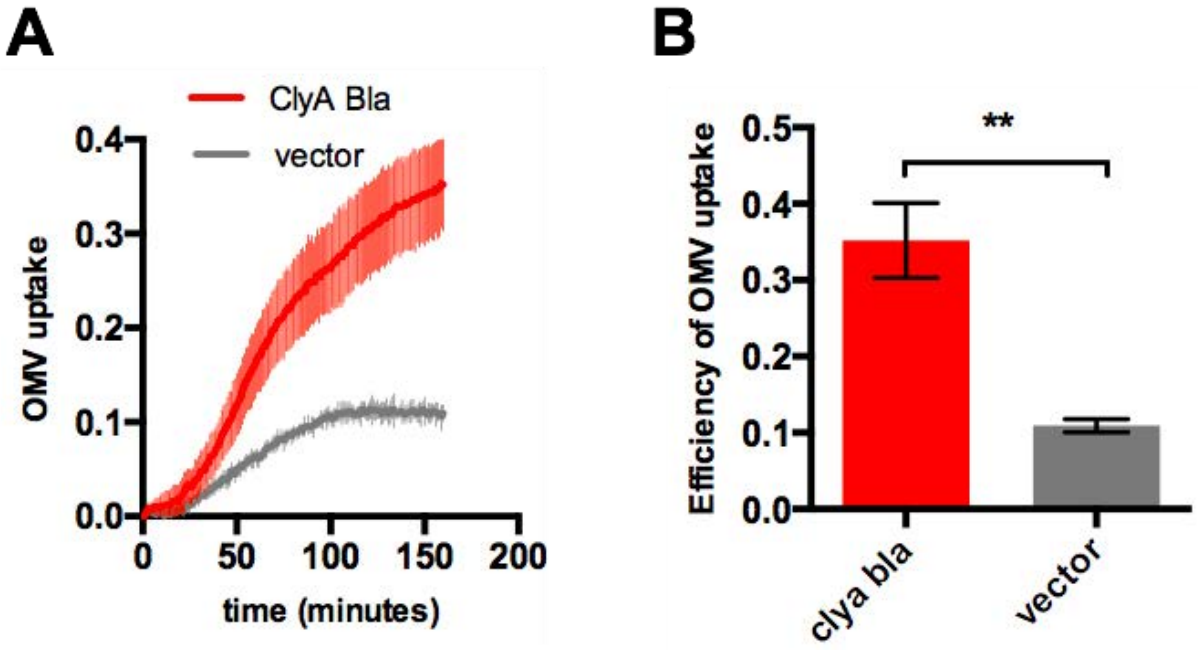

Figure 3.6 Entry of reporter OMVs can be detected in intestinal epithelial cells.
OMV uptake (Y axis) was defined as the ratio of blue:green fluorescence in RKO cells measured over 3h as an indication of entry of β-lactamase from the reporter OMV into the cells, where cleavage of CCF2-AM substrate then occurred (A). Data shown are means with SD, from three biological replicates with three technical replicates each. Efficiency of uptake was defined as the total change over 3h and was highest in cells infected with ClyA-Bla OMVs (B). A student's T test was used to analyse results, with replicates treated as 9 independent replicates with $p < 0.05$ considered significant. $P < 0.01$ (**).

3.2.6 Reporter OMVs can be delivered into host cells from whole bacterial cultures.

Methods used to isolate, quantify and characterise OMVs vary between studies. To determine if this method could be used without prior isolation of OMVs, a transwell experiment was conducted. Whole cultures of the ClyA Bla or empty vector strains were added to transwell inserts with pores of 0.4 μm to allow passage of vesicles into the HeLa cells cultured below the inserts. These experiments were conducted over a longer time period (15 hours) to allow production of OMVs from the bacterial cultures, and their movement through the transwell into the cell culture. After 15 hours, there was a clear difference in the blue vs green fluorescence intensity detected in the cells, suggesting passage of reporter OMVs and entry into the HeLa cells without prior isolation or concentration (Figure 3.7). This method can therefore be adapted for use without the time consuming OMV isolation processes, and potentially remove discrepancies in observations that may be attributable to differences in isolation methods.

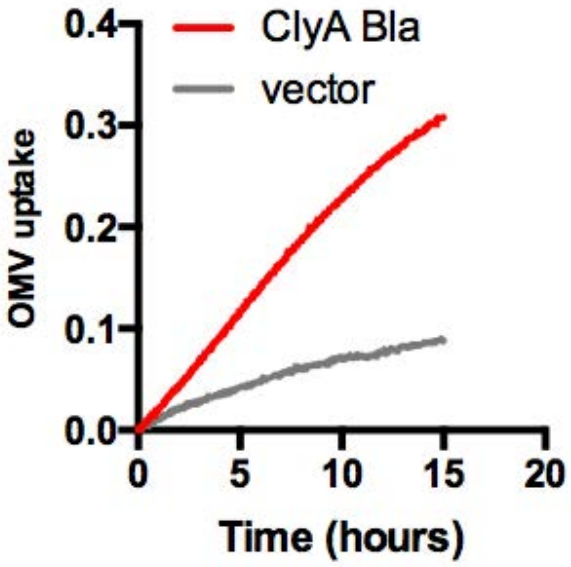

Figure 3.7 Reporter OMVs can be delivered into host cells without prior isolation.
Cultures of reporter and empty vector strains were added to 0.4 μm transwell inserts, above cultured HeLa cells loaded with CCF2-AM. OMV uptake (Y axis) represents the ratio of blue vs green fluorescence, monitored over 15 hours using a plate reader, as an indication of OMV entry and delivery of β-lactamase.

3.3 Discussion

This chapter describes a novel assay that can be used to determine the kinetics of interactions between OMVs and host cells. The affinity of OMVs for host cells during infection is well established, and many bacterial factors involved in adherence to host cells have been identified in OMVs (Kesty et al, 2004; Kuehn and Kesty, 2005). Underpinning the bacterial and host factors involved in these processes has been made more challenging by the small size of OMVs, and the need for high powered imaging or electron microscopy to visualise their presence in infected tissue. It is likely that their role in infections has been underestimated, as they would not be visible in typical biopsies, and they can also be present in sites distant to the origin of infection (Dorward, Schwan and Garon, 1991; Kuehn and Kesty, 2005; Ellis and Kuehn, 2010).

Current methods for studying the mechanisms of OMV-mediated delivery of bacterial cargo into host cells have produced inconsistent and often contradictory findings, often relying on less quantitative approaches, such as membrane labelling and immunofluorescence, or using changes in cytotoxicity or host cell phenotype as an indicator of OMV cargo delivery. This method provides a consistent platform for further studying the behaviour of OMVs in the context of infection and has overcome the difficulties associated with many of these methods, by utilising a sensitive, adaptable and quantifiable approach to the kinetics of OMV entry into host cells.

This work has shown that OMVs which display active β-lactamase can be used as reporter vesicles that indicate host cell entry through the efficient real-time cleavage of

dye molecules loaded into the host cells, removing the need for membrane or antibody labelling of specific OMV proteins or lipids, which may ignore subpopulations, or become very diluted when in the context of infection.

Using the colour change in the host cells to detect delivery allows for more robust quantification of OMV delivery, as the change in emission is readily detectable for a much higher number of cells compared with quantifying individual OMVs present in a host cell using selected fields of view in light microscopy. Other studies have also used changes in host cell phenotype or cytotoxicity to correspond to OMV entry, but typically these methods result in considerable delays in detection; this work indicates that significant OMV entry occurs within 10 minutes. This also establishes that there is very little lag between OMV entry and dye cleavage, making it appropriate as an indicator of such rapid events.

It also removes the need to fix the cells after pre-designated time points, which may miss the kinetics of OMV-host cell associations and delivery. This work allows the study of OMVs without altering the morphology, size or charge of the vesicles, and since ClyA is naturally targeted into vesicles, the expression of the ClyA-Bla fusion protein should not interfere with their behaviour. It will also potentially allow investigation and comparison of OMVs from a wide range of Gram negative species, simply by expression of the fusion protein, rather than relying on species-specific or unique characteristics of certain OMV populations. OMVs contribute to communication and interactions between

bacterial cells, and by modification of the CCF2-AM molecule, (as bacteria do not possess the esterases required to modify the dye substrate and retain it within the cell) this method could also be adapted to study delivery of OMVs between bacterial cells (Nord et al, 2005).

This method was able to detect rapid changes in fluorescence in the host cell as result of entry of β-lactamase and cleavage of the CCF2-AM substrate. This indicated that not only do OMVs enter and deliver cargo to host cells within minutes of infection, the method was sensitive enough to detect this, with little or no delay. This suggests that spatial or temporal separation of OMVs from the CCF2 substrate is not a limitation of the assay. This method has been established in several different viral infections, where the kinetics observed are very similar to those seen here. The size of viruses and OMVs can overlap, and this unique approach allows for the rapid entry of these small particles to be determined and quantified (Cavrois et al, 2003; Landowski et al, 2014).

This method is also adaptable to high throughput analysis, and has been previously used for drug screening of compounds to identify inhibition of entry of Ebola virus into HeLa cells (Kouznetsova et al, 2014). The use of a plate reader in these experiments allowed continuous monitoring of blue and green emission simultaneously to detect disruption of FRET, without the need for any manual interference with the experiment. It allowed for more robust data analysis as technical replicates could be performed and analysed in a much less time consuming manner than interpretation of microscopy data. It also means

that several different conditions can be screened at once, through the use of the multi well plate format.

This method has also been used to detect delivery of OMV cargo without the need for prior isolation of OMVs, by using the whole bacterial cultures in a transwell assay. This removes the time consuming isolation and characterisation steps, and also provides insight into the real-time dynamics of OMV release from the bacterial cell into the host. This approach could help to resolve some of the contradictory findings between studies, that may be due to variations in methods used to isolate, quantify and characterise vesicles prior to infections experiments.

Isolation methods can cause variations in the numbers of OMVs produced, so in this work, OMV numbers were carefully monitored using a combination of the protein content, β-lactamase activity and the nanoparticle tracking analysis to ensure the concentrations in the samples were normalised prior to infection of cells. In these experiments, an MOI of 1000 OMVs per HeLa cell was used, equivalent to an OMV protein concentration of 10 μg/ml. This is considerably lower than concentrations used in other studies, which have used up to 200 μg/ml, and for periods of up to 24 h, compared with 3h used in these experiments (Chitcholtan et al, 2008). It was determined that an average of 41 OMVs were isolated per bacterial cell for EHEC ClyA Bla, and therefore this would be equivalent to a bacterial MOI of 25. This is potentially lower than a physiological MOI of OMVs per cell, and so it is encouraging that a relatively low

number of OMVs is able to deliver sufficient β-lactamase to cleave the dye molecules in the HeLa cells (Namork and Brandtzaeg, 2002). The entry of OMVs was detected in HeLa cells, but also in the biologically relevant intestinal epithelial cell line, RKO. Other cell lines including HT29 and Caco-2 intestinal epithelial cells were found to be incompatible with this system, although it is unclear whether it is an issue with dye uptake or dye retention.

Exploring OMV entry processes will allow new insight into potential means of attenuating infections by preventing OMVs delivering their toxic cargo into hosts, but could also aid in the use of OMVs as designer delivery vehicles, due to their high affinity for host cells. The potential applications of OMVs in medicine and research will be broadened by new understanding of their ability to enter host cells and allow exploitation of these mechanisms.

The next chapters will utilise the assay to determine bacterial factors which influence the rate and efficiency of uptake, and to elucidate the mechanisms which facilitate or enhance OMV entry into host cells.

4. Presence of O polysaccharide in OMVs enhances their uptake by host cells

4.1 Introduction

The previous chapter developed an assay that can detect and quantitatively measure the kinetics of OMV uptake into host cells, by monitoring the change in fluorescence in host cells upon delivery of β-lactamase from engineered reporter vesicles into the cell cytoplasm.

The role of O polysaccharide in the interactions of OMVs with host cells has not been explored until now. Since OMVs are derived from the outer membrane, they contain LPS, and therefore also O polysaccharide (Park et al, 2010). OMV production also appears to be linked to modifications of LPS under different environmental conditions (Bonnington and Kuehn, 2016). The immunogenicity of OMVs cannot be attributed to the LPS content alone, as inoculation with equivalent or 2-fold higher amounts of purified LPS elicited far less lethality in a sepsis model (Park et al, 2010). OMVs are around 20-200 nm in diameter, but O polysaccharide alone can protrude over 30 nm (Strauss et al 2009). The O polysaccharide on OMVs may therefore represent an initial point of contact between OMVs and the host cell surface during infection.

This chapter sought to identify whether presence or absence of O polysaccharide on OMVs has an effect on their uptake by host cells. To check the correlation between LPS

and OMV uptake, OMVs with and without O polysaccharide, from different serotypes and pathovars, were used. OMVs from pathogenic strains of *E. coli*, EHEC and EAEC, and from the laboratory strain *E. coli* K12 were compared using this assay.

4.2 Results

4.2.1 EHEC OMVs enter host cells more rapidly and efficiently than OMVs from E. coli K12.

OMVs were isolated from EHEC and K12 ClyA-Bla strains. The number of OMVs was measured using Nanosight nanoparticle tracking analysis, with an average concentration of 1.1 $x10^{12}$ OMVs per ml for EHEC, and 1.96 x 10^{11} OMVs per ml for K12 (Figure 4.1A). This is in accordance with other data indicating that pathogenic strains can release up to 10 fold more OMVs than non-pathogenic strains (Horstmann and Kuehn, 2000). The mean protein concentration in EHEC OMV samples was 86 +/- 11μg/ml, and 14 +/- 3 μg/ml for K12. EHEC OMVs were significantly smaller than K12 OMVs. The average diameter was 79 +/- 35 nm for EHEC, and 86 +/- 38 nm for K12 (Figure 4.1B). The median sizes were 75 nm for EHEC, and 86 nm for K12 (Figure 4.1B). Prior to use in infection experiments, the OMV concentrations were normalised by diluting EHEC OMVs 6 fold to be equivalent to K12. The protein concentration was measured after normalisation and was found to be 15 μg/ml. 50 μl of normalised samples were added to nitrocefin, which is hydrolysed in the presence of β-lactamase, resulting in a colour change from yellow to red, to determine the specific β-lactamase activity. After normalising the number of OMVs in the samples, the β-lactamase activity was equivalent (Figure 4.1C).

HeLa cells infected with reporter OMVs from EHEC showed a higher and faster change in blue:green fluorescence intensity ratio (representing exposure of the OMV-derived

ClyA Bla probe to the CCF2-AM FRET substrate in the cytoplasm) after 3h compared with cells infected with reporter OMVs from *E. coli* K12 (Figure 4.2A). The change in gradient was estimated (Figure 4.2B) and there was a higher maximum rate of change from green to blue (Figure 4.2C) and a significantly higher total change in the blue:green fluorescence intensity ratio for cells infected with EHEC OMVs (Figure 4.2D). This indicated a higher rate and efficiency of uptake of EHEC OMVs compared with OMVs from *E. coli* K12.

These differences were independent of OMV concentration and β-lactamase activity which were equivalent for both strains (Figure 4.1C). Protein concentration in OMV samples was measured, and 5, 10 and 20 μg/ml were tested in the infection experiments. The efficiency of uptake of OMVs increased when the concentration was increased from 5 μg/ml to 10 μg/ml, but the maximum rate appeared to stop increasing at 20 μg/ml for EHEC, suggesting a partial concentration dependency but a possible saturation of entry mechanisms above this level (Figure 4.3A, B). For K12, the maximum rate appeared to continue to increase (Figure 4.3B)

Taken together, these results suggest EHEC OMVs contain cargos absent from K12 OMVs that accelerate the rate and increase the efficiency of vesicle uptake by host cells.

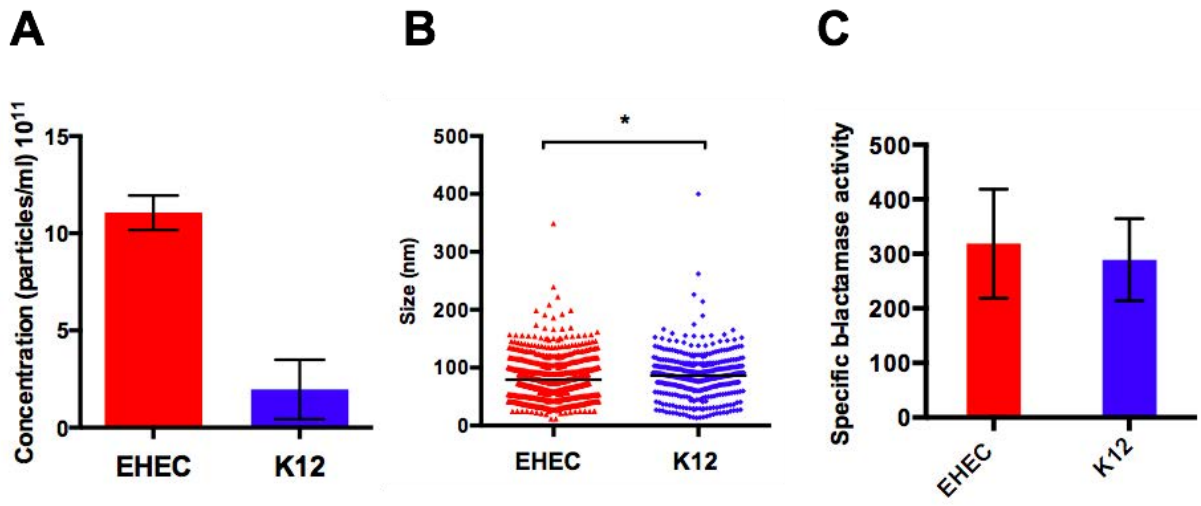

Figure 4.1. Comparison of OMVs isolated from EHEC and K12 reporter strains

The concentration and diameter of OMVs was measured with Nanoparticle tracking analysis (A, B) for two biological replicates and three technical replicates with a minimum of 100 tracks. EHEC released more OMVs than K12 (A) with an average concentration of 1.1 x10^{12} OMVs per ml for EHEC, and 1.96 x 10^{11} OMVs per ml for K12. Error bars represent standard deviation. The mean protein concentration in EHEC OMV samples was 86 +/- 11 μg/ml, and 14 +/- 3 μg/ml for K12. Student's T test determined EHEC OMVs were significantly smaller than K12 OMVs, $p < 0.05$ (*) (B) Black lines represent mean size values (79 +/- 35 nm for EHEC, and 86 +/- 38 nm for K12). The concentrations of OMVs were normalised, and β-lactamase activity was determined. Normalised samples showed equivalent β-lactamase activity, calculated by ability to hydrolyse nitrocefin (C). Data shown are means of three technical replicates for two biological replicates, analysed as 6 independent replicates. Error bars represent standard deviation.

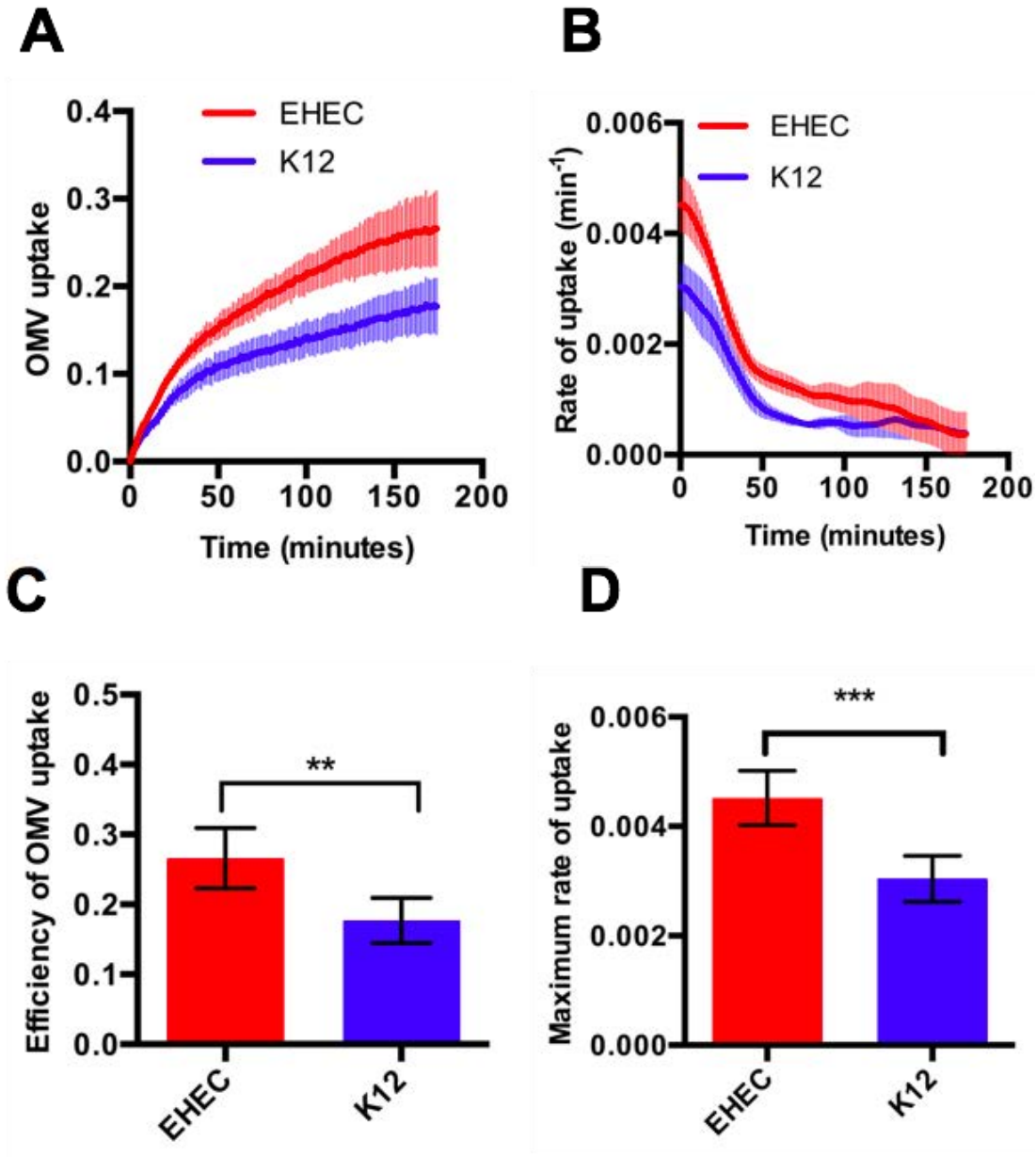

Figure 4.2. EHEC OMVs enter host cells more rapidly and efficiently than OMVs from* E. coli *K12.

OMV uptake (Y axis) was defined as the ratio of blue:green fluorescence intensity in HeLa cells infected with reporter OMVs from EHEC and K12 measured over 3h (A) This was normalised, with the fluorescence in uninfected cells used as a baseline, and data at 0h normalised to zero. The gradient over time (rate) was then estimated (B) to calculate the maximum rate of uptake (D). The total change in blue:green over 3h (efficiency of uptake) was significantly higher in cells infected with EHEC reporter OMVS compared with cells infected with reporter OMVs from K12 (C). Student's t-test was used to compare samples, which were from three biological replicates with three technical

replicates each. These were treated (Figure 4.2 continued)...as 9 independent replicates. $p<0.05$ was considered statistically significant (*), $p < 0.01$ (**), $p < 0.001$ (***).

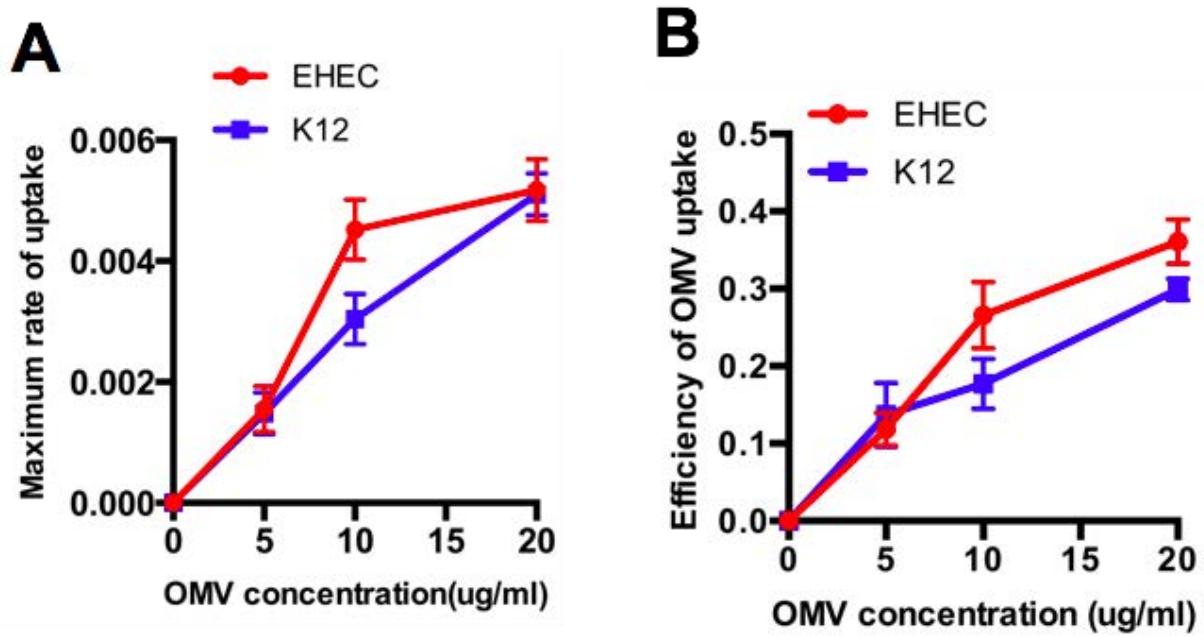

Figure 4.3. Uptake of OMVs is partially concentration dependent.
Increased OMV concentration (from 5 μg/ml-20 μg/ml) increased uptake, but the maximum rate of uptake appeared to saturate above 10 μg/ml (A, B) for EHEC (red), whilst the efficiency of uptake did not saturate for either EHEC (red) or K12 (blue) (B). Data shown are means from three technical replicates, with error bars representing standard deviation.

4.2.2 O polysaccharide increases efficiency of uptake of OMVs

Since OMVs are derived from the outer membrane of Gram negative bacteria, they contain lipopolysaccharides (LPS), (Cahill et al, 2015). Whilst lipid A and the core oligosaccharide regions are well conserved, many species including EHEC contain a highly variable polysaccharide domain known as O polysaccharide (Strauss et al, 2009). The O polysaccharide constitutes the outermost region of LPS, and due to its length of up to 30 nm (Strauss et al, 2009), likely the first component in contact with host cells. These characteristics led to the hypothesis that the O polysaccharide moiety present on EHEC OMVs may be a determinant for OMV recognition and uptake by host cells.

To test this hypothesis, FRET assays were carried out with Hela cells exposed to ClyA-Bla reporter OMVs harvested from three pairs of strains, reflecting different *E. coli* serotypes and pathovars and O polysaccharide deficient isogenic mutants, to determine how the presence or absence of O polysaccharide would impact OMV uptake kinetics in each case.

OMVs were derived from two pathogenic strains of *E. coli*, EHEC (serotype O157) Sakai and enteroaggregative *E. coli* (EAEC, serotype O42), and from the non-pathogenic K12 lab strain MG1655 (serotype O16). Prior to infection, OMVs were analysed for their size, concentration and surface charge (Figure 4.4A-C). The average size of EHEC OMVs with O polysaccharide was 79 +/- 36 nm vs 76 +/- 35 nm for those without O

polysaccharide. For K12, the average size for O polysaccharide OMVs was 91 +/- 34 nm vs 86 +/- 39 nm for O polysaccharide-lacking OMVs. There were no significant differences between the size or concentration of OMVs with or without O polysaccharide for EHEC or K12, although O polysaccharide positive strains produced more OMVs: 1.1 $x10^{12}$ particles per ml and 5.3 $x10^{11}$ particles per ml for EHEC, 2.7 $x10^{11}$ particles per ml and 1.9 $x10^{11}$ particles per ml for K12. In contrast, EAEC OMVs lacking O polysaccharide were significantly smaller than the O42 counterparts: the average size of OMVs with O polysaccharide was 102 nm, compared with 74 nm for OMVs without O polysaccharide (Figure 4.4A). OMVs lacking O polysaccharide also had a significantly higher concentration: 8.7 $x10^{11}$ particles per ml, compared with 1.5 $x10^{11}$ particles per ml for O polysaccharide OMVs (Figure 4.4B). Surface charge was measured using Zeta analysis. Presence of O polysaccharide for K12 and EHEC resulted in reduced negative charge, but for EAEC, the presence of O polysaccharide increased the negative charge of the membrane, to -11.9 mV from -8.68 mV (Figure 4.4C). However, zeta potential values between -10 mV and + 10 mV are considered uncharged (Clogston and Patri, 2010).

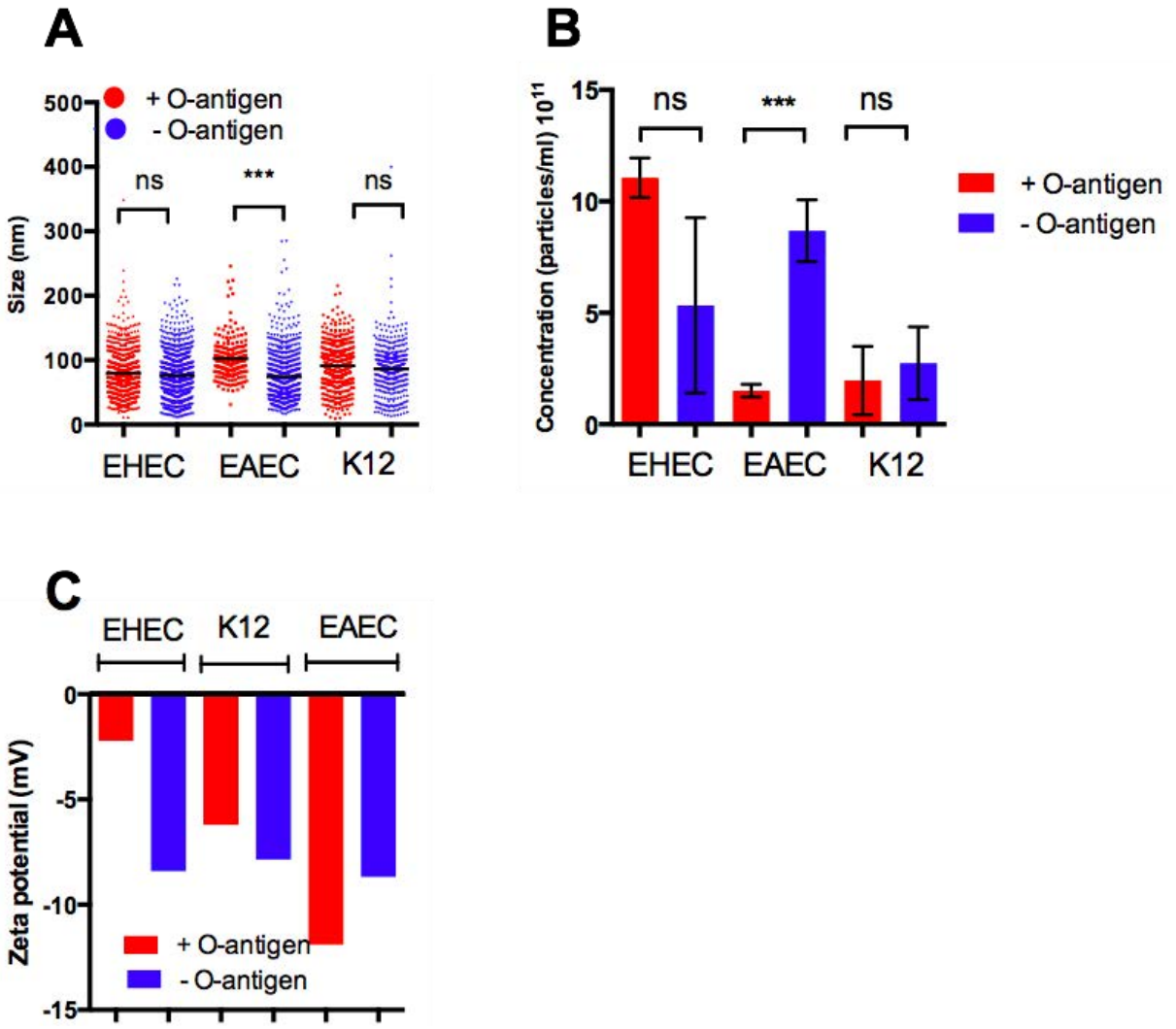

Figure 4.4 Size, concentration and charge of OMVs with or without O polysaccharide. *Isolated OMVs were diluted 1x10^{-6} and nanoparticle tracking analysis was used to determine the size distribution (A) and concentration with error bars showing standard deviation (B). Black lines in (A) represent median size from at least 100 tracks acquired per sample. Statistical significance was determined by Student's t-test, between OMV samples with or without O polysaccharide, with 6 technical replicates treated independently. EAEC OMVs without O polysaccharide were significantly smaller than their O polysaccharide counterparts. (***) p≤0.005, (ns) not significant. (C) ζ-potentials of isolated OMVs. Values represent means from 30 readings per sample.*

The OMV concentration was normalised using the nanosight data, and OMVs were added to HeLa cells at an MOI of 1000 OMVs/host cell. The change in fluorescence from green to blue was measured over 3h as an indicator of OMV uptake. For EHEC, OMVs from EHEC O157 wild type cells and an isogenic strain lacking the O157 O polysaccharide (gne::IS629) were compared (Rump et al, 2010). The O polysaccharide deficient mutant gne::IS629 carries a 1310 bp insertion in *gne*, disrupting the epimerase required for synthesis of the oligosaccharide repeating unit in the O polysaccharide (Figure 1.2, Rush et al, 2010; Rump et al, 2010). The maximum rate of uptake for ClyA-Bla reporter OMVs derived from this O polysaccharide deficient EHEC strain and the isogenic wild type O157 strain were not significantly different (Figure 4.5C). However, OMVs derived from wild type EHEC with intact O polysaccharide sustained a higher entry rate over a longer period (Figure 4.5B), and thus entered host cells ~ 43% more efficiently than those derived from O polysaccharide deficient EHEC (Figure 4.5A, D).

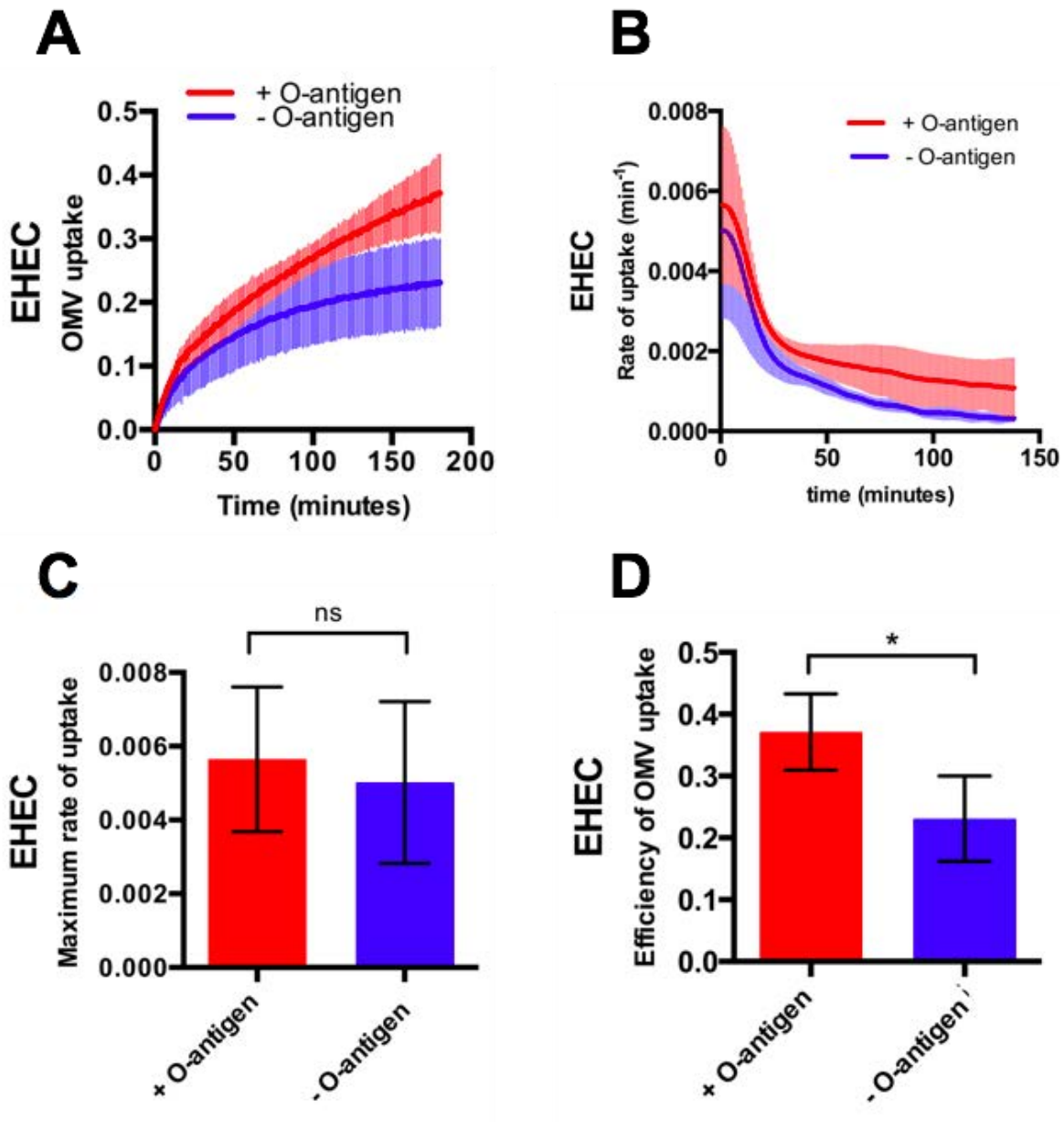

Figure 4.5. Presence of O polysaccharide increases efficiency of uptake of OMVs from EHEC.

CCF2-AM loaded Hela cells were exposed to ClyA-Bla OMVs isolated from EHEC containing O polysaccharide (red), or lacking O polysaccharide (blue) at an MOI of 1000 for 3 hours. OMV uptake (Y axis) is defined as the ratio of blue:green fluorescence over 3h as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred (A). Data shown are means with SD, with three technical replicates for each of three separate experiments. The gradient of the colour change was estimated to determine rate of colour change over time (B) and

(Figure 4.5 continued)...the maximum rate of change was determined as the highest change in gradient over the 3h (C). Efficiency of uptake (D) was defined as the total change in blue:green ratio over 3h from (A). ANOVA was used to analyse results, with a Brown Forsythe test for equal variance. Replicates were treated as 9 independent replicates, and corrected for multiple comparisons between samples. A p value < 0.05 was considered significant (*) and p > 0.05 was not considered statistically significant (ns). EHEC OMVs with O polysaccharide entered HeLa cells significantly more efficiently than the non O polysaccharide containing OMVs.

OMVs from wild type EAEC (serotype O42, intact O polysaccharide) were compared with OMVs from an isogenic O polysaccharide deficient mutant (Δ*wbaC*, lacking a glycosyltransferase necessary for O polysaccharide synthesis; Figure 1.2, Browning et al, 2013). EAEC OMVs with intact O polysaccharide entered host cells ~66% more efficiently than OMVs without O polysaccharide, due to a 77% higher maximum rate of uptake (Figure 4.6A-D) and a higher sustained rate over time (Figure 4.6B). This was in contrast with EHEC OMVs, which showed no significant difference in r max between those with or without O polysaccharide.

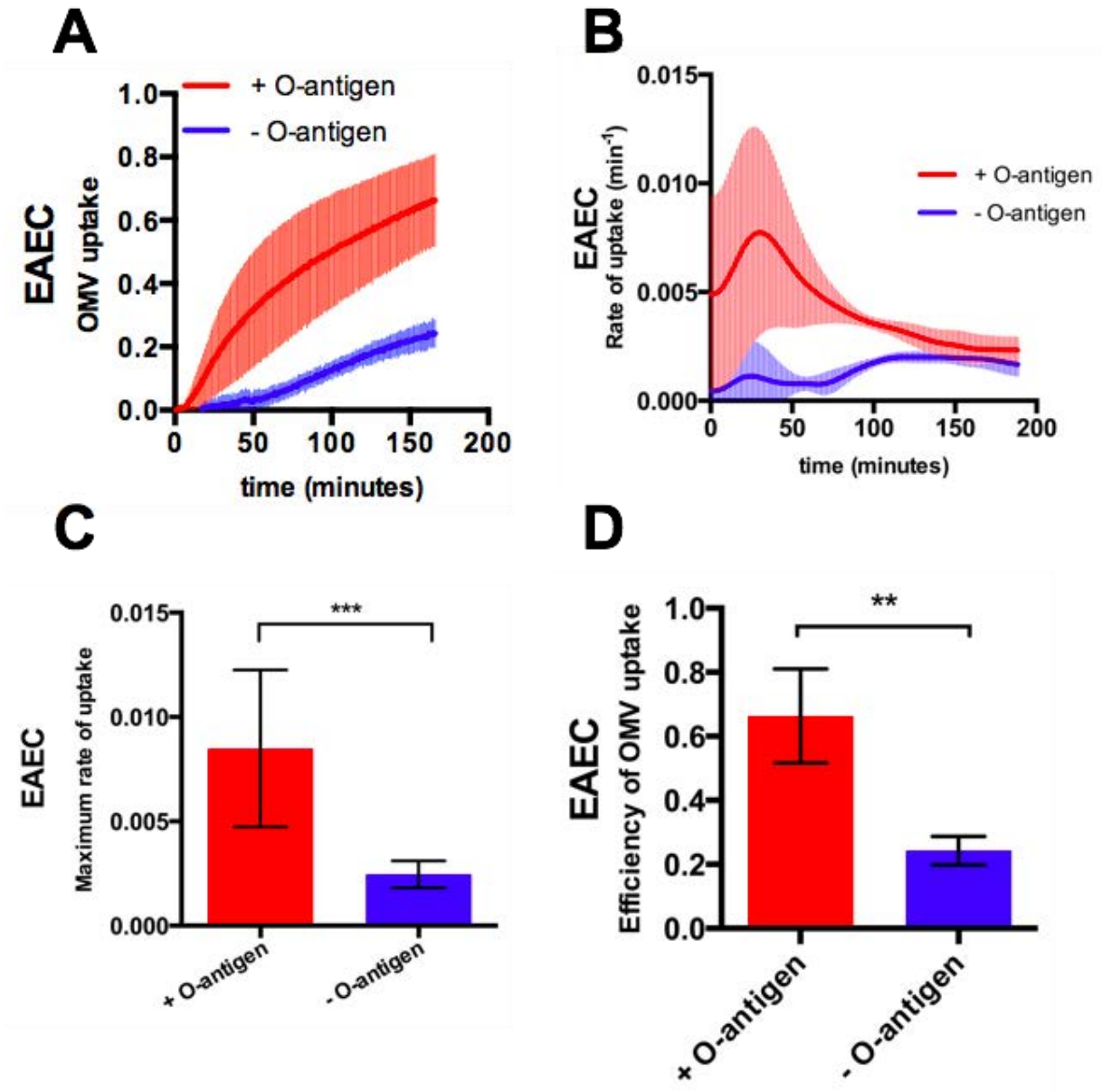

Figure 4.6. Presence of O polysaccharide increases rate of uptake of OMVs from EAEC.

CCF2-AM loaded Hela cells were exposed to ClyA-Bla OMVs isolated from EAEC containing O polysaccharide (red), or lacking O polysaccharide (blue) at an MOI of 1000 for 3 hours. OMV uptake (Y axis) is defined as the ratio of blue:green fluorescence over 3h as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred (A). Data shown are means with SD, with three technical replicates for each of three separate experiments. The gradient of the colour change was estimated to determine rate of colour change over time (B) and the maximum rate of change was determined as the highest change in gradient over the

(Figure 4.6 continued)...3h (C). Efficiency of uptake (D) was defined as the total change in blue:green ratio over 3h from (A). ANOVA was used to analyse results, with a Brown Forsythe test for equal variance. Replicates were treated as 9 independent replicates, and corrected for multiple comparisons between samples. A p value < 0.05 was considered significant, (**) indicates p≤0.01, (***) indicates p≤0.001.

The non-pathogenic laboratory strain *E. coli* K12 MG1655 has lost its ability to produce O polysaccharide due to a disruption in *wbbL* encoding the rhamnosyltransferase required for O polysaccharide synthesis (Figure 1.2, Liu and Reeves, 1994). These experiments compared entry of OMVs from this O polysaccharide deficient strain, to those from an isogenic strain (DFB 1655 L9), where wild type *wbbL* has been restored, allowing expression of the strain's original O16 O polysaccharide (Browning et al, 2013) (Figure 4.7A). Similar to O157, the presence or absence of O polysaccharide did not significantly alter the maximum rate (Figure 4.7C) but the presence of O polysaccharide allowed for a higher rate to be sustained for longer (Figure 4.7B), leading to a ~ 22% higher efficiency of OMV uptake overall (Figure 4.7D).

Taken together, these results suggest that the presence of the LPS O polysaccharide increases the entry efficiency of OMVs into host cells, independent of the specific mutation leading to O polysaccharide deficiency. Depending on the serotype used, this is caused by enhancing the maximum rate and/or by sustaining a higher uptake rate over a longer period, compared to OMVs lacking O polysaccharide. These variations may be due to differences in physicochemical features and/or other vesicle cargos between the different serotypes.

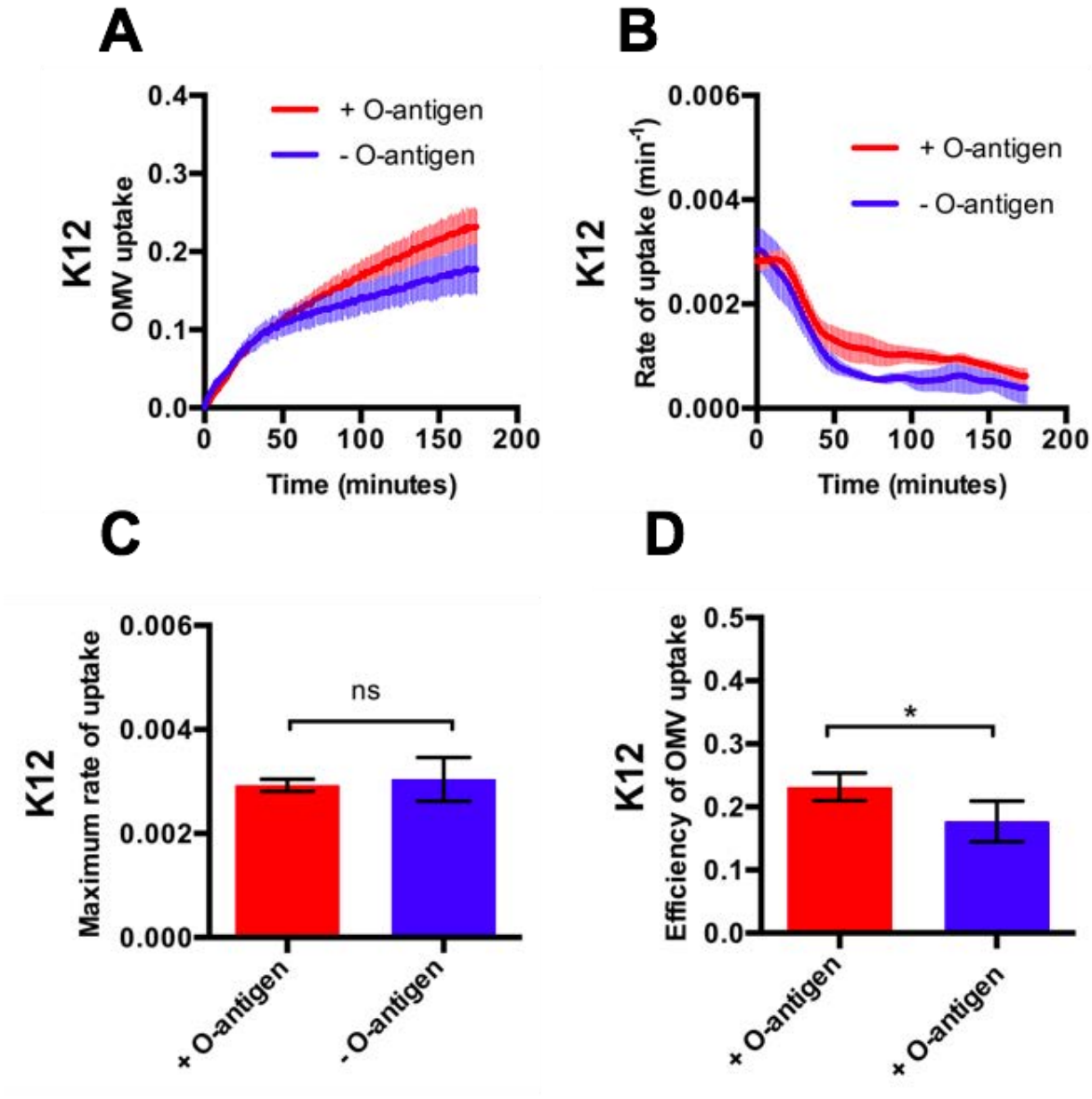

Figure 4.7. Presence of O polysaccharide increases efficiency of uptake of OMVs from K12

CCF2-AM loaded Hela cells were exposed to ClyA-Bla OMVs isolated from EAEC containing O polysaccharide (red), or lacking O polysaccharide (blue) at an MOI of 1000 for 3 hours. OMV uptake (Y axis) is defined as the ratio of blue:green fluorescence over 3h as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred (A). Data shown are means with SD, with three technical replicates for each of three separate experiments. The gradient of the colour change was estimated to determine rate of colour change over time (B) and the maximum rate of change was determined as the highest change in gradient over the 3h (C). Efficiency of uptake (D) was defined as the total change in blue:green ratio over

(Figure 4.7 continued)...3h from (A). ANOVA was used to analyse results, with a Brown Forsythe test for equal variance. Replicates were treated as 9 independent replicates, and corrected for multiple comparisons between samples. A p value < 0.05 was considered significant(*), while a p value > 0.05 was not considered statistically significant (ns).

4.2.3 O polysaccharide increases efficiency of OMV uptake by intestinal epithelial cells.

The previous experiments determined that presence of O polysaccharide can increase either the rate or the overall efficiency of OMV uptake in HeLa cells. Pathogenic *E. coli* strains are able to colonise the intestinal epithelium, and O polysaccharide has previously been implicated in contributing to this process (Ho and Waldor, 2007; Sheng et al, 2008). Conducting these FRET assays in an intestinal epithelial cell line is therefore more biologically relevant. For this reason, the experiments were repeated in RKO cells, a rectal carcinoma cell line (Ahmed et al, 2013).

As observed in HeLa cells, the OMVs with O polysaccharide showed more efficient entry and disruption to FRET in RKO cells (Figure 4.8A-D). This was the case for OMVs for EHEC, EAEC and K12. Whilst the overall fluorescence was lower than detected in HeLa cells (Figure 4.8A-C), which may be due to a difference in the ability of esterases within the cell to allow retention of the dye, the general effect was the same as for HeLa cells; entry of reporter OMVs was able to cleave the CCF2-AM substrate, and this occurred more efficiently when the cells were incubated with OMVs containing O polysaccharide.

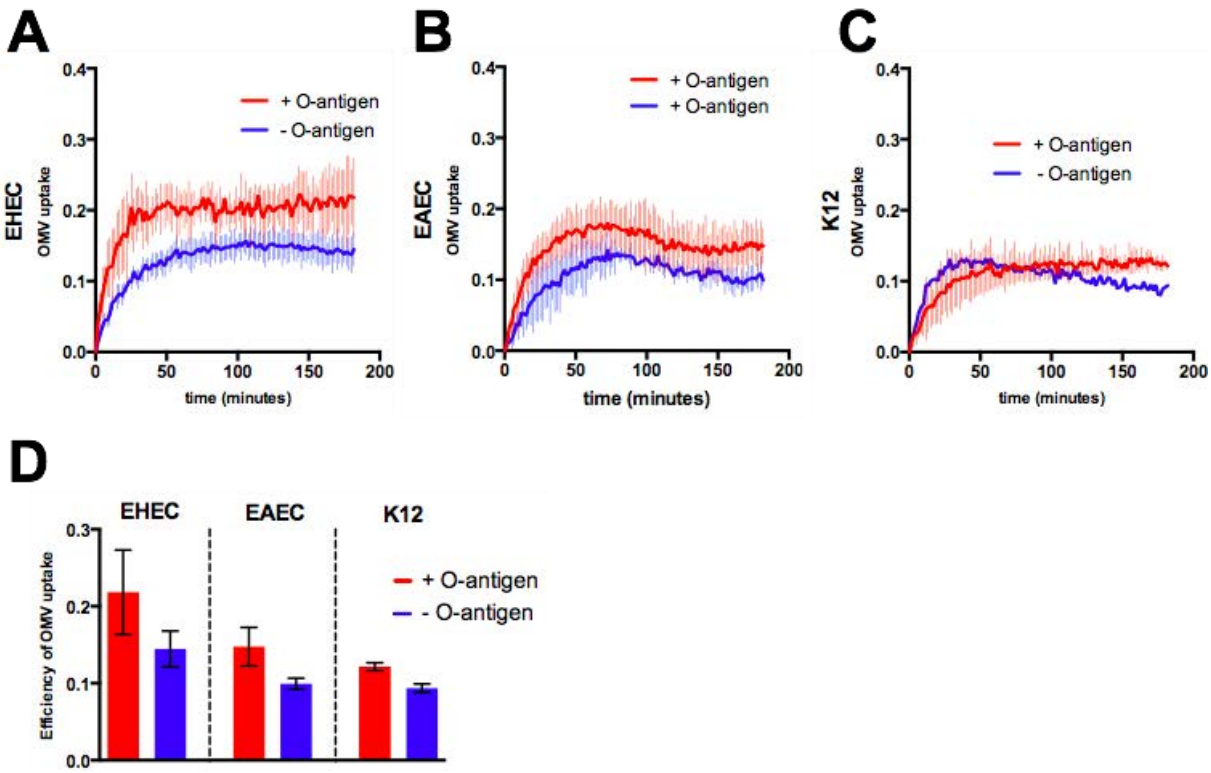

Figure 4.8. O polysaccharide increases efficiency of OMV uptake by intestinal epithelial cells.

CCF2-AM loaded RKO cells were exposed to ClyA-Bla OMVs isolated from EHEC (A), EAEC (B) or K12 (C) containing O polysaccharide (red), or lacking O polysaccharide (blue), at an MOI of 1000 for 3 hours. OMV uptake (Y axis) was defined as the ratios of blue:green fluorescence over time, representing cleavage of CCF2-AM substrate by OMV cargo within RKO cells. Ratios (A-C) were plotted as means ± stdev (n=3).Total FRET changes after 3 hrs (D) were determined from data shown in (A-C), with error bars representing standard deviation.

4.3 Discussion

Interactions between bacterial outer membrane vesicles and epithelial cells are now recognized as an important driver of bacterial pathogenesis. Yet, the ability to study vesicle-host cell interactions has been limited by a lack of methods to capture the rapid kinetics of vesicle entry and dismantling in real-time, and without altering the physicochemical properties of the vesicle. The assay developed here fulfils these requirements and allowed the study of the kinetics of OMV uptake with enough temporal resolution to reveal critical differences in rate and uptake efficiency of vesicles derived from different *E. coli* serotypes and pathovars.

The method uses a genetically encoded, OMV targeted probe and a cell-permeable dye, resulting in a change in FRET upon reporter uptake and dye cleavage. An advantage of this system is its high sensitivity, as 5 μg/ml OMVs, the lowest concentration reported in the literature, produced a reproducible trace with good signal/noise ratio. A rapid response was detected with this method, as changes in the fluorescence signal were detected within seconds. The use of this system can be extended to a high-throughput format, allowing further study of bacterial and host factors determining OMV uptake and trafficking. Using a transwell format, the method can be applied to cell-based assays consisting of bacteria releasing OMVs, and host cells without the need for OMV isolation. Although the specific probe used here was functional across a range of *E. coli* isolates and different host cell types, its use in other bacterial species will require further

characterization to determine if it is correctly targeted to OMVs and remains enzymatically active.

EHEC and EAEC OMVs were selected for this study, since OMVs have been shown to play a crucial role in toxin stabilization and delivery for both species and have been considered as a means to vaccinate and protect against hemolytic uremic syndrome, a severe complication of EHEC infection (Aldick et al, 2008; Choi et al, 2014; Kunsmann et al, 2015). It is clear that LPS, and specifically O polysaccharide, contributes to bacterial fitness within a host and pathogenicity, by enhancing resistance to complement, modulating phagocytosis and phage infection (Liang-Takasaki et al, 1982; Van der Ley et al, 1986). The O polysaccharide of most *E. coli* strains has 10-18 repeats, whilst EHEC O157 can exceed 80 repeats (Franco, Liu and Reeves, 1998; Kalynych et al, 2011). The length of the O polysaccharide in EHEC can be over 30 nm, with the length of the O polysaccharide positively correlated with the ability of the bacterial cell to adhere to host cells and tissues, while loss of O polysaccharide results in defects in colonisation, biofilm formation, and increased pathogen clearance (Murray, Attridge and Morona, 2006; Sheng et al, 2008; Strauss et al, 2009; Hathroubi et al, 2016). It is compelling that many lab strains lose their O polysaccharide, suggesting an advantage in survival in natural settings that is lost in an artificial growth conditions (Stevenson et al, 1994).

Studies into the contribution of O polysaccharide to bacterial fitness have typically focused on whole bacterial cells, while comparatively little research has been conducted

into its possible role in the behaviour of OMVs, and their affinity for host cells. Recent work showed that EHEC OMVs allow efficient delivery of LPS into the host cell cytoplasm, resulting in inflammatory responses, caspase-11 activation and cell death, but did not explore the role of LPS in uptake (Kunsmann et al, 2015; Vanaja et al, 2016). Other work has suggested that modifications to the composition of the LPS can increase the production of OMVs in order to increase survival during unfavourable conditions, such as low pH (Bonnington and Kuehn, 2016). This data suggest that O polysaccharide has an additional role during bacteria-host interactions, which is to accelerate the uptake and delivery of vesicle associated virulence factors such as hemolysins and Shiga-like toxins to host cells and enhancing pathogenicity (Bielaszewska et al, 2017).

For EHEC and K12, presence of O polysaccharide did not have a significant effect on production of OMVs, but for EAEC the absence of O polysaccharide resulted in a significant increase in the concentration of OMVs isolated. Unlike K12 and EHEC, the presence of O polysaccharide resulted in a more negative surface charge. EAEC expresses fimbriae at the outer surface which enhance aggregation. The fimbriae are highly positively charged, therefore without the O polysaccharide the membrane may have become more positively charged, and this may have affected membrane stability and OMV production, and also the interaction of the OMVs with the host. In the other strains, the opposite was true, as since the O polysaccharide is generally un charged, its presence can reduce the overall negative charge of the membrane. The largest change in rate of

uptake was observed for the EAEC OMVs, and so it may be that the effect of the O polysaccharide is more significant in this strain.

A limitation of this work is that all three mutants used were constructed differently, as the genes to synthesise O polysaccharides vary. As complementations of the mutants were not used, it is possible that these deletions have other effects that were not controlled for and this may alter the OMV cargo and behaviour. It would be necessary to use OMVs from complemented strains in the future, and also to express the same O polysaccharide in all three to control for variations in the O polysaccharide structure affecting their ability to enter cells.

HeLa cells were primarily used in this work, as they are well established for use with the CCF2 substrate, as well as infection studies, but the same effect was also observed in the rectal carcinoma cell line, RKO. These cells are more relevant to the study of *E. coli* pathogenesis, which typically occurs in the intestine, and so it is encouraging that the results were replicated in these cells. Other intestinal cell lines, such as HT29, were also investigated, but preliminary experiments showed that they were unable to retain the CCF2-AM substrate. Zebrafish embryos were also injected with the substrate, but no dye uptake was observed, even at a 10 fold higher concentration of the substrate. This may be due to differences in esterase activity, which is required to decrease the hydrophobicity of the FRET probe, thus decreasing its membrane permeability and trapping the probe in the host cell cytoplasm. Whilst it is disappointing that this system did not work in a zebrafish

model, it still represents a useful high throughput approach to studying kinetics of vesicle entry into host cells in tissue culture. In the future, this format could be used to identify particular chemicals or treatments which impair the ability of OMVs to enter host cells. Since OMVs are able to deliver toxic cargo and promote pathogenesis, inhibition of their entry and cargo delivery may become a useful means of attenuating infection. In contrast, it could also be used to find molecules or receptors which enhance delivery, and facilitate the development of 'designer vesicles' which could deliver molecules such as dyes or drugs to specific cells.

It is well known that OMVs contain different cargos, depending on pathovar and serotype (Bonnington and Kuehn, 2016). OMVs are a dynamic form of export from the bacterial cell, and their cargo reflects the needs of their cell of origin, and therefore varies with growth phase, environmental conditions and stress responses (McBroom and Kuehn, 2007). OMVs are able to transport a wide variety of cargo molecules from the bacterial cell into its external environment, and thus the roles that OMVs have in enhancing bacterial fitness are equally diverse (Haurat et al, 2015). OMVs from K12 with restored O polysaccharide were able to enter host cells and deliver their cargo more efficiently than their 'rough' counterparts. However, the speed and efficiency of this process was still lower than that observed with OMVs from the pathogenic strains of EHEC or EAEC, suggesting that there are likely to be other adaptations that maximise the OMV-host cell interactions during infection, or this may be due to differences in the O polysaccharide structure. Although all strains used are from *E. coli*, there are significant genetic

differences between the strains, and they have also adapted to different environments. K12 has been a lab strain for almost 100 years, whilst EHEC is a serious human pathogen rarely found in healthy people, and EAEC is able to persist and colonise the human intestinal tract. It would therefore be expected that OMVs from non pathogenic strains would be less efficient at entering host cells. It is intriguing that O polysaccharide is able to contribute to this process across all three strains studied.

In order to try and ensure the OMV cargo was the same between the 'rough' and 'smooth' OMVs, it was attempted to deplete the O polysaccharide using glycoside hydrolases to cleave the bonds between subunits. When the LPS of the enzyme treated strains was analysed using silver staining, it was apparent that the core regions of the outer membrane had also been modified, and the number of repeat units of O polysaccharide, and thus it would not be a fair comparison: however future work may enable characterisation of these 'semi-rough' OMVs in order to further elaborate on the requirement of the O polysaccharide for efficient uptake of OMVs. Other studies have found that even a single O polysaccharide unit can enhance virulence 100 fold compared to a complete 'rough' strain, and so it would be interesting to determine the minimum amount of O polysaccharide necessary (Bengoechea et al, 2004).

All three strains used have different O polysaccharide serotypes; O16, O157 or O42. It may be that the particular sugar composition alters the affinity for host cells, or that strains express more or less repeats altering the interaction kinetics. It is interesting that

presence of O polysaccharide did not affect all strains in the same manner; for EHEC and K12, the maximum rate was not significantly affected, but for EAEC, there was a significant increase in the maximum rate, leading to an overall increase in uptake efficiency. Therefore other differences in either the cargo or the membrane composition may also be contributing to the ability of OMVs to enter cells. It would be useful to express the same O polysaccharide in all three strains to examine the role of the specific structure in interactions with host cells, but due to the large number of unique genes in the O polysaccharide synthesis operon required for expression of O polysaccharides, it was experimentally difficult to transfer these genes into other strains. It would also be difficult to control the number of repeats added to each O polysaccharide, which may also affect its ability to interact with the host cell surface; a longer O polysaccharide may result in more rapid internalisation.

Previous work has indicated that the cargo of OMVs can play a role in facilitating entry, and whilst this is certainly likely to be the case for many virulence factors or adhesins, this work has identified a generic component of OMVs that can contribute to OMV interactions with host cells, even in non-pathogenic strains.

The comparison of O polysaccharide deficient mutants with wild type OMVs as well as comparison of different pathovars has the pitfall that other vesicle cargo may be modulated and alter uptake kinetics. However, in this study there is a reproducible correlation between O polysaccharide containing and –lacking strains, across three

different serotypes and pathovars, suggesting for the first time that O polysaccharide is, if not the only factor, at least a main driver of uptake kinetics.

The next area to be explored in this work is unravelling how exactly the O polysaccharide contributes to enhancing OMV uptake, and exploring the mechanisms of entry that may be exploited by OMVs.

5. Composition of OMVs affects their preferred route of uptake by host cells

5.1 Introduction

Previous chapters used a new and sensitive OMV uptake assay to reveal a role for O polysaccharide in enhancing the ability of OMVs to enter host cells, either by increasing the overall efficiency of uptake, or significantly increasing the maximum rate. This occurred for OMVs derived from pathogenic strains, EHEC and EAEC but also the laboratory strain, K12.

This chapter utilised chemical inhibition of endocytic uptake processes to determine how O polysaccharide influences the route of entry into host cells, using the sensitive CCF2-AM plate reader assay developed earlier in this work.

5.2 Results

5.2.1 Uptake of OMVs is dynamin dependent.

Dynamin is a 100 kDa GTPase protein that facilitates invagination of the plasma membrane, by polymerising around the neck of a vesicle, allowing it to pinch off and become separated from the membrane (El-Sayed and Harashima, 2013; Preta et al, 2015). Dynamin activity is required for both clathrin and caveolin mediated endocytosis (El-Sayed and Harashima, 2013).

HeLa cells pre-treated with 80 μM of the dynamin GTPase inhibitor Dynasore (Girard et al, 2011; Preta et al, 2016) for 1h prior to infection with reporter OMVs from EHEC, EAEC and K12 showed reduced blue fluorescence compared with untreated infected cells, indicating an impairment of OMV entry and subsequent β-lactamase cleavage of the CCF2-AM substrate (Figure 5.1A-E). This impairment occurred for OMVs with and without O polysaccharide, and for the pathogenic strains EHEC and EAEC, and the non-pathogenic lab strain K12. This indicated that dynamin is a requirement for OMV entry, irrespective of the presence of O polysaccharide in the outer membrane.

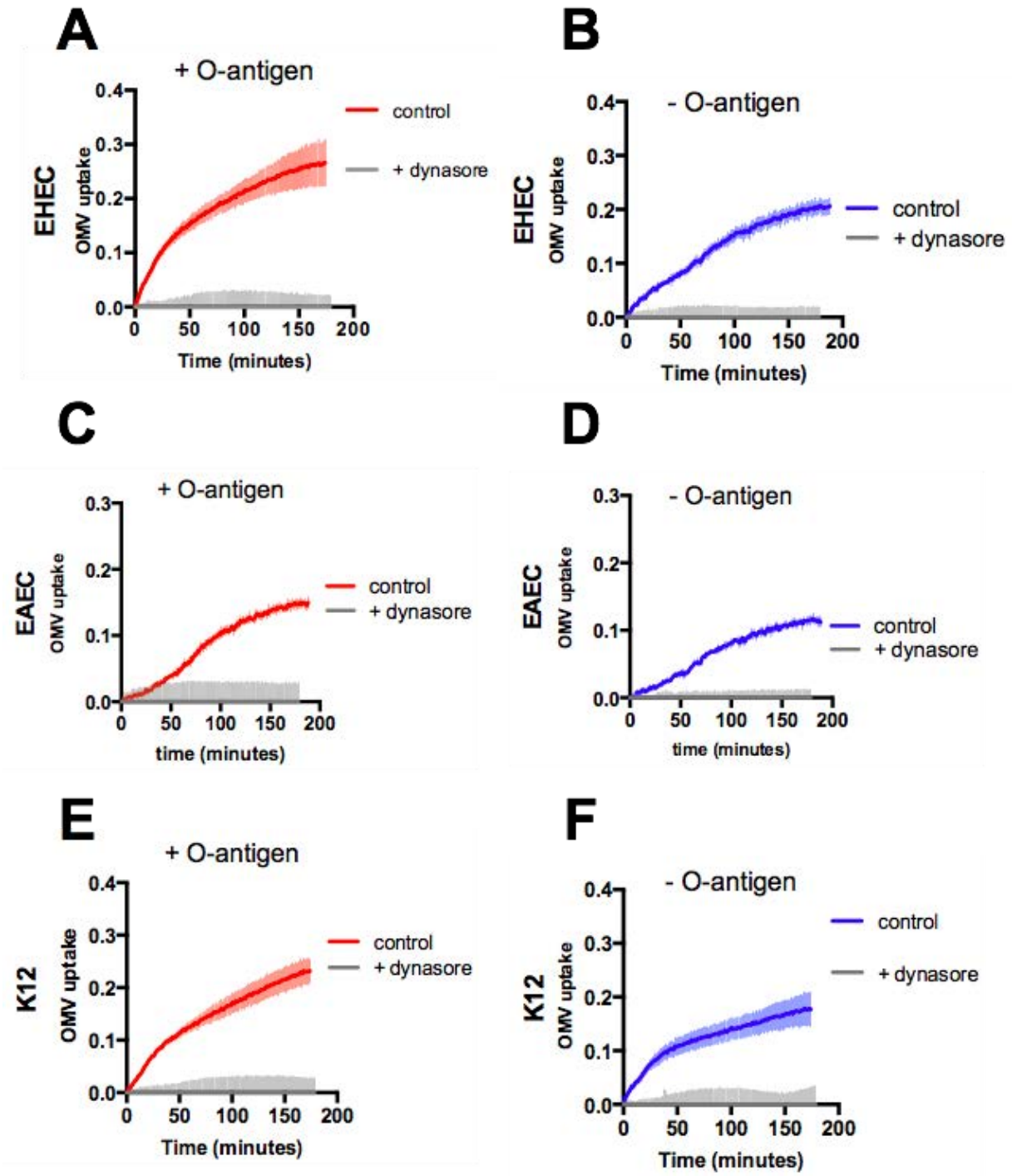

Figure 5.1. OMV uptake is dynamin dependent.
HeLa cells pre-treated with 80 µM dynasore for 1h prior to incubation with reporter OMVs from EHEC, EAEC and K12 at an MOI of 1000 all showed a reduction in OMV uptake (A-E, grey line) compared with the untreated controls (A-E, red: presence of O

(Figure 5.1 continued) ..antigen, blue: lacking O polysaccharide). OMV uptake (Y axis) was defined as the ratio of blue:green fluorescence over 3h as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred (A-E). Data shown are means with SD, with three technical replicates for each of three separate experiments.

5.2.2 Macropinocytosis is not a major route of uptake of OMVs.

Macropinocytosis has been suggested as a possible entry mechanism for extracellular vesicles into host cells (Bomberger et al, 2009). Inhibition of macropinocytosis following treatment of host cells with 20 μM blebbistatin (Ramanathan et al, 2015) to inhibit the formation of macropinosomes enhanced the efficiency of uptake of EHEC OMVs containing O polysaccharide, but had no effect on the maximum rate of uptake (Figure 5.1A-D). There was no effect on uptake for EHEC OMVs without O polysaccharide (Figure 5.2E-H). For EAEC, there was no effect on either the efficiency or the rate, for OMVs with and without O polysaccharide (Figure 5.3).

In contrast, for K12 there was a significant increase in efficiency and rate of uptake of OMVs, particularly for those without O polysaccharide (Figure 5.4). This indicates that inhibition of macropinocytosis causes these OMVs to use an alternative, faster pathway. These data suggest that only a small fraction of OMVs usually enters cells by macropinocytosis, and inhibition of this relatively slow uptake route either does not affect or accelerates uptake.

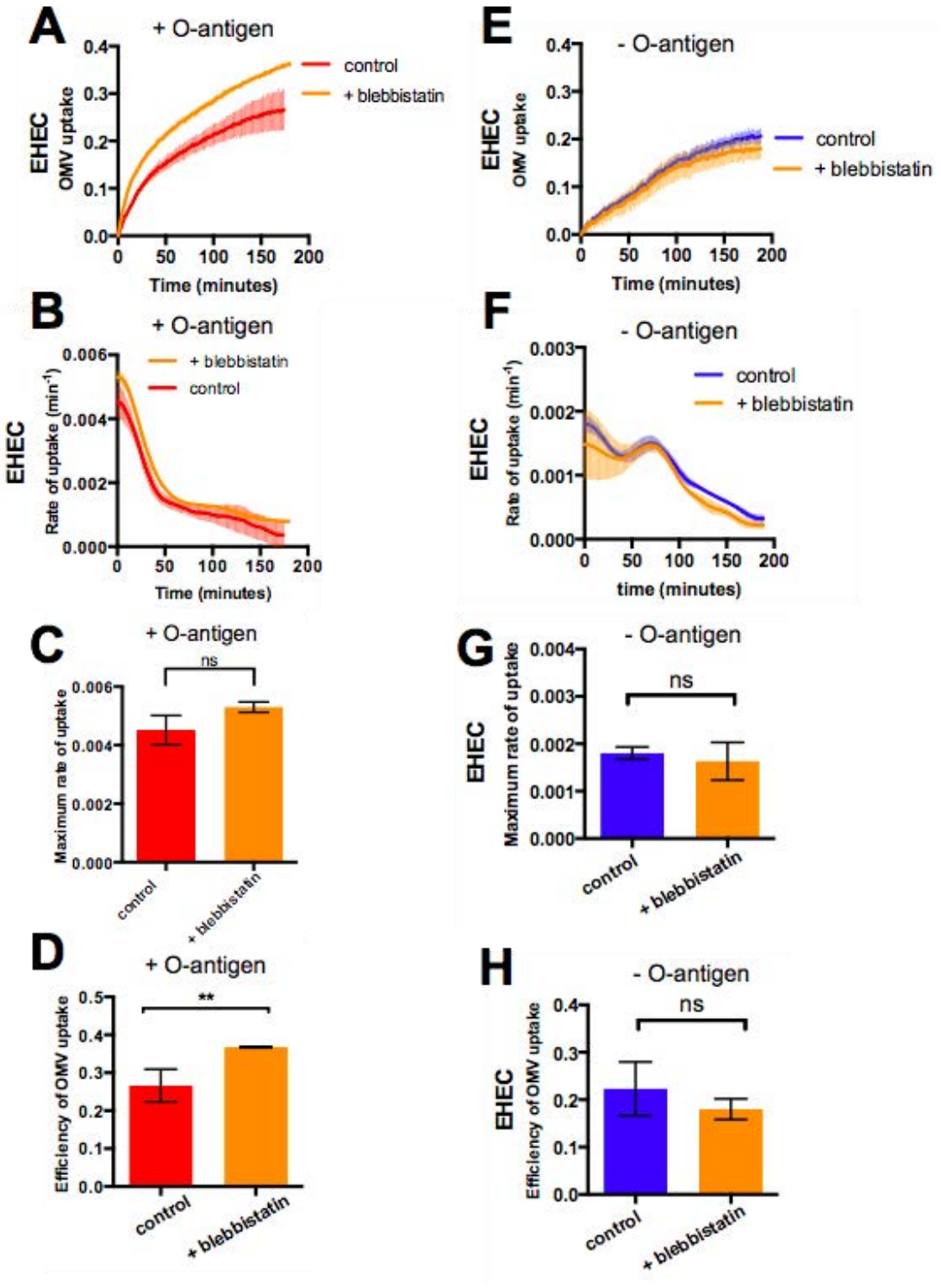

Figure 5.2. Macropinocytosis is not a major route of uptake of OMVs from EHEC.

(Figure 5.2. continued) ...HeLa cells treated with 20 μM blebbistatin for macropinocytosis inhibition (orange) for 1h at 37 °C and exposed to ClyA-Bla OMVs isolated from EHEC with O polysaccharide (red) or without (blue) at an MOI of 1000 for 3 hours. OMV uptake (Y axis) is defined as the ratio of blue:green fluorescence over 3h as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred. Ratios were plotted as means ± stdev for 3 biological replicates with three technical replicates. (A, E) The gradient of the colour change was estimated to determine the rate of colour change over time (B, F) and the maximum rate of change was determined as the highest change in gradient over the 3h (C, G). Efficiency of uptake (D, H) was defined as the total change in blue:green ratio over 3h from (A and E). Replicates were treated as 9 independent replicates. Significance was determined using student's t-test, with a p value <0.05 considered statistically significant. (**) p≤0.01, and (ns) not significant.

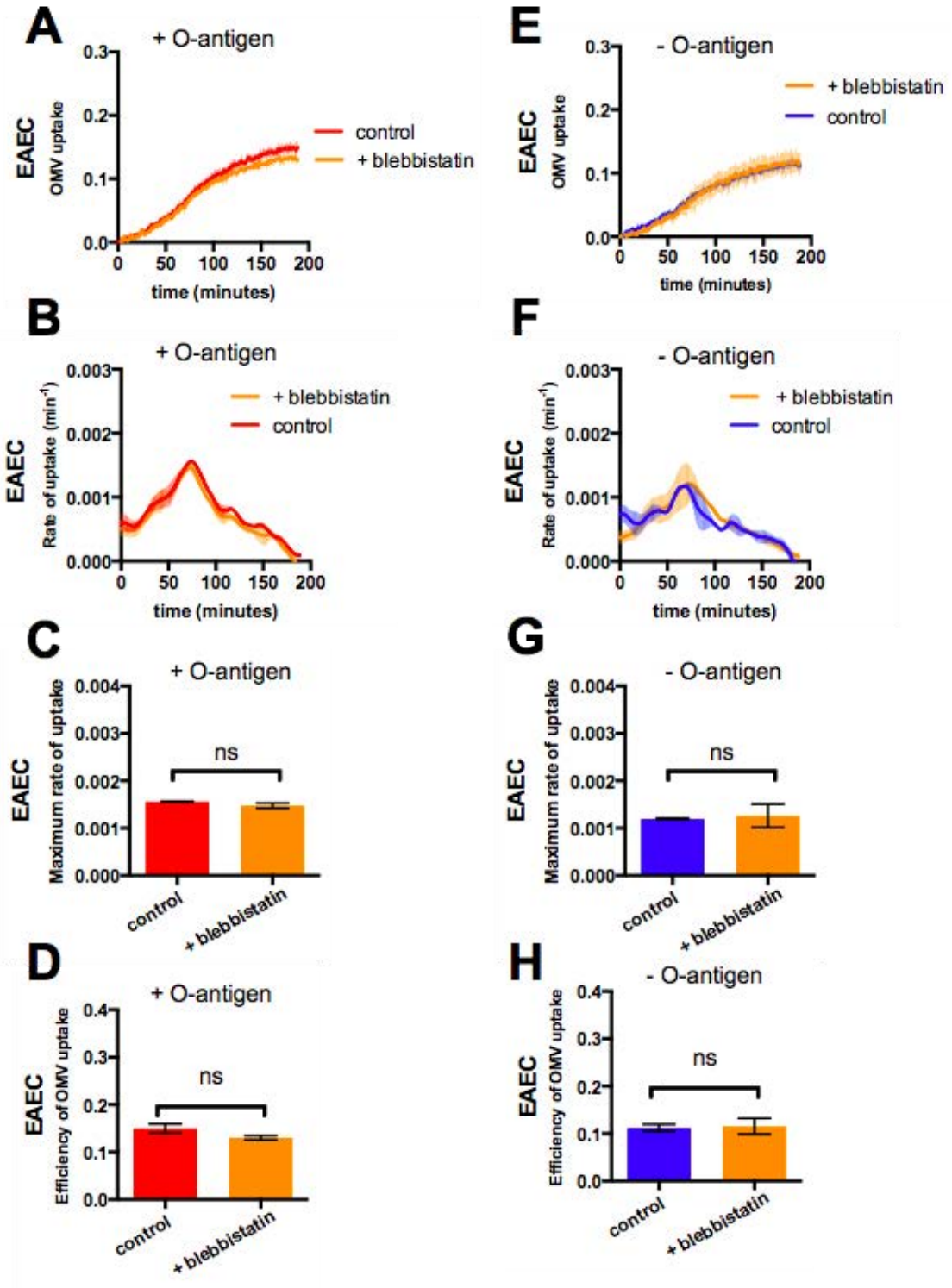

Figure 5.3. Macropinocytosis is not a major route of uptake of OMVs from EAEC.

(Figure 5.3. continued) HeLa cells treated with 20 μM blebbistatin for macropinocytosis inhibition (orange) for 1h at 37 °C and exposed to ClyA-Bla OMVs isolated from EAEC with O polysaccharide (red) or without (blue) at an MOI of 1000 for 3 hours. OMV uptake (Y axis) is defined as the ratio of blue:green fluorescence over 3h as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred. Ratios were plotted as means ± stdev for 3 biological replicates with three technical replicates. (A, E) The gradient of the colour change was estimated to determine the rate of colour change over time (B, F) and the maximum rate of change was determined as the highest change in gradient over the 3h (C, G). Efficiency of uptake (D, H) was defined as the total change in blue:green ratio over 3h from (A and E). Replicates were treated as 9 independent replicates. Significance was determined using student's t-test, with a p value > 0.05 not considered statistically significant.

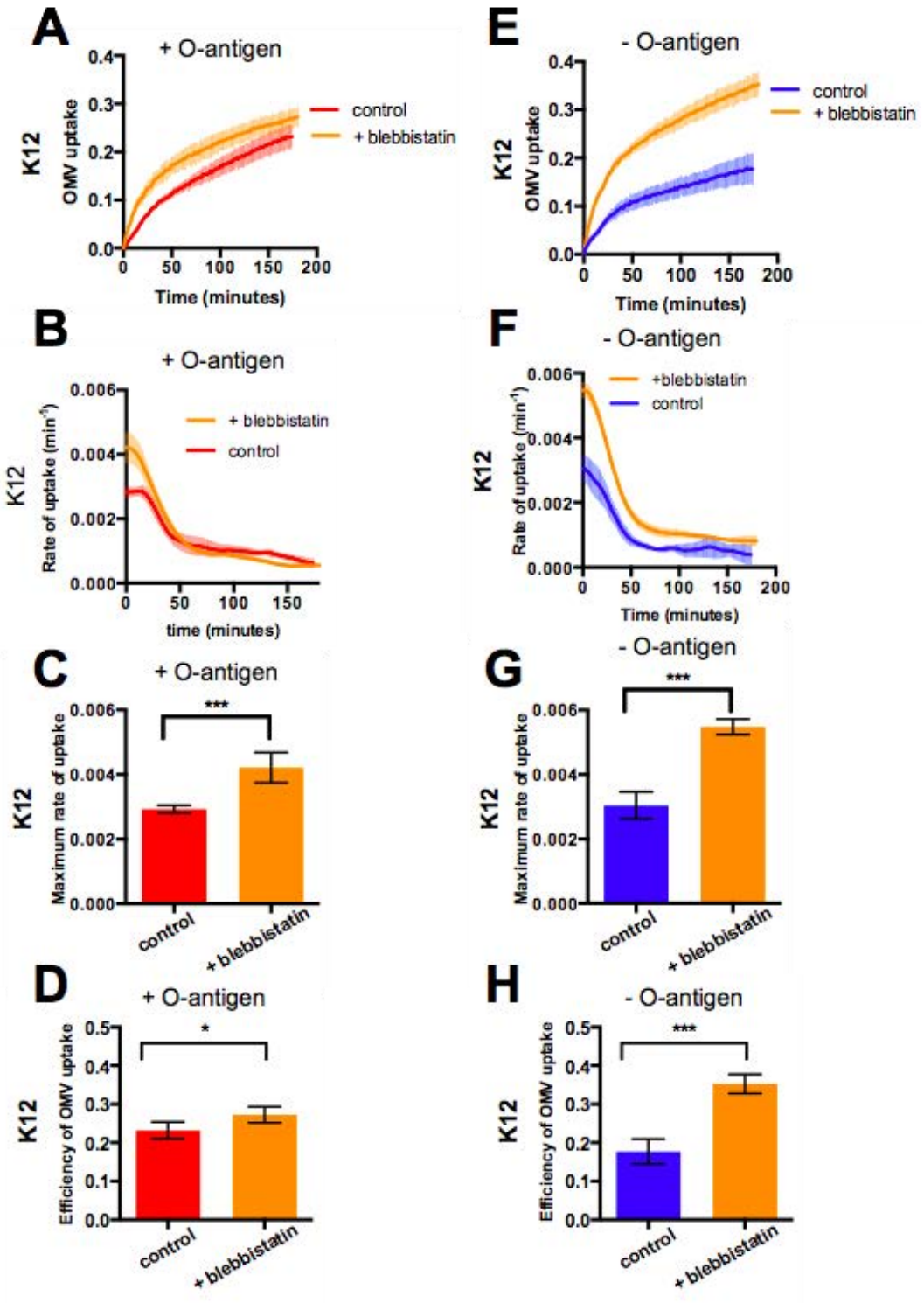

Figure 5.4 Macropinocytosis is not a major route of uptake of OMVs from K12.

(Figure 5.4 continued)...HeLa cells treated with 20 μM blebbistatin for macropinocytosis inhibition (orange) for 1h at 37 °C and exposed to ClyA-Bla OMVs isolated from K12 with O polysaccharide (red) or without (blue) at an MOI of 1000 for 3 hours. OMV uptake (Y axis) is defined as the ratio of blue:green fluorescence over 3h as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred. Ratios were plotted as means ± stdev for 3 biological replicates with three technical replicates. (A, E) The gradient of the colour change was estimated to determine the rate of colour change over time (B, F) and the maximum rate of change was determined as the highest change in gradient over the 3h (C, G). Efficiency of uptake (D, H) was defined as the total change in blue:green ratio over 3h from (A and E). Replicates were treated as 9 independent replicates. Significance was determined using student's t-test, with a p value < 0.05 considered statistically significant. (***) p≤0.001, (*) p≤0.05 and (ns) not significant.

5.2.3 OMVs lacking O polysaccharide are dependent on receptor mediated endocytosis

The previous experiments revealed the dynamin dependent entry of OMVs into host cells, but since dynamin is implicated in numerous uptake routes, it was necessary to further elucidate the mechanisms of vesicle entry (Preta et al, 2015). Clathrin mediated endocytosis has been described previously as a route of entry for OMVs (Olofsson et al, 2014). It was next determined whether the entry of OMVs was dependent on clathrin mediated endocytosis, which requires dynamin and utilises protein receptors on the host cell surface (Vercauteren et al, 2010).

Clathrin or receptor mediated endocytosis was inhibited either by proteolytic removal of all protein receptors from the surface of HeLa cells with 5μg/ml papain prior to incubation with OMVs, or by blocking clathrin-coated pit assembly using 1μg/ml chlorpromazine (Larrick et al, 1985; Papatheodorou et al, 2010; Vercauteren et al, 2010). Removal of protein receptors from the host cell surface had no effect on the efficiency of

uptake for OMVs with O polysaccharide, for EHEC, EAEC and K12 but significantly decreased efficiency of uptake of O polysaccharide deficient OMVs, particularly for K12 (Figure 5.5). Pre-treatment of cells with chlorpromazine had a similar effect; but significantly increased the overall uptake for EHEC and K12 OMVs with O polysaccharide (Figure 5.5).

Treatment with papain did not alter the maximum rate of uptake for EHEC or EAEC OMVs, but caused a significant increase in rate for K12 OMVs with O polysaccharide, and additionally a significant decrease in rate of uptake for those without O polysaccharide (Figure 5.6). Similarly, chlorpromazine treatment did not affect the maximum rate for EHEC or EAEC OMV uptake, but again caused a significantly increased rate of uptake for OMVs from K12 with O polysaccharide (Figure 5.6). Both papain and chlorpromazine negatively affected uptake of OMVs without O polysaccharide, and although produced variable effects in O polysaccharide positive OMVs, they did not inhibit uptake.

This suggests that OMVs lacking O polysaccharide require protein receptors for uptake and use clathrin-mediated endocytosis as a main route of entry. In contrast, OMVs with intact O polysaccharide do not rely on protein receptors for entry, and inhibition of clathrin-mediated endocytosis further accelerates their uptake into host cells.

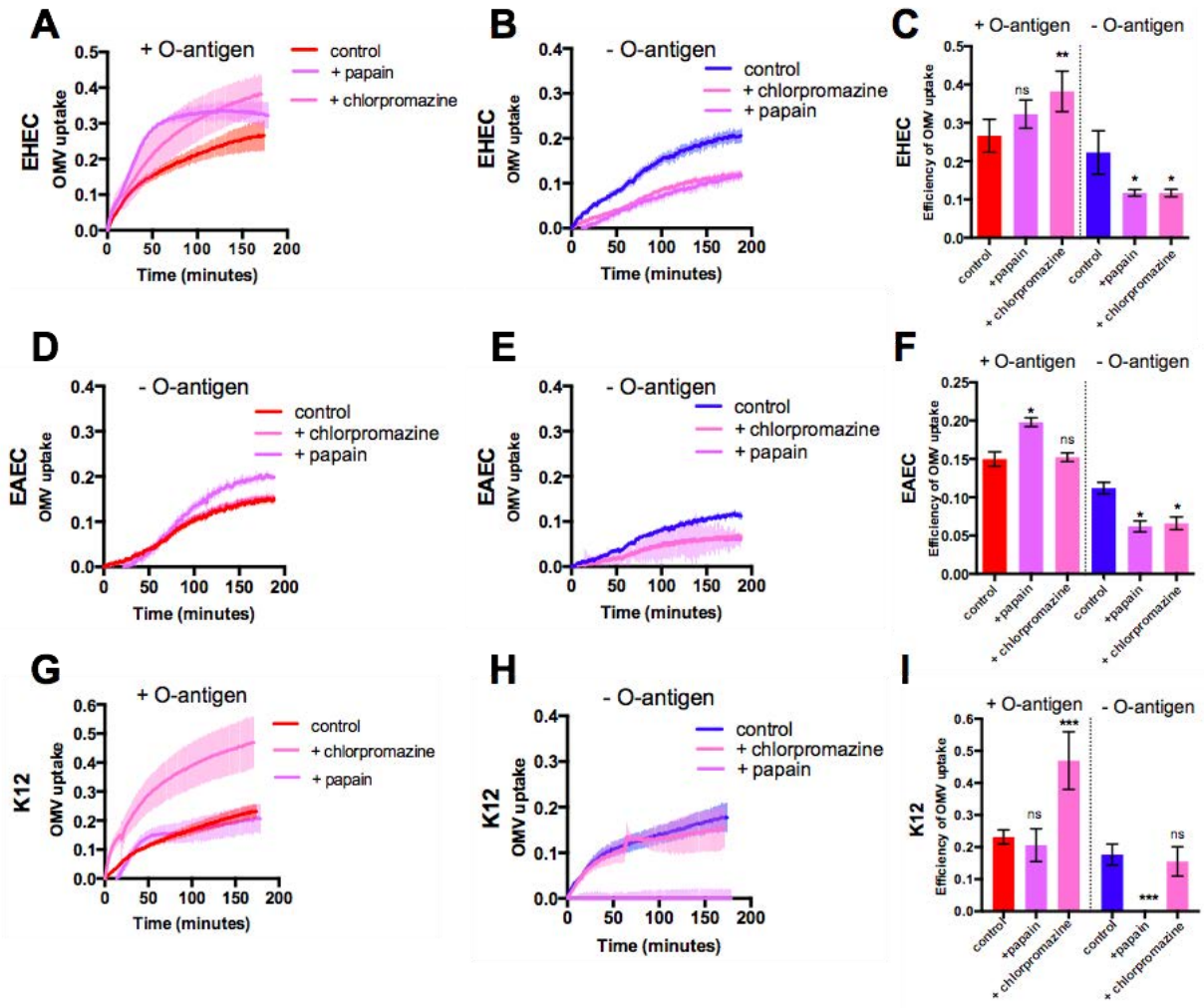

Figure 5.5. OMVs lacking O polysaccharide require clathrin mediated endocytosis for entry into host cells.

Hela cells were either left untreated (control, red), or pre-treated with 5 µg/ml papain (lilac), 1 µg/ml chlorpromazine (pink) and exposed to ClyA-Bla OMVs isolated from EHEC (A), EAEC (D) or K12 (G) with (red) or without (blue) O polysaccharide at an MOI of 1000 for 3 hours. OMV uptake (Y axis) is defined as the ratio of blue:green fluorescence over 3h as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred. Ratios were plotted as means ± stdev for 3 biological replicates with three technical replicates. (A,B,D,E,G,H). Efficiency of uptake (C, F, I) was defined as the total change in blue:green ratio over 3h from (A,B,D,E,G,H). ANOVA was used to analyse results, with a Brown Forsythe test for equal variance. Replicates were treated as 9 independent replicates, and corrected for multiple comparisons between samples. P value < 0.05 was considered statistically significant. (***) indicates p≤0.001, (**) p≤0.01, (*) p≤0.05, (ns) not significant.

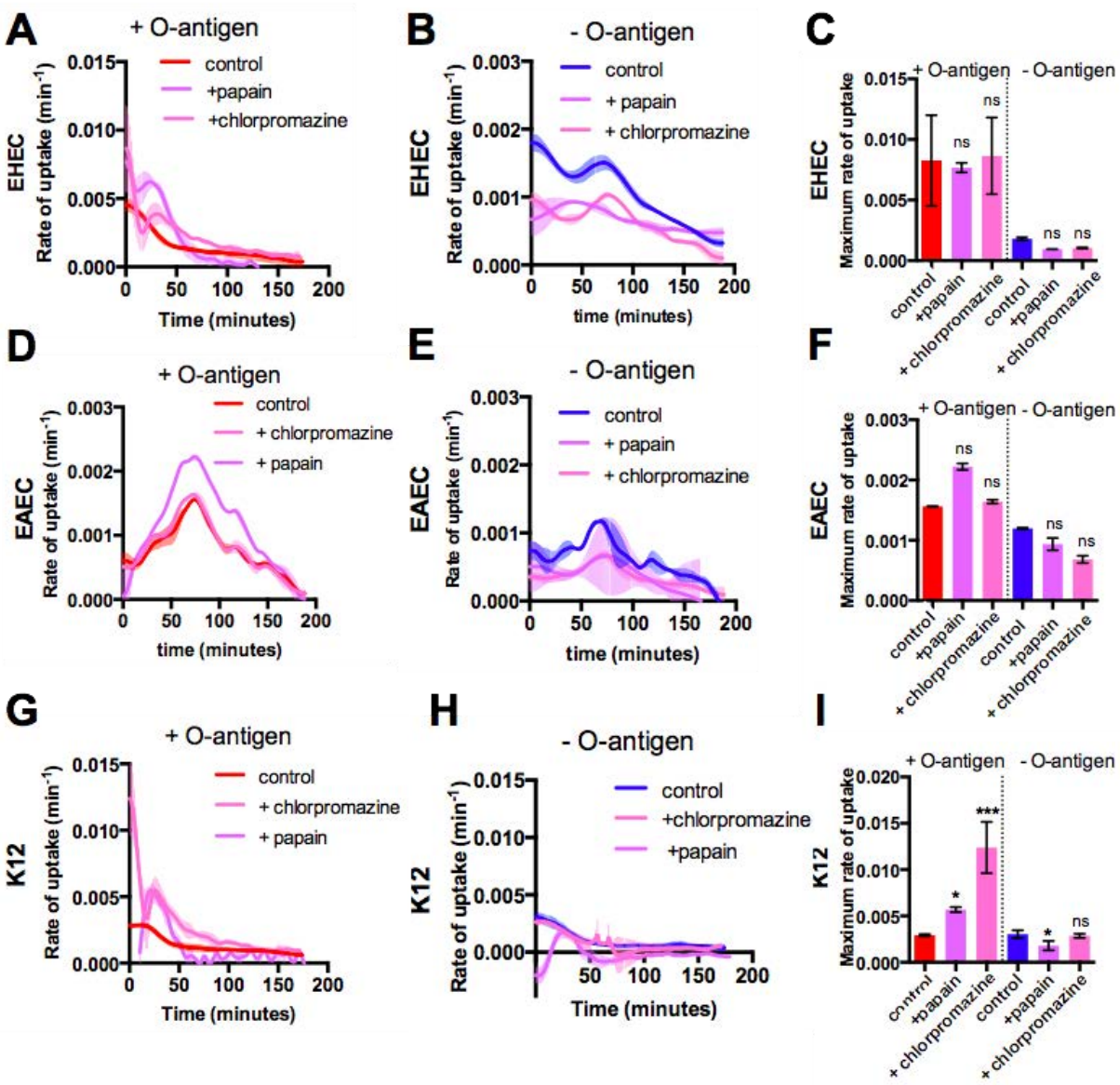

Figure 5.6. Rate of entry for OMVs with O polysaccharide increases in the absence of clathrin mediated endocytosis.

To determine rate of OMV entry, polynomials were fitted to each FRET data set in Figure 5.5 (A,B,D,E,G,H) using the cubic spline function csaps in Matlab. Numerical estimates of the gradients of the resulting polynomials were determined using the gradient function. Data shown are means ± stdev for 3 biological replicates with three technical replicates. Maximum rate of uptake (C, F and I) were determined from data in (A,B,D,E,G,H) as the maximum change in gradient over 3h. Data shown are means ± stdev, with replicates treated as 9 independent replicates. Significance was determined by ANOVA, with a Brown Forsythe test to determine equal variance, and corrected for multiple comparisons

(Figure 5.6 continued)...between samples and with their relevant control. A p value < 0.05 was considered statistically significant. (***) p≤0.001, (*) p≤0.05, (ns) not significant.

5.2.4 OMVs with O polysaccharide enter host cells faster because they can access raft-mediated endocytosis more efficiently.

Since OMVs displaying O polysaccharide on their surface accessed host cells faster in the absence of clathrin-dependent endocytosis, it seemed that they were not dependent on this route and must be using an alternative entry pathway. The next experiments investigated whether this was mediated by raft-dependent pathways, which use cholesterol rich microdomains of the plasma membrane, and in the case of caveolin mediated entry, also require dynamin (Rewatkar et al, 2015). Disruption of raft-mediated endocytosis, either by sequestration of membrane cholesterol from membrane microdomains via methyl-β-cyclodextrin (mbcd) (Danthi and Chow, 2004; Vercauteren et al, 2010; Contreras et al, 2010) or by disrupting raft dynamics with filipin (Maxfield and Wustner, 2012; Ilnytska et al, 2013) led to a reduced r_{max} (Figure 5.8) and uptake efficiency (Figure 5.8).

For EHEC OMVs, efficiency of uptake was reduced in the absence of lipid rafts, irrespective of the presence of O polysaccharide, but this effect was more pronounced for the OMVs containing O polysaccharide (Figure 5.7 A-C). However, the maximum rate of uptake was significantly reduced for EHEC O polysaccharide OMVs, whereas the maximum rate of uptake was unaffected for the OMVs lacking O polysaccharide (Figure

5.8 A-C). This suggests that the EHEC OMVs with O polysaccharide are more adversely affected by the loss of lipid rafts as an entry pathway than their non-O polysaccharide counterparts.

For EAEC OMVs, the efficiency of uptake was significantly reduced by filipin, but only the O polysaccharide negative OMVs were significantly inhibited by mbcd (Figure 5.7 D-F). The maximum rate was not affected for either O polysaccharide or non-O polysaccharide OMVs (Figure 5.8 D-F). The effect of filipin treatment was more significant on the O polysaccharide containing OMVs.

In the case of K12 OMVs, mbcd did not reduce efficiency of uptake for the O polysaccharide containing OMVs, but significantly reduced entry of non O polysaccharide OMVs (Figure 5.7 G-I). It did not affect the maximum rate of uptake (Figure 5.8 G-I). Filipin treatment significantly inhibited the efficiency of entry, and the maximum rate of uptake of both sets of OMVs (Figure 5.8 G-I). The difference in the apparent efficacy of filipin treatment between EAEC and K12 may be due to the higher variability in the filipin treated cells, which showed more error compared with controls or mbcd treatment.

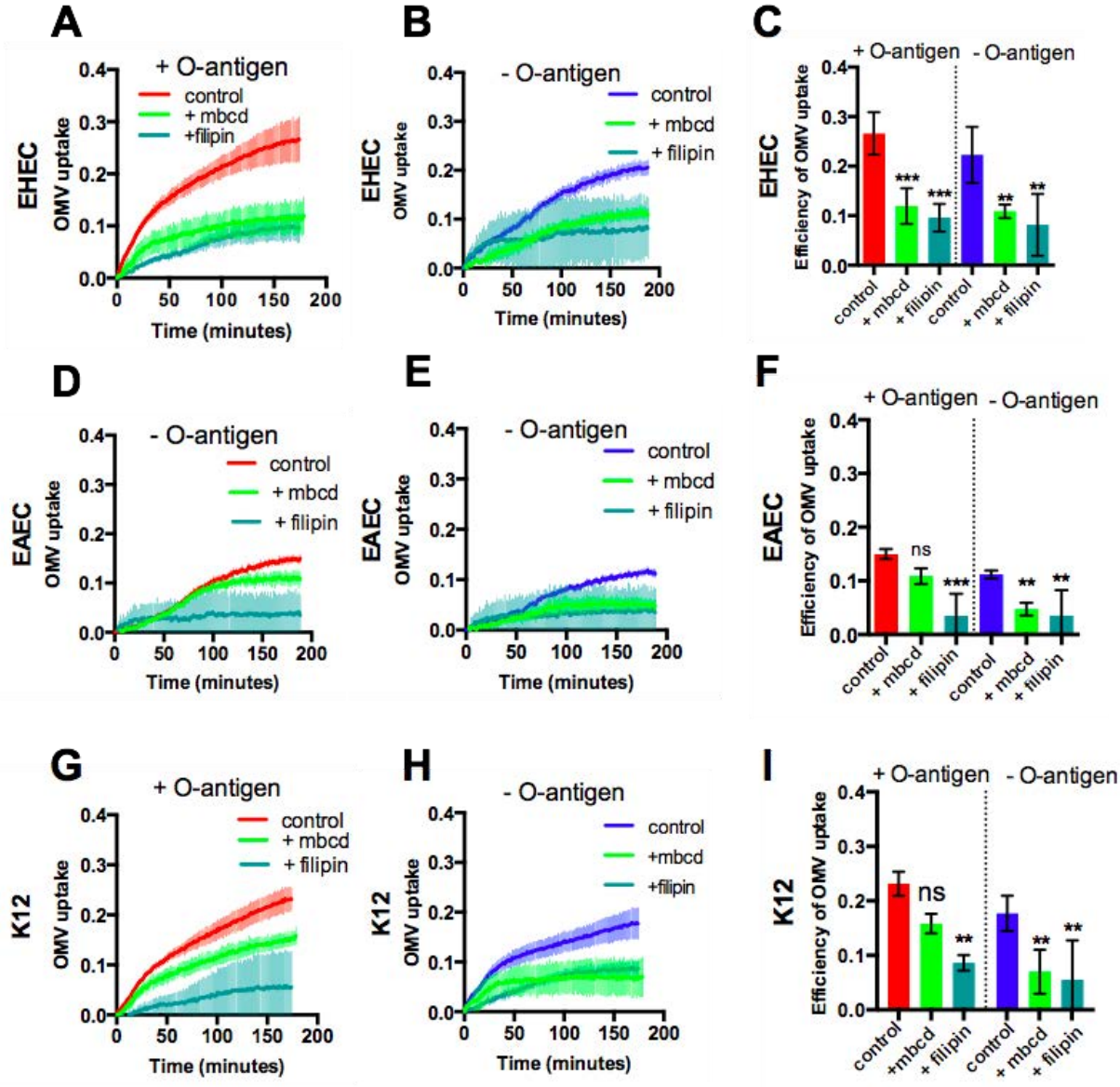

Figure 5.7. OMVs can efficiently access host cells via lipid rafts.

Hela cells were either left untreated (control, red), or pre-treated with 5 mM methyl-β-cyclodextrin (light green) or 1 µg/ml filipin (turquoise)and exposed to ClyA-Bla OMVs isolated from EHEC (A-C), EAEC (D-F) or K12 (G-I) with (red) or without (blue) O polysaccharide at an MOI of 1000 for 3 hours. OMV uptake (Y axis) is defined as the ratio of blue:green fluorescence over 3h as an indication of entry of β-lactamase from the reporter OMVs into host cells, where cleavage of CCF2-AM substrate then occurred. Ratios were plotted as means ± stdev for 3 biological replicates with three technical replicates. (A,B,D,E,G,H). Efficiency of uptake (C, F, I) was defined as the total change in blue:green ratio over 3h from (A,B,D,E,G,H). ANOVA was used to analyse results, with a Brown Forsythe test for equal variance. Replicates were treated as 9 independent replicates, and corrected for multiple comparisons between samples. P value < 0.05 was

(Figure 5.7 continued)...considered statistically significant. (***) indicates p≤0.001, (**) p≤0.01, (*) p≤0.05, (ns) not significant.

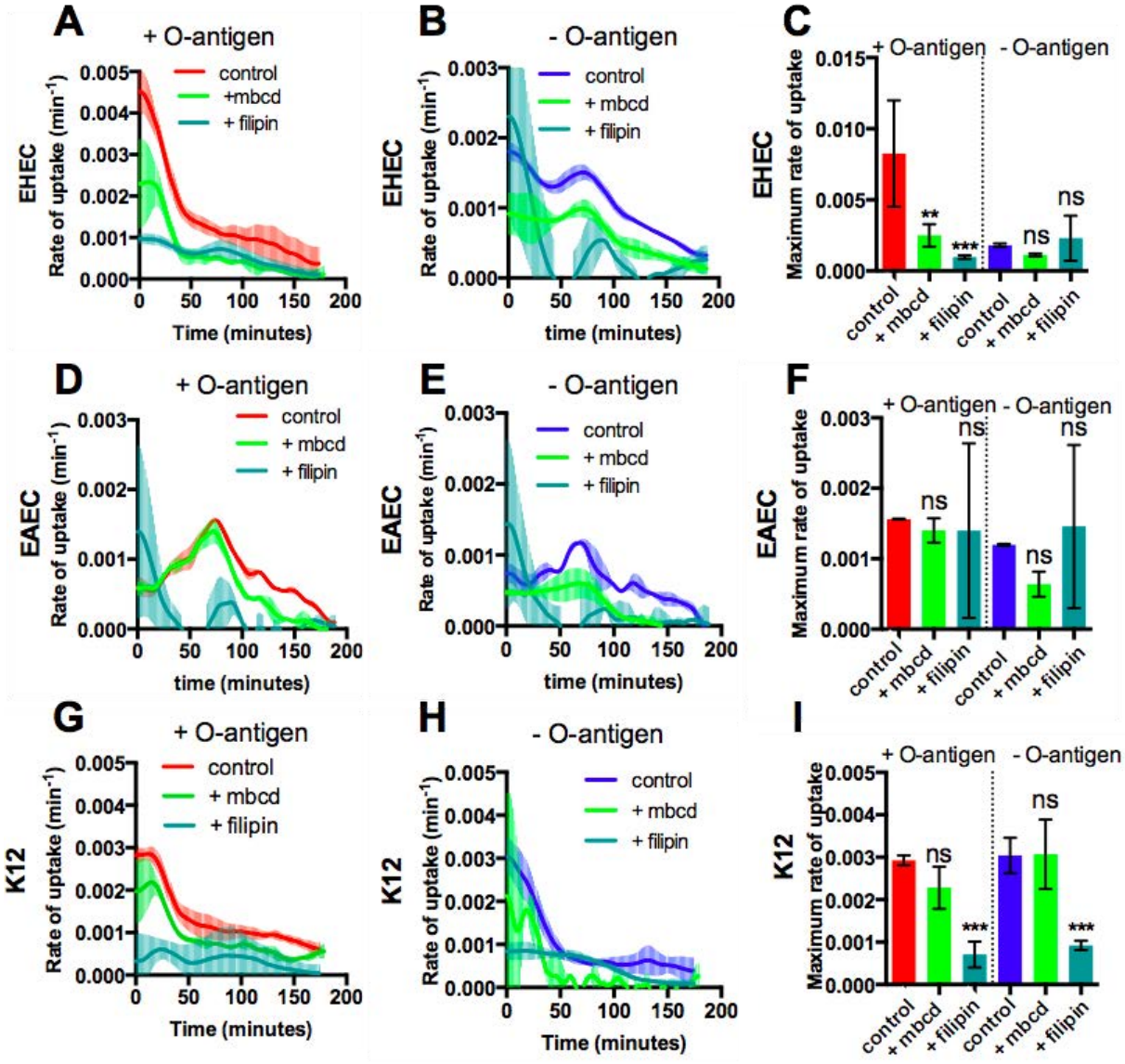

Figure 5.8. Rate of entry for OMVs is reduced in the absence of lipid raft mediated endocytosis

To determine rate of OMV entry, polynomials were fitted to each FRET data set in Figure 5.7 (A,B,D,E,G,H) using the cubic spline function csaps in Matlab. Numerical estimates of the gradients of the resulting polynomials were determined using the gradient function. Data shown are means ± stdev for 3 biological replicates with three technical replicates. Maximum rate of uptake (C, F and I) were determined from data in (A,B,D,E,G,H) as the maximum change in gradient over 3h. Data shown are means ± stdev, with replicates treated as 9 independent replicates. Significance was determined by ANOVA, with a Brown Forsythe test to determine equal variance, and corrected for multiple comparisons between samples and with their relevant control. A p value < 0.05 was considered statistically significant. (***) $p \leq 0.001$, (**) $p \leq 0.01$ (*) $p \leq 0.05$, (ns) not significant.

These data show that, while OMVs are able to access different uptake routes including macropinocytosis, clathrin-dependent and raft-dependent endocytosis, OMVs displaying O polysaccharide on their surface are able to utilise raft-dependent endocytosis more efficiently, and are more affected by its inhibition, while OMVs lacking O polysaccharide are more reliant on clathrin-mediated uptake. Inhibition of receptor or clathrin mediated endocytosis shifts a larger fraction of these O polysaccharide positive OMVs to use raft-mediated endocytosis which further accelerates their uptake, whereas the O polysaccharide lacking OMVs are less able to use this alternative pathway (Figure 5.9).

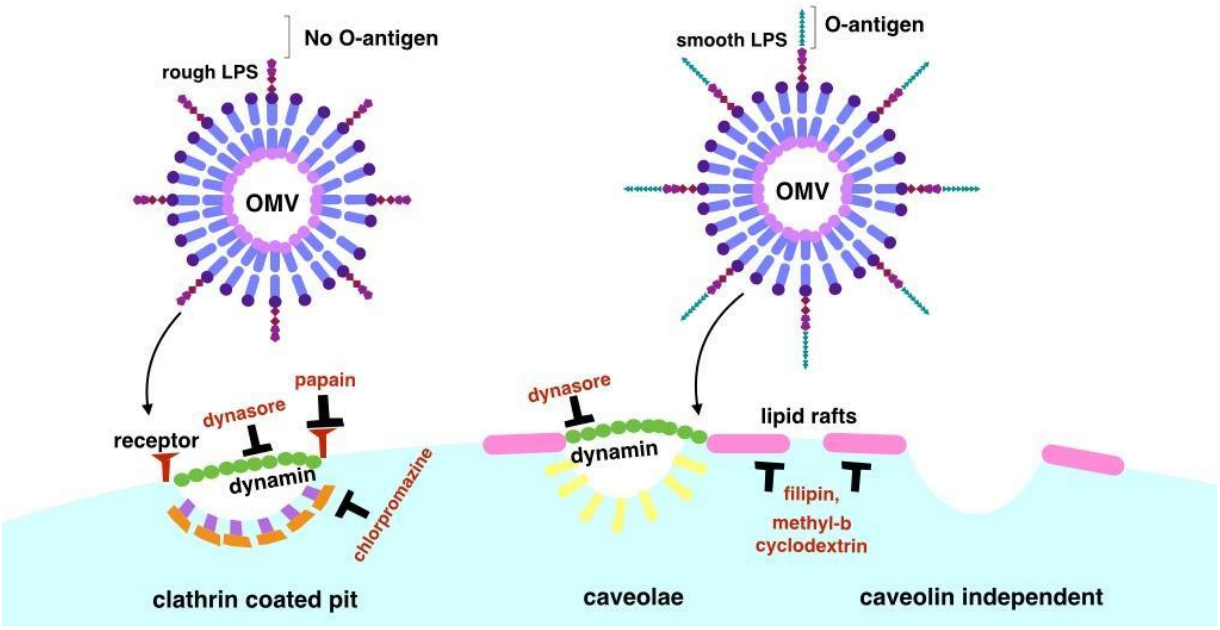

Figure 5.9. LPS composition determines major route and kinetics of OMV entry into host cells

5.2.5 Purified LPS competes with EHEC OMVs for lipid raft mediated endocytosis.

Next it was considered whether purified LPS would compete with the OMVs for binding sites on the host cell surface. Prior to infection with OMVs, HeLa cells were pre-incubated with 1 μg/ml LPS from either smooth (O55:B5, core type R3) or rough strains (EH100, no O polysaccharide, core type R2). Supplementation with LPS inhibited the efficiency of uptake of EHEC OMVs, but had no significant effect on uptake of OMVS from EAEC or K12 (Figure 5.10).

Since LPS is known to associate with the host via TLR4, including in the HeLa tissue culture cell line, inhibition of TLR4 by pre-treatment of cells with C34, a 2-acetamidopyranoside which competitively binds in the inner hydrophobic region of the MD2 TLR4 co-receptor (Neal et al, 2013; Jiang et al, 2017) was used to determine if the EHEC OMVs required TLR4 binding in order to enter cells. C34 treatment did not inhibit uptake of EHEC OMVs, suggesting that they are not competing for TLR4 (Figure 5.11) (Neal et al, 2013). TLR4 activation also requires binding of CD14, a protein localised in cholesterol rich lipid raft domains (Plociennikowska et al, 2015). EHEC OMVs were previously shown to be highly dependent on the presence of host cell membrane cholesterol for entry into host cells, more so than the other strains (Figure 5.7-8). As it was demonstrated that entry of EHEC OMVs is strongly dependent on cholesterol rich regions of the membrane, it suggests that the inhibition of uptake observed with the addition of purified LPS, but not with the TLR4 inhibitor, is due to competition with OMVs for binding in the cholesterol rich domains, where TLR4 is also present.

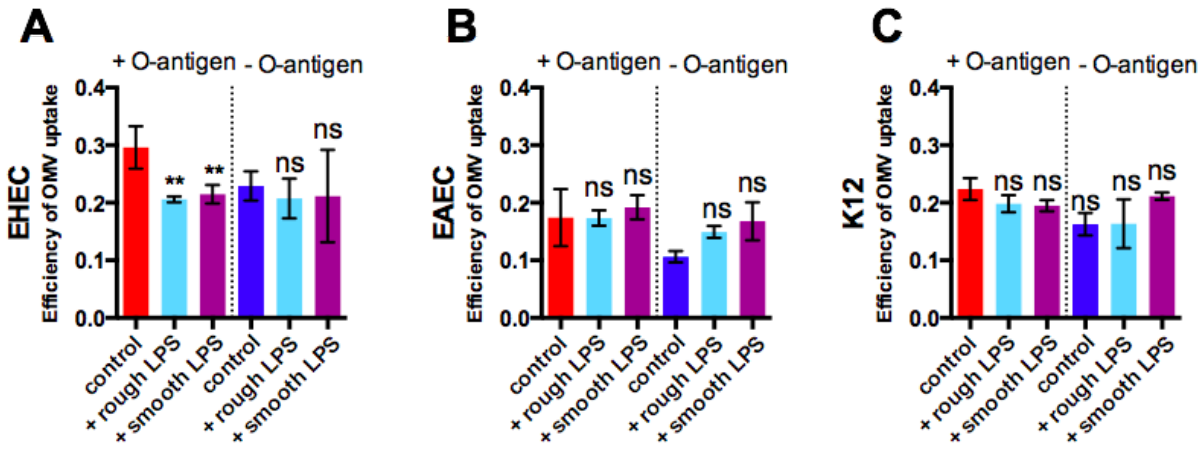

Figure 5.10. Supplementary LPS inhibits entry of EHEC O polysaccharide OMVs.

(A) Hela cells were either left untreated (red), or pre-treated with 1μg/ml rough LPS (from EH100 Ra mutant strain, Sigma) (pale blue) or 1μg/ml smooth LPS (from E. coli O55:B5, Sigma) (purple) and then exposed to ClyA-Bla OMVs with and without O polysaccharide isolated from EHEC (A), EAEC (B) or K12 (C) at an MOI of 1000 for 3 hours. Total FRET changes after 3 hrs were determined and plotted to visualize overall efficiency of uptake into untreated Hela cells. Data shown are means ± stdev (n=3) and significance was determined by ANOVA, with a Brown Forsythe test for equal variance, and a correction for multiple comparisons (**) indicates p≤0.01, (ns) not significant.

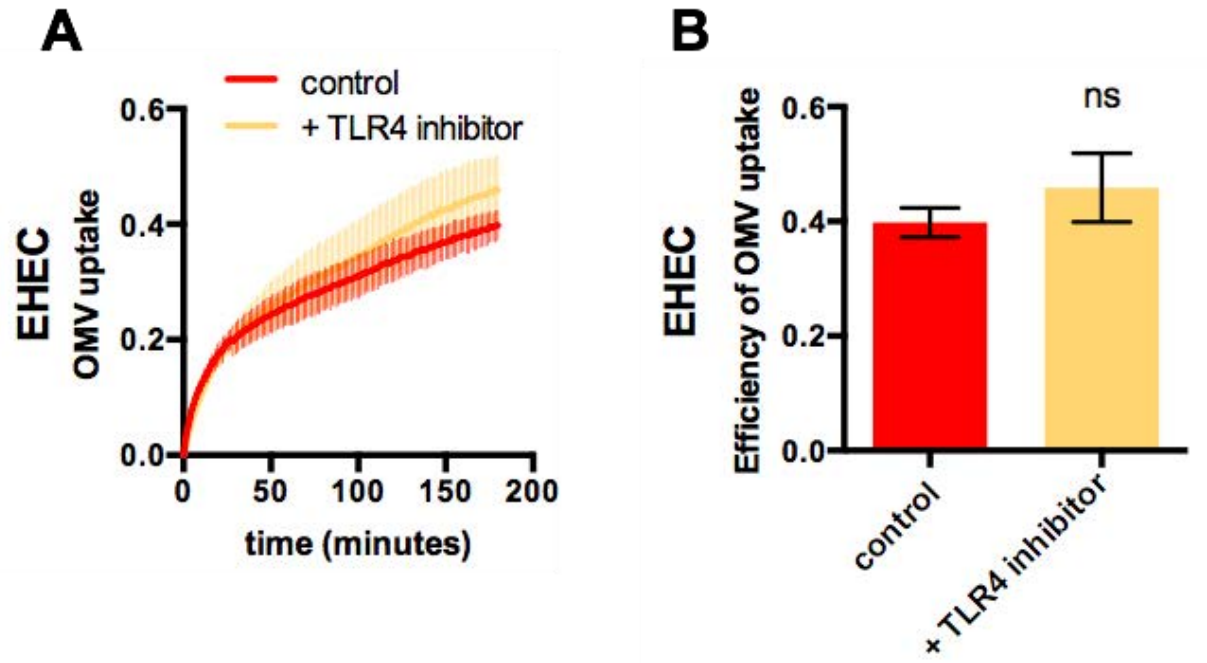

Figure 5.11. Inhibition of TLR4 does not affect entry of EHEC OMVs.

(A) Hela cells were either left untreated (red), or pre-treated with 10 μM TLR4 inhibitor C34 (orange) and exposed to ClyA-Bla OMVs isolated from EHEC with O polysaccharide at an MOI of 1000 for 3 hours. OMV uptake (Y axis, (A),) is defined as the ratio of blue:green fluorescence over time, representing delivery of bla from OMVs into HeLa cells where it can cause cleavage of CCF2-AM dye substrate. Data plotted as mean ± stdev (n=3).
(B) Total FRET changes after 3 hrs were determined from data in (A) and plotted to visualize overall efficiency of uptake into untreated Hela cells (red), or cells pre-treated with C34. Data shown are means ± stdev (n=3) and significance was determined by ANOVA, with a Brown Forsythe test for equal variance, and corrected for multiple comparisons. (ns) indicates p value > 0.05.

5.3 Discussion

The newly-devised assay was used to identify the relative contribution of cellular uptake pathways to OMV entry into host cells. Assays using pharmacological inhibitors to block specific endocytic pathways, showed that while all OMVs use multiple uptake routes, their surface structure biases them towards different pathways. For example, O polysaccharide deficient OMVs had a stringent requirement for surface protein receptors for their uptake, while O polysaccharide containing OMVs were able to access protein-receptor independent pathways. Depletion of such receptors actually allowed them to access protein-receptor independent pathways more efficiently and utilize raft-mediated endocytosis, a more rapid mode of uptake, as main route of entry. While raft-mediated endocytic routes are not as well characterized as clathrin-mediated endocytosis, it is clear there are multiple pathways, including caveolin and non-caveolin dependent raft-mediated endocytosis. Many previous studies have used co-localisation microscopy experiments to determine the route of OMV entry; this assay demonstrates that internalisation occurs within minutes, and that visualisation of these processes would require fixation of these cells almost immediately after infection, meaning that microscopy is unsuitable for fully understanding the kinetics of OMV entry.

Inhibition of receptor-mediated endocytosis by treatment of cells with papain resulted in a significantly increased rate of uptake for EHEC, but a significant reduction in entry for OMVs from EHEC without O polysaccharide. For OMVs from K12, there was no change in rate but an increased total uptake, suggesting a change in the OMV entry phenotype in

the presence of O polysaccharide. This indicates that OMVs without O polysaccharide rely on protein receptors and clathrin- mediated endocytosis in order to gain access to the host cell, whilst OMVs with O polysaccharide are not inhibited by the loss of this pathway. It may be that the presence of the O polysaccharide could lead to possible ligands in the inner regions of the LPS being obscured, whereas these potential receptor binding regions are exposed in the absence of O polysaccharide, and interestingly, loss of the receptor-mediated pathway is beneficial to EHEC, and this may be due to a preference for the non-receptor mediated pathways which allow for more efficient cargo delivery from OMVs.

When lipid raft mediated endocytosis was inhibited with methyl-ß-cyclodextrin or filipin, total uptake of OMVs from all three strains was reduced, but the relative decrease in efficiency was largest for EHEC OMVs, which also showed a significant decrease in the rate of uptake (Figure 3.8-9). This indicated that in contrast to K12, EHEC OMVs preferentially utilize clathrin- independent entry routes. Entry was also shown to be strongly dynamin-dependent, suggesting OMVs containing O polysaccharide are also taken up by caveolin-mediated endocytosis. OMVs from EHEC are known to contribute to infection via delivery of diverse virulence factors, and this may be a further adaptation to pathogenesis for EHEC, as although the rate of internalization by caveolae is around 5 times slower than that of clathrin-dependent endocytosis, unlike clathrin-coated pits, caveolae do not enter endocytic trafficking routes, and thus are not targeted to lysosomes for processing or degradation (Ritter et al, 1995; Bielaszewska et al, 2013). Instead, the

contents of caveolae rapidly diffuse into the cytoplasm, consistent with the observation of a rapid increase in cytoplasmic dye cleavage and thus, rapid increase in FRET almost immediately after vesicle addition to cells. This suggests that internalization via caveolin-mediated endocytosis allows more efficient delivery of cargo into a host cell (Ritter et al, 1995). When clathrin-mediated endocytosis is inhibited, it becomes obligatory for OMVs to utilise the more efficient caveolin pathway, which would explain the increased total change in blue fluorescence observed for EHEC OMVs when cells are treated with papain or chlorpromazine. It may be that OMVs from K12 lacking O-antigen are less well adapted to using this route, and the presence of the O polysaccharide on OMVs from K12 enhances their ability to use the alternative lipid raft-mediated route of entry (Figure 3.8).

EAEC OMVs displayed an intermediate phenotype between EHEC and K12 OMVs; Loss of receptor mediated endocytosis caused an increased efficiency of uptake for O polysaccharide positive OMVs, and a reduction for O polysaccharide negative OMVs, but did not affect the maximum rate, similar to EHEC (Figure 3.6, 3.7). Treatment with raft inhibitors had a less pronounced inhibition of uptake compared with EHEC, and did not affect the maximum rate, unlike for both EHEC and K12 (Figure 3.8, 3.9). The difference in preference of route of uptake may be due to the variation in the size of OMVs between the strains; the average diameter of EAEC OMVs was considerably larger (102 nm) than EHEC or K12 OMVs (79 nm and 91 nm respectively), and since the diameter of caveolae is typically 50-80 nm, this may preclude larger vesicles from entry

via this pathway (Wang et al, 2009). Caveolae are able to accommodate larger (100-200 nm) particles, but their uptake is 5-10 times less efficient than for particles of <40 nm, and they can internalise several smaller particles simultaneously (Wang et al, 2009). Clathrin coated pits can readily endocytose particles of up to 200 nm (Vercauteren et al, 2010). Therefore the size distribution of OMVs may bias them towards entry through certain uptake pathways.

Chemical inhibition of endocytic pathways are commonly used, but these inhibitors are also known to have a wide range of off target effects and can also cause cytotoxicity (Preta et al, 2015). No positive controls were used for these inhibitors in this work, but the concentrations used were previously verified in HeLa cells (Papatheodorou et al, 2010; Contreras et al, 2010; Girard et al, 2011; Ilnytska et al, 2013). Mycoplasma contamination was also only checked twice throughout the project; this may also have had a wide range of effects on cellular behaviour.

Previous work with *H. pylori* OMVs had indicated an inhibitory role of extracellular LPS on OMV uptake (Parker et al, 2010). In these experiments, it was found that addition of purified LPS, from either rough or smooth strains, resulted in significant inhibition of uptake of EHEC OMVs, but had no effect on uptake of OMVs from K12 or EAEC. LPS is recognised by the TLR4 receptor on the host cell membrane, which can then drive internalisation, but activation of this receptor requires binding of LPS to the CD14 protein which is associated with lipid raft microdomains and caveolae (Shuto et al, 2005;

Plociennikowska et al, 2015). EHEC OMVs were found to be strongly dependent on lipid rafts for their entry, and were most affected by inhibition of this pathway with cholesterol sequestering agents (Figure 5.7). Inhibition of TLR4 using C34, a small molecule which competitively binds the TLR4-MD2 complex, had no significant effect on uptake of OMVs (Figure 5.11) (Neal et al, 2013). This indicates that the reduction in uptake observed for EHEC OMVs in the presence of supplementary LPS is not due to competition for TLR4 binding. In addition, it is also shown that removal of surface receptors resulted in an increased rate of uptake for EHEC OMVs, suggesting they are not dependent on receptor mediated endocytosis (Figure 5.6). It is concluded that EHEC OMVs are competing with purified LPS for cholesterol rich domains of the cell membrane, rather than competing for the TLR4 receptor. Although HeLa cells are known to express TLR4, and the C34 inhibitor is verified in HeLa cells, no positive control was used to confirm the activity of the inhibitor (Neal et al, 2013; Jiang et al, 2017; Dowaidar et al, 2017).

Other studies examining the routes of entry for OMVs and subsequent delivery on their cargo have often produced contradictory findings; this may be a result of discrepancies between the methods used in these studies. The methods developed earlier in this work have provided a platform for study of OMV entry kinetics that overcomes the issues with many of the methods used previously, by allowing real time, sensitive analysis of technical replicates. A single well in a 96 well plate can contain up to 20,000 cells, and the use of the plate reader allows for averaging of the fluorescence emissions from these

cells every 90 seconds. Generating this amount of data using microscopy would be unfeasible.

The previous chapter identified a role for O polysaccharide in increasing the efficiency of OMV uptake. The methods used here have demonstrated that O polysaccharide enhances OMV uptake and cargo delivery due to creating a bias towards the non-receptor mediated routes of cellular entry. In all cases, loss of receptor mediated endocytosis in the presence of O polysaccharide OMVs caused an increase in their uptake, suggesting a shift towards lipid raft or caveolin mediated endocytosis that enhanced the efficiency of OMV uptake and delivery of their contents. This is independent of TLR4 binding. This suggests that the presence of O polysaccharide on the OMVs of pathogenic strains is a useful adaptation that increases their ability to deliver virulence factors and enhance pathogenicity.

Understanding these uptake processes is vital in order to be able to inhibit the delivery of their toxic cargo during infection, but their affinity for host cells can also be used to our advantage. Artificial liposomes are already being explored as a drug delivery tool; OMVs are essentially naturally produced liposomes, and they too could be repurposed for delivery of beneficial cargo, instead of the toxic contents they are associated with in an infection setting (Gujrati et al, 2014).

6. Discussion

The data presented in this work has developed a much-needed novel method for monitoring the entry of bacterial outer membrane vesicles into host cells, and this revealed that the O polysaccharide component of OMVs created a bias towards non receptor mediated endocytosis, resulting in enhanced efficiency of uptake. The entry of OMV cargo directly into the host cell is associated with pathogenesis, via the delivery of numerous bacterial virulence factors (Kesty et al, 2004; Yoon et al, 2016). Many of these virulence factors are preferentially exported by OMVs, rather than via other bacterial secretion systems (Kato et al, 2002; Wai et al, 2003).

In the method described here, the preferential targeting of the pore forming cytolysin, ClyA, into OMVs is exploited as a means to export the reporter component, the β-lactamase enzyme (Kim et al, 2008). Entry of β-lactamase into host cells pre-loaded with CCF2-AM dye allows it to cleave the cephalosporin ring linking the fluorescein and coumarin molecules, resulting in their spatial separation, a disruption to FRET and a detectable change in emission from 530 nm to 460 nm. The use of the CCF2-AM FRET system has been described previously to monitor secretion of bacterial effector molecules, and in viral infections and drug screening, but this is the first time it has been adapted to study OMVs (Cavrois et al, 2002; Mills et al, 2008; Landowski et al, 2014).

Use of this assay has allowed continuous, real-time monitoring of OMV entry into host cells, in a sensitive manner. The method also generates robust and repeatable data,

through the use of a multi-well plate format, which unlike other methods of study has allowed multiple conditions and repeats to be tested simultaneously. Whilst this assay has been tested in various strains of *E. coli*, further work will be necessary to determine whether ClyA can also be successfully targeted into other distantly related strains, which do not naturally express ClyA (Kim et al, 2008).

The small size of OMVs has made study of their entry problematic; their size range of 20-200 nm means that conventional methods of light microscopy are unable to detect the whole population of vesicles. The resolution limit of confocal microscopy is approximately 180 nm (Heintzmann and Ficz, 2013). This could result in conclusions being made which do not reflect the full repertoire of interactions between OMVs and the host cell; this work showed that OMVs can use multiple entry pathways. The size of OMVs is likely to influence their mechanism of uptake. Macropinocytosis can facilitate ingestion of particles up to 1 μm, whilst clathrin mediated endocytosis can internalise vesicles with a diameter of up to 120 nm. Caveolae and lipid rafts generally allow entry of molecules smaller than 100 nm in diameter (Parton et al, 2007; Amano et al, 2010). This method allowed monitoring of a wider population of vesicles, as detection required entry of β-lactamase rather than visualisation of vesicles, and used measurement of the intracellular change in fluorescence in the host cell instead. Future work could isolate OMVs on the basis of their size and determine if this has an effect on their preferred route of uptake.

This method relies on delivery of β-lactamase cargo from the vesicles into the host cells, rather than the labelling of the OMVs. It is not clear how many molecules of β-lactamase were exported into a single vesicle, and whether this value would change if the size of the OMV was altered. Immunogold labelling of the OMVs would be necessary to determine the number of β-lactamase molecules present per vesicle. It is also unknown how many β-lactamase molecules need to enter the host cell in order to cause sufficient dye cleavage for a detectable change in emission. It is likely that the efficiency or rate of OMV entry varies amongst the host cells. The use of the plate reader allows for averaging of the fluorescence in each well, where up to 20,000 cells are present, and so this generates a large amount of data to determine the average level of OMV entry. It would be unfeasible to obtain the equivalent amount of data from microscopy alone.

In addition, methods which use labelling of specific OMV epitopes may neglect some subpopulations of OMVs, and the use of lipophilic dyes could affect the behaviour of the OMVs themselves, by interference with the lipid membrane (Lulevich et al, 2009). OMVs were shown to enter the cells rapidly, with disruption to FRET occurring within minutes. This suggested that there is no significant delay between the entry of β-lactamase, cleavage of the substrate and its detection by the plate reader, but also that other means of determining the kinetics of OMV uptake are impractical; fixing cells at these early time points would be difficult, whilst live imaging may result in only the later-entering OMV populations being detected or quantified, meaning that the overall kinetic data may be skewed.

With modifications to allow bacterial expression of the esterases required to retain the FRET substrate, this system could also be adapted to monitor the delivery of OMVs between bacterial cells (Nord et al, 2005). Inter-bacterial delivery of OMVs is a process which is observed in mixed bacterial communities and can enable bacteria to compete within a niche by release of toxins and enzymes, and accelerate the spread of antibiotic resistance through the transfer of resistance genes (Berleman and Auer, 2013; Chatterjee et al, 2017). Expanding this method to characterise the interactions between OMVs and bacterial cells could help to elucidate the roles of OMVs in bacterial communities.

It was attempted to use this method *in vivo* in a zebrafish model of infection, but there was no evidence of dye uptake in the embryo. Despite this, this assay still represents a useful *in vitro* method of screening for factors which affect OMV uptake, and could also be used to assist in developing OMV-based drug delivery vehicles. Fusion to ClyA represents a verified method of targeting other molecules into OMVs, such as anti-cancer drugs or siRNA, and therefore this assay could be used to observe their delivery into tumour cells *in vitro*, prior to use *in vivo* (Kim et al, 2008; Gujrati et al, 2014). Transwell experiments also demonstrated that this method could be used without purification of vesicles, which would overcome many of the discrepancies in data caused by variations in OMV isolation techniques.

This work showed that OMVs have a high affinity for mammalian host cells, as the MOI used was equivalent to a bacterial MOI of 25, and the vesicles were able to enter and deliver cargo to HeLa cells. Efficient OMV delivery was also observed in RKO cells, a human rectal carcinoma cell line, which represents a more biologically relevant setting as *E. coli* is commonly found in the intestinal tract (Savageau, 1983; Ahmed et al, 2013). The data presented here indicated that the presence of O polysaccharide, the variable component of the Gram negative outer membrane, is implicated in enhancing the ability of OMVs to enter the host, which may be due to the presence of O polysaccharide creating a bias towards different pathways of endocytosis. Presence of O polysaccharide, in either the pathogenic (EHEC, EAEC) or non pathogenic (K12) strains used, resulted in an increased uptake, in both HeLa and RKO cells. As cell surface proteins and the abundance of lipid rafts can vary between different cell lines, it is compelling that the effect of O polysaccharide was maintained across these two cell lines (Duncan et al, 2002).

OMVs from EHEC and EAEC, both human pathogenic species of *E. coli*, entered more efficiently than those from the lab strain, K12. It may be that OMVs from pathogenic species are better adapted to entering and delivering their cargo into host cells, and whilst most research has focused on the behaviour of OMVs from pathogenic strains of bacteria, previous studies have identified entry of OMVs from non pathogenic strains into host cells (Canas et al, 2016). Restoration of the native O16 O polysaccharide in K12 was demonstrated to enable it to become a pathogen of the nematode *Caenorhabditis elegans*

(Browning et al, 2013). In this work, the OMVs from the K12 parent strain were able to enter host cells, but when O polysaccharide expression in K12 was restored, the rate and efficiency of entry was significantly increased.

Many strains readily lose their O polysaccharide when cultured in a laboratory environment, indicating its presence is not necessary or advantageous in this setting, in contrast to the natural environment, where the vast majority of Gram negative species express O polysaccharide (Stevenson et al, 1994). Alongside its other described roles in bacterial fitness in the wild, this work suggests that O polysaccharide is also involved in enhancing the ability of OMVs to interact with the host. It seems likely that the role of OMVs in non pathogenic species is underestimated, particularly in the case of commensals or probiotic species (Muraca et al, 2015). OMVs from the probiotic strain *E. coli* Nissle 1917 have been shown to reduce inflammation and symptoms of inflammatory bowel disease (Canas et al, 2016; Fabrega et al, 2017). Bacteria such as *Salmonella* species that can replicate and colonise in the necrotic tumour environment have been explored as a potential anti-cancer treatment, but they themselves cause side effects due to their endotoxicity, and use of modified LPS has been identified as a means of reducing their immunogenicity (Stritzker et al, 2010). While much work has focused on reducing endotoxicity, this work suggests that in order to maximise the affinity of OMVs for host cells, O polysaccharide is required. Understanding the requirements for OMV entry may help to enhance their ability to reduce tumour growth, as they can be

targeted to deliver specific molecules into tumour cells (Stritzker et al, 2010; Gujrati et al, 2014).

The enhanced uptake observed in the presence of O polysaccharide was maintained for the three different O polysaccharide serotypes tested (O157, O42 and O16). The presence of O polysaccharide has been previously reported to enhance adhesion and virulence of various species of Gram negatives, but its role had not been explored in the context of OMVs before. As it comprises a significant structural component at the outer surface of OMVs, it seems logical that its presence could influence the interactions with host cells. The specific structure of these O polysaccharides was not analysed in this work, and it would be useful to identify whether there are specific components of these O polysaccharides that influence the interactions of OMVs with the host cell membrane. It would also be interesting to express different O polysaccharides in these species, such as expressing O157 in K12 to rule out the possibility of the OMV cargo affecting their uptake.

The fate of the O polysaccharide within the host cells was not studied in this work. Delivery of LPS from EHEC OMVs into the cytosol has been reported, which led to activation of the inflammatory caspase-11, resulting in cell death (Vanaja et al, 2016). Their study showed that LPS from the OMVs is not exclusively detected via its interactions with TLR4, and instead was detected in the cytosol via inflammatory caspases. Similarly, our work demonstrated that entry of OMVs was TLR4 independent,

with no effect on uptake observed with the use of a TLR4 inhibitor. The behaviour and effect of OMVs on the host is not just a consequence of the high concentration of LPS; inoculation of mice with equivalent or higher amounts of LPS did not elicit the same level of lethality as OMVs (Park et al, 2010). It may be that the presence of LPS in the context of OMVs does not interact with the host in the same way as purified or cell-associated LPS. The use of OMVs in vaccine preparations and as adjuvants requires a balance between immunogenicity and toxicity, and therefore understanding how the specific components of LPS, including O polysaccharide, influence these responses is vital in order to further their potential medical applications (Collins, 2011; Vanaja et al, 2016).

The assay developed in this work revealed that OMVs can utilise multiple modes of entry into the host cell, but also that their LPS composition alters their affinity for these pathways. OMVs without O polysaccharide were more reliant on receptor mediated endocytosis, whilst those with O polysaccharide were unaffected by inhibition of receptor mediated endocytosis, and instead were dependent on lipid rafts. The protrusion of O polysaccharide from LPS can obstruct the exposure of surface antigens, such as PgtE in *S. enterica*, and Pla in *Y. pestis*, affecting their ability to adhere and invade host cells (Kukkonen et al, 2004). It may be that the presence of O polysaccharide on OMVs has a similar effect, and obscures potential host cell binding ligands, and so alternative entry routes are used instead. Entry via non receptor mediated endocytosis can be advantageous for pathogens as, although generally a slower route of entry, some evidence suggests that

the cargo entering the cell via this method is more likely to be released into the cytoplasm without degradation (Ritter et al, 1995; Bielaszewska et al, 2013; Lim et al, 2014). This may explain why the rate and efficiency of uptake for O polysaccharide containing OMVs was increased in the absence of receptor mediated endocytosis, as it forced a larger proportion of vesicles to utilise alternative routes, resulting in increased cytosolic exposure of β-lactamase to the FRET substrate.

Chemical inhibition of endocytic uptake routes was used in this study. Although these methods are well-established, it is possible that use of inhibitors against dynamin, clathrin and cholesterol have other off-target effects, and it may be useful in the future to use siRNA to silence the relevant genes in the host cell instead (Awasthi-Kalia et al, 2001; Vercauteren et al, 2010). However, if there were side effects of the inhibitor treatments, it would be expected to affect all the cells equally, which does not explain why differences in uptake were observed between cells infected with OMVs with or without O polysaccharide.

Presence of O polysaccharide may not be the only factor affecting OMV entry, but it had a significant effect for all the serotypes studied, and considering the heterogeneity of OMV production, cargo and behaviour, it is compelling that this single factor had such a profound effect. It remains to be seen if this is conserved for species other than *E. coli*, or if the effect of O polysaccharide on OMVs is limited to this species or others occupying a similar niche.

6.1 Future work

This assay represents a platform for future studies into OMVs and their interactions with host cells. This work did not explore the fate of the OMVs upon entry into host cells, or whether this differed for those containing O polysaccharide. Imaging flow cytometry is a relatively new technique, combining the quantitative sorting power of flow cytometry with microscopy, to allow for counting and analysis of colocalisation simultaneously, and has begun to be utilised for study of extracellular vesicles (Lannigan and Erdbruegger, 2017). It has yet to be used to study bacterial vesicles, but could be compatible with the reporter assay used here. CCF2 loaded cells used in the infection experiments could be isolated afterwards, and stained for O antigen in conjunction with particular organelles, such as lysosomes or mitochondria, and quantitatively determine the fate of OMV components within infected cells.

A role for O polysaccharide was determined in this work, but three different structures were used. As the O polysaccharides are highly variable, it would be useful to explore whether there are particular sugar components or linkages that are more advantageous for cell entry, and to determine the effect of the number of repeat units. Engineering K12 to express O157 would also elucidate whether the effect on cell entry is influenced by the specific polysaccharide, or whether it simply being present is the main factor for enhancing OMV uptake.

One theory of OMV biogenesis suggests that the membrane composition influences OMV production and cargo (Li et al, 1996); analysis of the OMV cargo would also allow insight into whether the O polysaccharide affects the molecules packaged into the vesicles.

ClyA is used in this system in order to target the β-lactamase enzyme into the OMVs. ClyA is conserved amongst many *E. coli* and *Shigella* strains, with homologs in *Salmonella* and *Klebsiella*, but it is unclear whether its use in other distantly related strains would still allow for targeting into vesicles (Fahie et al, 2013). The use of this assay would be significantly expanded if it was compatible with more Gram negative species, to gain further appreciation of the interactions between OMVs and the host, but also to determine how universal the effect of the O polysaccharide is.

One of the most promising results in this work was the transwell assay, which showed that OMVs could be delivered and detected entering host cells without prior isolation from the bacterial culture. This removes a significant challenge of OMV research, and a source of the potential discrepancies observed between studies, and also represents a more realistic infection model. Future work could determine the ability of OMVs to enter cells from cultures grown in different media, or from different growth phases. Since OMVs are considered to be important contributors to pathogenicity, it could also be used to examine the effect of antibiotics or stress on OMV delivery, and determine whether specific conditions experienced by bacteria during infection of a host can influence the behaviour of their OMVs (Kesty et al, 2004).

Although this work focused on the delivery of OMVs from bacteria to the host, OMVs are also delivered between bacteria, and play numerous roles in communities, such as removing competing species, or delivering antibiotic resistance enzymes and genes (Li et al, 1998; Chatterjee et al, 2017). Bacteria lack the esterases required for CCF2 dye retention in the cytoplasm, but engineering strains to express these enzymes may allow use of this system, and monitoring of colour change to determine OMV entry (Nord et al, 2005).

Type VI secretion is used by many Gram negatives to target bacteria occupying the same niche (Ho et al, 2017). Preliminary experiments alongside this work showed that EHEC mutants lacking a Type III secretion system produced more OMVs, and that T3SS effector molecules were detected in the isolated OMV fractions from these mutants, suggesting that OMVs can act as a compensatory secretion system. Future work to develop this assay to detect delivery of reporter OMVs into bacterial cells could subsequently determine the role of OMVs in enabling competition in mixed communities, and whether it can also compensate for loss of Type VI secretion.

6.2 Summary

This work has developed a novel method for studying the entry kinetics of OMVs, and the endocytic mechanisms which facilitate these processes. The role of OMVs in health and disease has been under explored, often due to methodological limitations or

inconsistencies in the data. This method overcomes these limitations and provides a platform for further study of OMVs and their interactions with host cells, but it could also be expanded, with modifications, to studying the role of OMVs within bacterial communities, where they are known to contribute to biofilm formation, quorum sensing and DNA transfer (Haurat et al, 2015).

This approach has identified a role of O polysaccharide in influencing the route of uptake, and subsequently the efficiency of cargo delivery from OMVs into the host cell. During infection, delivery of toxic cargo from OMVs is a significant contributor to pathogenesis (Kesty et al, 2004; Park et al, 2010). Further understanding of the mechanisms employed by OMVs in pathogenesis could allow development of inhibitors or vaccines which target this process. It could also enable expansion of the uses of OMVs in medical applications through the development of designer delivery vesicles which exploit their high affinity for host cells.

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