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Extracellular Vesicles from Cod (*Gadus morhua* L.) Mucus contain Innate Immune Factors and Deiminated Protein Cargo

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Abstract

Extracellular vesicles are released from cells and participate in cell communication via transfer of protein and genetic cargo derived from the parent cells. EVs play roles in normal physiology and immunity and are also linked to various pathological processes. Peptidylarginine deiminases (PADs) are phylogenetically conserved enzymes with physiological and pathophysiological roles. PADs cause post-translational protein deimination, resulting in structural and, in some cases, functional changes in target proteins and are also linked to EV biogenesis. This study describes for the first time EVs isolated from cod mucosa. Mucosal EVs were characterised by electron microscopy, nanoparticle tracking analysis and EV-specific surface markers. Cod mucosal EVs were found to carry PAD, complement component C3 and C-reactive proteins. C3 was found to be deiminated in both whole mucus and mucosal EVs, with some differences, and further 6 deiminated immune and cytoskeletal proteins were identified in EVs by LC-MS/MS analysis. As mucosal surfaces of teleost fish reflect human mucosal surfaces, these findings may provide useful insights into roles of EVs in mucosal immunity throughout phylogeny.

Highlights

- Extracellular vesicles (EVs) are described for the first time in cod mucosa
- EVs from cod mucosa contain complement component C3, CRP-I and CRP-II
- Deiminated forms of complement component C3 are exported in cod mucosal EVs
- Deiminated protein cargo of cod mucosal EVs includes cytoskeletal and immune proteins

Key words**:** extracellular vesicles (EVs); mucosal immunity; peptidylarginine deiminase (PAD); protein deimination; complement C3; CRP; cod (*Gadus morhua* L.).

1. Introduction

In teleost fish, the first critical barrier against infection consists of the mucosa-related epithelial tissues which contain amongst other complement proteins (Lange et al. 2004a; Lovoll et al., 2007), lectins, (Jørndrup and Buchmann 2005; Rajan et al., 2011), pentraxins (Tsutsui et al., 2009; Magnadottir et al., 2018a), lysozyme (Fernandes et al., 2004; Rajan et al., 2011) and IgT/IgZ (Zhang et al., 2010; Zhang et al., 2017). Recently, novel roles for peptdylarginine deiminases (PADs) were described in mucosal immunity of Atlantic cod (*Gadus morhua* L.), identifying a range of deiminated cytoskeletal, nuclear, metabolic and immune proteins in skin mucosa of adult cod (Magnadottir et al., 2018a), as well as identifying for the first time deiminated forms of C-reactive protein (CRP, Magnadottir et al., 2018b) in cod mucosa.

Peptidylarginine deiminases (PADs) are preserved throughout phylogeny from bacteria to mammals (Vossenaar et al., 2003) and have various physiological roles in embryonic development, cell differentiation, cell death and gene regulation (Wang and Wang 2013; Witalison et al., 2015). PADs cause post-translational conversion of protein arginine to citrulline in target proteins in a Ca^{2+} -dependent manner, sometimes resulting in target protein's structural and functional changes (Vossenar et al., 2003, György et al., 2006). PADs and protein deimination were recently described in cod and halibut ontogeny and immunity (Magnadottir et al., 2018a; Magnadottir et al., 2018b; Magnadottir et al., 2019a), shown to be present in multiple tissues during larval development and to form part of innate immune defences in cod, including mucosal tissues (Magnadottir et al., 2018a; Magnadottir et al., 2018b). By post-translational deimination, PADs cause for example neo-epitope generation, related to various autoimmune and neurodegenerative diseases (Witalison et al., 2015; Lange et al., 2017), but may also be an important factor in tissue remodelling through protein moonlighting, which allows proteins to exhibit more than one physiologically relevant biochemical or biophysical function within one polypeptide chain (Henderson and Martin, 2014). Importantly, PADs have been shown to play key roles in the regulation of extracellular vesicle (EV) release (Kholia et al., 2015, Kosgodage et al., 2017; Gavinho et al., 2019), to affect composition of EV cargo (Kosgodage et al., 2018) and to regulate EV-mediated host-pathogen interactions in intestinal tissue (Gavinho et al., 2019).

EVs are small (30-1000 nm) lipid bilayer structures released from parent cells and participate in cell communication via transfer of cargo proteins, enzymes and genetic material (Inal et al., 2013; Colombo et al., 2014; Kosgodage et al., 2018; Turchinovich et al., 2019; Vagner et al., 2019). EVs play important physiological and pathophysiological roles including in immunity and host-pathogen interactions (Inal et al., 2013; Gavinho et al., 2018; Gavinho et al., 2019). As EVs are related to a number of pathophysiological processes they are also regarded as useful biomarkers (Inal et al., 2013; Hessvik and Llorente, 2018; Kosgodage et al., 2018; Ramirez et al., 2018; Wu et al., 2019). While EVs are widely studied in human pathologies, studies on EVs in teleost fish are scarce (Faught et al., 2017; Lagos et al., 2017; Iliev et al., 2018). Diverse roles for EVs in mucosal tissues are gaining increased attention and their relevance in various mucosal-related diseases and function of mucosal surfaces in being realised. Important roles for EVs have been implicated in oral mucosa and wound healing (Sjöqvist et al., 2019), intestinal inflammation and repair (Bui et al., 2018), host-pathogen interactions in intestinal infections (Ma'ayeh et al., 2017), including via PAD-mediated pathways (Gavinho et al., 2019), in intestinal mucosal immunity (Xu et al., 2016), airway tissue and allergies (Lässer et al., 2016; Nazimek et al., 2016; Mueller et al., 2018).

Comparative studies on mucosal immunity in teleosts are an important research topic as these share many characteristics with type I mucosal surfaces of mammals and are therefore also translatable to mucosal surfaces of the respiratory tract, intestine and uterus (Zhang et al., 2010; Gomez et al., 2013; Xu et al., 2013; Zhang et al., 2017). Understanding of mucosal EVs in immune defences in teleosts may shed novel light on roles for EVs in mucosal tissues for innate immune defences; wound healing and host-pathogen interactions.

This study characterises for the first time EVs in cod mucus and describes EV-mediated export of PAD, complement component C3, CRP and deiminated protein cargo in mucosal EVs.

2. Materials and Methods

2.1. Fish and sampling

Mucus was isolated from adult experimentally farmed cod (*Gadus morhua* L), kept at the Marine Institute's Experimental Fishfarm Stadur, Grindavik, Iceland. Mucus was collected from the dorsal side of the fish's body, gently using a glass slide to avoid contamination with epithelium cells or blood. Mucus from 10 individual fish was pooled, immediately frozen on dry ice and stored at -80 °C until used.

2.2 Extracellular vesicle isolation

EVs were isolated from cod mucus using step-wise centrifugation as follows: First, the mucus pool was diluted 1:5 (200 µl mucus plus 800 µl Dulbecco's phosphate buffered saline (DPBS) per isolation) and centrifuged at 4,000 *g* for 30 min at 4 °C to remove cell debris and aggregates. Thereafter the supernatant was ultracentrifuged at 100,000 *g* for 1 h at 4 °C. The EV pellets were resuspended and washed in DPBS (sterile filtered in 0.22 µM filters) and centrifuged again at 100,000 *g* for 1 h at 4 °C. The resulting EV pellet was then either used immediately or stored at -80 °C for further analysis.

2.3 Nanoparticle tracking analysis (NTA) and characterisation of EVs

For NTA analysis, an EV pellet, isolated as described above, was solubilised in 100 µl DPBS and then diluted 1/50 before quantification by NTA analysis, to assess particle size based on Brownian motion, using the Nanosight NS300 (Malvern U.K.). Samples were applied to the Nanosight using a syringe pump to ensure even flow of the sample, with numbers of particles in the window being 40-60 and individual videos were recorded for 60 sec to create a size distribution histogram. EVs were further characterised using Western blotting and the EV-specific markers CD63 and Flot-1, which are phylogenetically conserved in bony fish (Iliev et al., 2018). Mucosal EVs were also morphologically analysed by transmission electron microscopy (TEM). In brief, EVs were fixed with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.0) for 1 h at 4 °C, re-suspended in 100 mM sodium cacodylate buffer (pH 7.0), placed on to a grid with a glow discharged carbon support film, stained with 2 % aqueous Uranyl Acetate (Sigma-Aldrich) and thereafter viewed in TEM.

2.4 Immunoprecipitation and protein identification

For extraction of protein, EV pellets derived from cod mucosa were resuspended in RIPA+ buffer (Radioimmunoprecipitation assay buffer containing 10% protease inhibitor complex; Sigma-Aldrich, U.S.A), pipetting gently at regular intervals for 2 h on ice. Protein was isolated by centrifugation at 16,000 *g* for 20 min and collecting the supernatant. For isolation of total deiminated proteins from the EV protein preparation, immunoprecipitation was performed using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Merck, U.K.) according to the manufacturer's instructions in conjunction with the monoclonal F95 pan-deimination antibody (MABN328 Merck, U.K.), which is raised against a deca-citrullinated peptide and specifically detects protein citrulline (Nicholas and Whitaker, 2002). Incubation was performed overnight at 4 °C on a rotating platform. F95 bound proteins were thereafter eluted under reducing conditions according to the manufacturer's instructions (Merck) and the F95 enriched eluate was analysed by liquid chromatography–mass spectrometry (LC-MS/MS; performed by Cambridge Centre for Proteomics, U.K.) with peak list files submitted to in-house Mascot (Cambridge Centre for Proteomics), using the following database: Gadus_morhua_20190405 (1283 sequences; 308668 residues), and with setting set at significance threshold $p<0.05$ and cut-off at Ions score 20.

2.5 Western blotting

Mucosal EVs were analysed by Western blotting for detection of the EV specific markers CD63 (ab68418, Abcam, U.K.) and Flotillin-1 (ab41927, Abcam), which have been shown to be conserved throughout phylogeny in bony fish (Iliev et al., 2018). Western blotting was also carried out for total deiminated proteins (F95, MABN328 Merck, U.K.), PAD2 (ab50257, Abcam), deiminated histone H3 (citH3; ab5103, Abcam), complement component C3 (Lange et al., 2004b), CRP-I and CRP-II (Gisladottir et al., 2009; Magnadottir et al., 2018b). The samples were reconstituted in 2 x Laaemmli sample buffer (BioRad, U.K.) containing 5 % beta-mercaptoethanol (Sigma, U.K.), boiled for 5 min at 100 °C and separated on 4-20 % TGX gels (BioRad, U.K.). Approximately 5 μg of protein was loaded per lane and even load was assessed using Ponceau S staining (Sigma, U.K.). Blocking of membranes was in 5 % bovine serum albumin (BSA, Sigma) in Tris buffered saline with 0.1% Tween20 (TBS-T) for 1 h, followed by incubation at 4 °C overnight with the primary antibodies in TBS-T (F95 1/1000; citH3 1/2000; CRP-I and CRP-II 1/1000; C3 1/1000; CD63 1/1000; Flot-1 1/2000). Membranes were then washed 3 times in TBS-T, followed by incubation at room temperature for 1 h with the HRP-conjugated secondary antibodies (anti-mouse IgM, anti-mouse IgG or anti-rabbit IgG; BioRad, U.K.; 1/4000 in TBS-T), followed by 6 washes in TBS-T before visualisation with enhanced chemiluminescence (ECL; Amersham, U.K.). Membranes were imaged using the UVP BioDoc-IT^{TM} System (U.K.).

3. Results

3.1 Characterisation of EVs from cod mucus

EVs from cod mucus were characterised by size exclusion using NTA, by morphological analysis using TEM and by Western blotting using the EV-specific markers CD63 and Flot-1 (Fig 1). NTA analysis revealed a poly-dispersed population ranging from 30-500 nm with peaks at 72, 142, 200 and 286 nm, with modal size 141.9 nm(Fig. 1A). The amount of EVs in mucus was approximately 5.8 x 10^9 particles/ml of mucus. The cod mucus EVs were positive for CD63 and Flot-1 (Fig. 1B) and morphological analysis using TEM confirmed a polydispersed EV population (Fig. 1C).

3.2 Innate immune protein cargo in mucosal EVs

EVs from mucosa were assessed for complement component C3 and CRP (CRP-I and CRP-II), which were all verified to be present as protein cargo in the EVs (Fig. 2). C3 was found at higher levels than CRP-I and CRP-II (Fig. 2A-C). In comparison, in total mucus C3 and both CRP forms were clearly detected (Fig. 2D-F). Notably, C3 was detected at quite high levels in the mucosal EVs (Fig. 2A).

3.3 Deiminated protein cargo in mucosal EVs

Mucosal EVs were assessed for total deiminated proteins, revealing a range of proteins from 15-250 kDa reacting with the F95 antibody (Fig. 3A) and a positive reaction with the PAD2 antibody was also detected in the EVs at the expected 70 kDa size (Fig. 3B). In comparison, total mucus also showed positive for F95 (Fig. 3C) and PAD was also strongly detected, similar as previously observed (Magnadottir et al., 2018a). The mucus-derived EVs did not show positive for deiminated histone H3 (not shown), compared to total mucus which showed strong positive citH3 (Fig. 3D). For identification of deiminated proteins, the F95 enriched eluates from the EVs and total mucus were assessed for C3, CRP-I and CRP-II, revealing that C3 is found in deiminated form in mucus EVs (Fig. 4), with a band representative of the C3 β-chain reacting with the F95 enriched eluate (Fig. 4A). Total mucus F95 enriched eluate also showed positive for C3, with both C3 α- and β-chains positive for F95 enrichment, as well as α-chain fragments (Fig. 4B). Neither of the CRP antibodies (anti-CRP-I or anti-CRP-II) reacted with the F95 enriched eluates of the EVs (not shown), but both forms have previously been shown to be deiminated in whole cod mucus (Magnadottir et al., 2018b). To identify further deimination candidates in the EVs, the F95 enriched eluate from the mucosal EVs was analysed by LC-MS/MS (Cambridge Proteomics, U.K.) and the peak files submitted to Mascot, identifying 6 immunogenic and cytoskeletal protein hits (Table 1). Only hits for cod peptides are included.

Table 1. Deiminated proteins identified by F95 enrichment in extracellular vesicles isolated from mucus of cod (*Gadus morhua* L.). Deiminated proteins were isolated by immunoprecipitation using the pan-deimination F95 antibody, the F95 enriched eluate was analysed by LC-MS/MS and peak list files were submitted to mascot. Only peptide sequence hits scoring with *G. morhua* are included. Peptide sequences and m/z values are listed.

Protein name	**m/z**	**Peptide sequence**	**Score ($p<0.05$)ǂ**	**Total score**
Q9PUG4_GADMO *Tubulin beta chain*	514.7648 660.8550 830.4506 1022.4645 1105.1826	*K.TAVCDIPPR.G* *R.IMNTFSVVPSPK.V* *R.ALTVPELTQQVFDAK.N* *K.FWEVISDEHGIDPTGSYNGDSDLQLDR.I* *K.EAESCDCLQGFQLTHSLGGGTGSGMGTLLISK.I*	26 49 53 37 24	188
A8CZC9_GADMO *Elongation factor 1-alpha*	974.5462 513.3090 433.5868 953.1371	*R.LPLQDVYK.I* *K.IGGIGTVPVGR.V* *R.EHALLAFTLGVK.Q* *K.IGYNPAAVPFVPISGWHGDNMLEASSK.M*	22 44 60 39	165
Q2PDJ0_GADMO *Beta-actin (Fragment)*	499.7473 566.7665 796.6581	*R.DLTDYLMK.I* *R.GYSFTTTAER.E* *R.TTGIVMDSGDGVTHTVPIYEGYALPHAILR.L*	36 21 66	123
Q78AY8_GADMO *Fast skeletal muscle alpha-actin*	398.2388 499.7473	*K.IIAPPER.K* *R.DLTDYLMK.I*	46 36	82
G8ENP0_GADMO *Galectin (Fragment)*	696.6854	*R.EEFLVILSDGSEVHFPNR.L*	59	59
A0A067XLH1_GADMO *Profilin*	689.3651	*R.VILDNLYKEDASVNLMTK.D*	42	42

ǂIons score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 16 indicated identity or extensive homology ($p < 0.05$). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits. Cut-off was set at Ions score 20.

Discussion

This is the first study to characterise extracellular vesicles (EVs) in mucus of Atlantic cod (*Gadus morhua* L). EVs isolated from cod mucus were characterised according to the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) guidelines (Théry et al., 2018), using NTA, TEM and Western blotting for EV-specific markers. A poly-dispersed EV population of 30-500 nm was observed by NTA and was positive for the EV-specific markers CD63 and Flot-1, previously described to be conserved throughout phylogeny in bony fish (Iliev et al., 2018). Numbers of EVs in mucus were found to be approximately 5.8×10^9 particles/ml of mucus, which is similar to what has been observed in human nasal mucus (Nocera et al.,2017). The present study reports for the first time deiminated protein cargo in EVs isolated from mucus of a teleost fish species. To our knowledge, for the first time, deiminated complement C3 is reported in mucosa and mucosal EVs. Acute phase proteins CRP-I and CRP-II are also described for the first time in cod mucus EVs. Six further deiminated proteins were identified in cod mucosa EVs using F95 enrichment and LC-MS/MS analysis. These overlapped with 38 proteins previously found to be deiminated in whole cod mucus (Magnadottir et al., 2018a; Figure 4C). Deiminated proteins identified in cod mucosal EVs in the present studies are discussed below:

Complement component C3 plays a central role in all pathways of complement activation (Dodds and Law, 1998; Dodds, 2002) and has in cod been described as a 2 chain glycoprotein with a 115 kDa α-chain and a 74 kDa β-chain (Lange et al., 2004b). The complement system forms part of the first lines of immune defence against invading pathogens and in the clearance of necrotic or apoptotic cells (Dodds and Law, 1998; Sunyer et al., 1998; Fishelson et al., 2001; Carrol and Sim, 2011). C3 is also implicated in regeneration (Del-Rio-Tsonis et al., 1998; Haynes et al., 2013) and related to tissue remodelling during cod ontogeny (Lange et al., 2004a; Lange et al., 2005). C3 was found to form part of the mucosal EV cargo at a high level, while C3 positive bands in EVs versus total mucus varied somewhat. A faint band for the C3 α-chain was seen in total mucus, one strong band for the C3 β-chain and lower molecular mass bands at approximately 42 and 25 kDa, indicative of C3 α-chain fragments. In EVs, a faint band was detected for C3 α-chain, similar to that seen in whole mucus, as well as a prominent band for the C3 β-chain. Particular to the EVs was the presence of several strong bands detected below the β-chain, which could be indicative of additional C3 α-chain fragments, or otherwise indicate some unknown deiminated proteins that bind to C3. In the EVs there were, similar as seen in whole mucus, two lower bands in the 42 and 25 kDa regions, indicative of C3 α-chain fragments. Deiminated forms of complement component C3 were seen in cod mucus-derived EVs, with the C3 β-chain being the only deimination positive band for C3 in the mucosal EVs. In whole mucus, the F95 enriched eluate showed positive for both the C3 α- and β-chains (Fig. 4B), similar to those seen in a previous study of C3 deimination in halibut serum (Magnadottir et al., 2019a). This indicates that C3 exported in EVs may differ in deimination compared to C3 in whole mucus. It remains to be considered though as due to overall C3 detection being lower in EVs than in whole mucus, the α-chain may not be detected in the EV blot for the F95 enriched eluate. In the F95 enriched eluates, for both whole mucus and mucus-derived EVs, the band representative of deimination positive β-chain is detected at slightly lower molecular weight than seen for C3 β-chain in the total protein extracts of EVs and mucus, indicating a putative change in migration due to this post-translational modification. Post-translational deimination of C3 may possibly influence its function including cleavage ability, binding, deposition and generation of the convertase, as well as facilitate its functional diversity (Magnadottir et al., 2019a).

C-reactive protein forms I and II, previously described in cod immunity (Gisladottir et al., 2009; Magnadottir et al., 2013; Gudmundsdottir et al., 2014) and ontogeny (Magnadottir et al., 2018b), and identified to be deiminated both in whole cod serum and mucus (Magnadottir et al., 2018b), were here shown to form part of the EV cargo in cod mucosa and both were detected at similar levels. Both CRP forms were also strongly detected in whole mucus as previously observed (Magnadottir et al., 2018b) and showed more oligomeric forms present in whole mucus (Fig. 2E-F) than in mucus derived EVs (Fig. 2B-C). This indicates some differences in CRP oligomer formation between whole mucus and CRP exported in EVs. CRPs are fluid phase pattern recognition molecules that form an important part of the innate immune defence and are conserved between fish and human (Gisladottir et al., 2009; Chen et al., 2015; Magnadottir et al., 2018b). Pentraxins have been shown in humoral defence in mucosa of a range of teleost fish (Tsutsui et al., 2009; Patel and Brinkmann, 2017; Valdenegro-Vega et al., 2014; Kovacevic et al., 2015; Shi et al., 2018), including cod (Magnadottir et al. 2018b), while to our knowledge pentraxins have not been reported in mucosal EVs before. While both cod CRP forms were detected in mucosal EVs, neither CRP form reacted with the F95 enriched eluates of the mucosal EVs, indicating that deiminated forms are only present in whole mucus (Magnadottir et al. 2018b) but not exported in mucosal EVs. Interestingly, circulating pentameric CRP localised to damaged tissue has recently been shown to bind to cell-derived EVs, enhancing leukocyte recruitment (Braig et al., 2017). A regulatory role of PADs exported in EVs and PAD-mediated EV release on CRP function in mucosal tissues and related pathologies may therefore be of some interest.

Histone H3 is a known deimination candidate and participates in anti-pathogenic functions via formation of neutrophil extracellular traps (NETosis) (Brinkmann et al., 2004; Urban et al., 2006;

Papayannopoulos et al., 2009; Li et al., 2010; Branzk et al., 2014). Fish mucosa is crucial for trapping of pathogens (Ellis, 2001; Gomez et al., 2013) and as recent studies highlighted roles for deiminated histones in cod mucus (Magnadottir et al., 2018a) its presence was assessed here in mucus derived EVs. Deiminated histone H3 was though here only seen in whole mucus (Fig. 3D), as previously observed (Magnadottir et al., 2018a) but not detected in the EVs (not shown). In mammalian mucosa, NETosis has for example been associated with gut mucosal inflammation (Al-Ghoul et al., 2014) and antimicrobial defence in oral mucosa (Mohanty et al., 2015).

Tubulin beta chain and ***beta-actin*** participate in cytoskeletal rearrangement, are linked to mucosal responses in cod following infection (Rajan et al., 2013a) and cod larval development (Sveinsdottir et al., 2008). Deimination of these proteins has also been linked to EV release and biogenesis (Kholia et al., 2015). Neither of these target proteins has been reported in mucosal EVs in deiminated form before.

Elongation factor 1-alpha has roles in cytoskeleton organisation (Khacho et al., 2008), regulation of cell growth and in the immune response, including in degranulation of neutrophils (Talapatra et al., 2002; Hamrita et al., 2011; Vera et al., 2014). It is reported here for the first time as deiminated in mucosal EVs.

***Fast skeletal muscle alpha-actin*,** identified here as deimination candidates in mucosal EVs, were also previously identified as deiminated in total cod mucosa (Magnadottir et al., 2018a). Differences in other post-translational modifications, but not deimination, have been suggested for four isoforms of fast skeletal muscle alpha-actin in early cod larval development (Sveinsdottir et al., 2008). It is here reported for the first time as deiminated in mucosal EVs.

Galectins are known to be strongly expressed in mucosal tissues in fish (Rajan et al., 2013a; Rajan et al., 2013b; Vasta et al., 2004; Zhou et al., 2016; Magnadottir et al., 2019b) and have a wide range of function in innate immunity, including against viral and bacterial infections (Chen et al., 2013; Nita-Lazar et al., 2016). Galectins are involved in many pathological processes, including acute and chronic inflammatory diseases, autoimmunity (Sciacchitano et al., 2018), tumours, as well as wound healing (McLeod et al., 2018). Deiminated galectin is here reported for the first time in mucosal EVs.

Profilin has diverse functions in cytoskeletal actin dynamics and has for example linked to mucosal responses of cod during infection (Rajan et al., 2013a). In sea urchin (*Echinoidea*), it has been shown to be increased under immune challenge and injury (Smith and Davidson, 1994). While previously identified as deiminated in cod mucosa, it has not been reported as deiminated in EV cargo before.

In addition to the range of deiminated target proteins described above in mucosal EVs, we also found that PAD enzyme itself forms part of the EV cargo in cod mucosa. This is the first report on PADs in teleost EVs, but previously, such lateral transfer of PADs has been reported in a range of cancer cells (Hurwitz et al., 2006) and shown to deiminate target proteins in plasma (Chang and Han, 2006). Lateral transfer of PADs via EVs, to modulate immune proteins of the host for immune evasion, has for example been shown in *P. gingivalis* (Bielecka et al., 2014). In Giardiasis, a parasitic infection of the gut, *Giardia intestinalis* host-pathogen interactions and cell adhesion to the host gut cells was recently shown to be PAD-dependent and related to PAD-mediated EV release (Gavinho et al., 2019). To what extent EV-exported PAD may affect deimination of target proteins at sites of EV uptake, modulate immune function of the host and regulate host-pathogen interactions via EV-mediated communication remains to be further investigated.

Research on mucosal EVs is gaining a momentum with increasing interest in roles in the aerodigestive mucosa (Mueller et al., 2018; Lässer et al., 2016) and in relation to a range of mucosal pathologies, including cystic fibrosis (Asef et al., 2018) as well as in host-pathogen interactions. In addition, pathogenic bacterial and commensal-derived outer membrane vesicles (OMVs) and their roles in interaction with the host are another topic of investigation (Nazimek et al., 2016; Nicholas et al. 2017; Patten et al., 2017). Studies on EVs are a new field in fish immunology and this is, to our knowledge, the first report of mucosal EVs and their deiminated protein cargo in teleost fish.

Conclusion

For the first time extracellular vesicles (EVs) are described in teleost fish mucus and deiminated protein cargo, including complement C3, cytoskeletal and metabolic proteins, are identified in EVs of cod mucus. As comparative studies on mucosal immunity in teleosts are translatable to human mucosal surfaces, our findings presented here highlight a novel tool to study mucosal EVs to further understanding of conserved roles for protein deimination and EVs in mucosal immunity throughout phylogeny.

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Fig. 1. Characterisation of extracellular vesicles (EVs) from cod mucus. A. Nanoparticle tracking analysis (NTA) showing a poly-dispersed population of EVs in the size range of 30 to 500 nm, with peaks at 72, 142, 200 and 286 nm. **B.** Cod mucus EVs are positive for the EV-specific markers CD63 and Flot-1. **C.** Morphological analysis of EVs from cod mucus by transmission electron microscopy (TEM); a composite figure is shown, representing a poly-dispersed EV population; scale bar is 200 nm in all figures.

Fig. 2. EV-cargo of cod mucus contains C3, CRP-I and CRP-II. A-C: EVs isolated from cod mucus are positive for innate immune proteins **A.** Complement component C3; **B.** CRP-I; **C.** CRP-II. **D-F:** In addition, cod total mucus was assessed for the same innate immune proteins: **D.** C3 in cod total mucus; **E.** CRP-I in cod total mucus; **F.** CRP-II in total mucus of cod. (C3 α- and β- chain, as well as α-chain fragment (α-f) are indicated. Arrows highlight CRP positive bands, including oligomeric forms).

Fig. 3. PAD and deiminated proteins are exported in cod mucosal EVs. A. PAD was detected in mucus-derived EVs, at the expected size of approximately 70 kDa. **B.** EVs from cod mucus are positive for deiminated proteins as assessed by the F95 pan-deimination specific antibody. **C-E:** In addition, total mucus was assessed for: **C.** PAD; **D.** Deiminated proteins; **E.** Deiminated histone H3, which was not detected in the EVs (not shown).

Fig. 4. Deiminated protein targets in EVs of cod mucus. A. Complement component C3 is exported in deiminated form in mucosal EVs. The F95 enriched protein eluate was tested against complement component C3, verifying a deimination positive C3 β-chain in mucosal EVs. **B.** F95 eluate of total mucus was also assessed for C3, verifying the presence of deiminated C3 α- and β-chain; C3 α-chain fragments (α-f) are indicated. **C.** Deiminated proteins identified by F95 enrichment and LC-MS/MS analysis revealed further 6 deiminated proteins found in mucosal in EVs, all of which have previously been identified in total mucus in deiminated form (as previously reported in Magnadottir et al., 2018a) and all deiminated proteins hitherto recognized in cod mucosa and EVs, including in the current study (including C3 and the two forms of CRP respectively) are represented in the Venn-Diagram. For details on hits identified by LC-MS/MS in mucosal EVs see **Table 1**.

Table 1. Deiminated proteins identified by F95 enrichment in extracellular vesicles isolated from mucus of cod (*Gadus morhua* L.). Deiminated proteins were isolated by immunoprecipitation using the pan-deimination F95 antibody, the F95 enriched eluate was analysed by LC-MS/MS and peak list files were submitted to mascot. Only peptide sequence hits scoring with *G. morhua* are included. Peptide sequences and m/z values are listed.

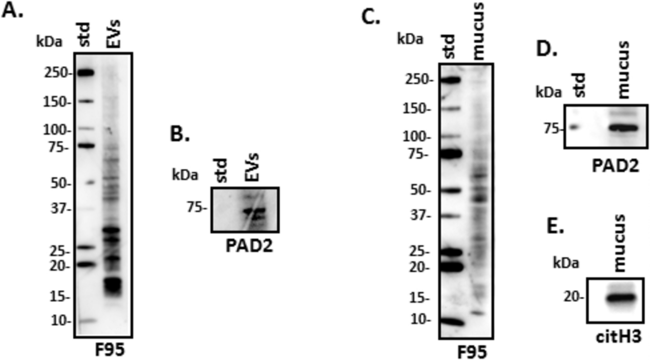
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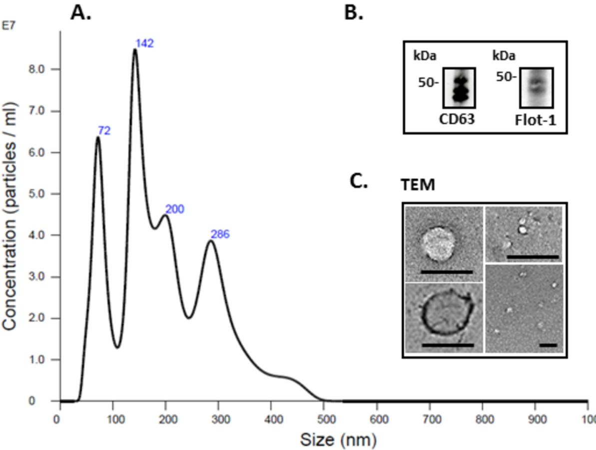
Fig. 1



A.
B.
C.
TEM
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Concentration (particles / ml)
Size (nm)
142
72
200
286
kDa
50-
CD63
kDa
50-
Flot-1
0
1.0
2.0
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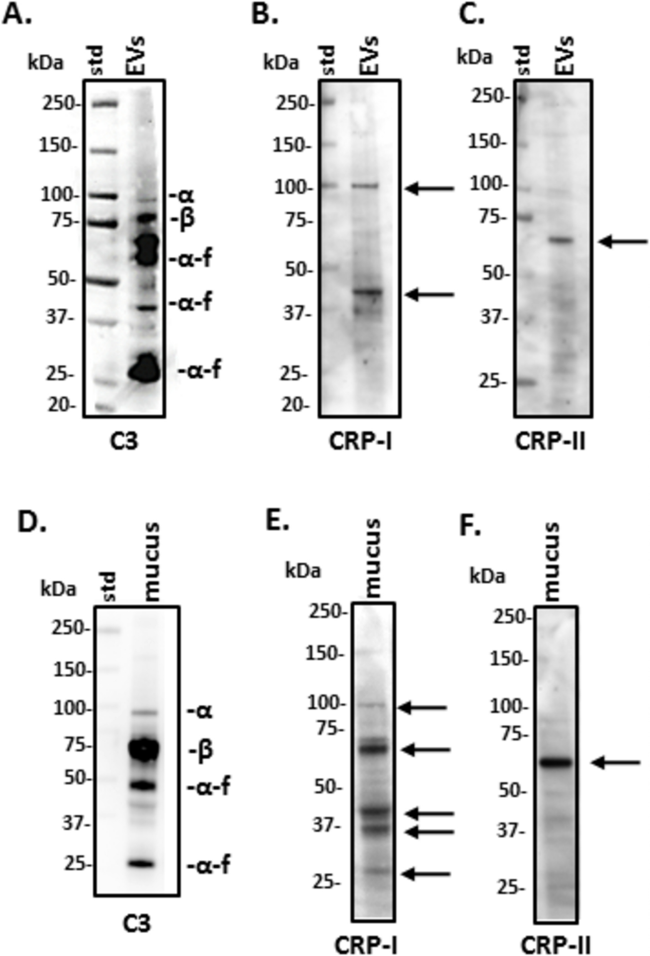

A.
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CRP-I
C.
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CRP-II
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-α
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kDa
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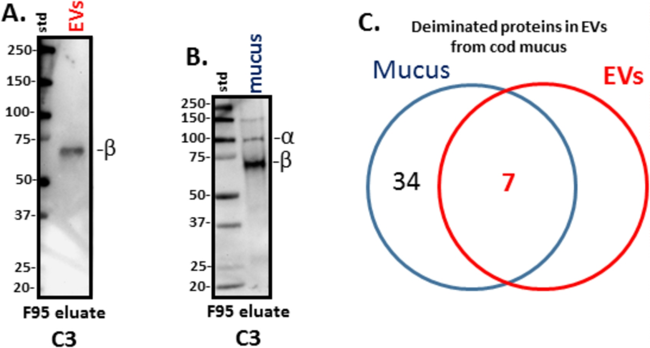
CRP-II


A.
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Deiminated proteins in EVs
from cod mucus
Mucus
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34
7