Chapter

# Extracellular Vesicles: Living Prototypal Communication System

*Paul A. Brown*

## Abstract

Communication is an ever-present part of our world. Such transfer of information occurs on many levels from the spoken natural languages, to artificial languages, to the cellular exchanges that govern the molecular world. Cells interact using various coded and non-coded molecules, which although not natural languages, could be considered types of biological language. These molecules are packaged into extracellular vesicles by cells from all three domains of life. Vesicles may then participate in intracellular trafficking of their cargo molecules. Or cells may secrete vesicles into the extracellular world, from where they are transported to, and taken up by, target recipient cells. Once delivered, extracellular vesicles exert a plethora of physiological and pathological effects, as well as an influence on recipient cell evolution. In executing their functions, both vesicles and their molecular cargo face evolutionary pressures over time and across habitats, forcing them to adapt to meet changing needs. This chapter will present extracellular vesicles as a highly conserved prototypal communication system.

**Keywords:** extracellular vesicles, exosomes, microvesicles, apoptotic bodies, outer membrane vesicles, membrane vesicles, biological network, cellular communication

## 1. Introduction

Communication is ubiquitous in our world and spans the range of human experience from social, to physical, to biological. In all these spheres, systems have been developed, or have evolved, to facilitate the transfer of information. All communication requires the delivery of a shared system of codes and signals between a source and a recipient. The information must be packaged, relayed and received for effective communication to occur.

We package our spoken languages by our choice of words and phrases (diction) from among our vast repertoire, as well as by how we arrange those words (syntax). But other types of information can also be packaged in different ways, like our choice of facial expressions, gestures and body postures. The information is then relayed either verbally or in non-verbal ways, to be received by a recipient who understands and can respond to the information received. If any of these stages is not properly executed, effective communication may not occur.

This chapter will describe an evolutionarily conserved biological method of communication that also packages, transports, and delivers intelligible information,

but between a donor and recipient cell. Recipient cells must also be capable of responding to the information received for effective communication to occur. At the heart of this communication system are microscopic lipid-bilayer-encapsulated structures called extracellular vesicles (ECVs) that are released from, and taken up by, cells from all three domains of life.

## 2. Communication systems

At its most basic level, communication can be thought of as a process of sending and receiving, involving source, conduit and destination [1]. Many different models of communication and communication systems have been proposed. In healthcare, communication may involve various people, their messages, communication channels, as well as regulatory protocols and policies, all of which facilitates several types of communication services using different communication devices [2]. Others describe the concepts of flow and interactivity. Information flows interactively as it is created, released, transferred, received and processed repeatedly, as applicable for example to computer systems [1]. Biological communication involves the reciprocally adaptive relationship between a signal and response; a signaler and a receiver who have each evolved to interact with each other [3].

Implicit in these descriptions is the transfer of meaningful information. To be effective, communication requires that the received message is processed and elicits an appropriate response on the part of the recipient [3]. Such activities are easily identified among higher animals, including humans. However, even among the latter, it is understood that much of this communication is non-verbal [4, 5].

Biological communication obviously falls into this latter category. There is a vast amount of interaction that occurs at the cellular and sub-cellular levels. This chapter will discuss one such communication system; extracellular vesicles. But before these are explored, it is important to come to some understanding of what is being communicated. What do ECVs transport?

### 2.1 The 'alphabets' of life

Our genes are comprised of only four different nucleotides, namely guanine, cytosine, adenine, and thymine (**Figure 1**). As reported by Watson and Crick [6], these are arranged sequentially along two antiparallel strands. Traditionally they have been represented by the letters G, C, A, and T, respectively, giving the impression they are part of some kind of alphabet. Each of the four interacts with a corresponding nucleotide in the adjacent strand, G with C and A with T, forming what is referred to as the double helix that characterizes a deoxyribonucleic acid (DNA) molecule [6].

The base adenine was first isolated from pancreatic tissue in 1885 by Albrecht Kossel. This was followed by his isolation of the other three bases over the next few years [7]. The base pairings were deduced from experiments beginning in the 1940s, involving the separation of individual bases by paper chromatography and their subsequent identification and quantification using ultraviolet spectroscopy [8]. The results demonstrated that the A:T and G:C molar ratios were fairly constant and close to unity [9, 10]. Together, these early experiments laid the foundation for our understanding of genetic material as a coded system; a biological alphabet.

At first glance, these four molecules that comprise the genetic code may not appear particularly impressive. English for example has 26 letters in its alphabet, Spanish has 27 and Greek has 24. However, when one considers the average size of a

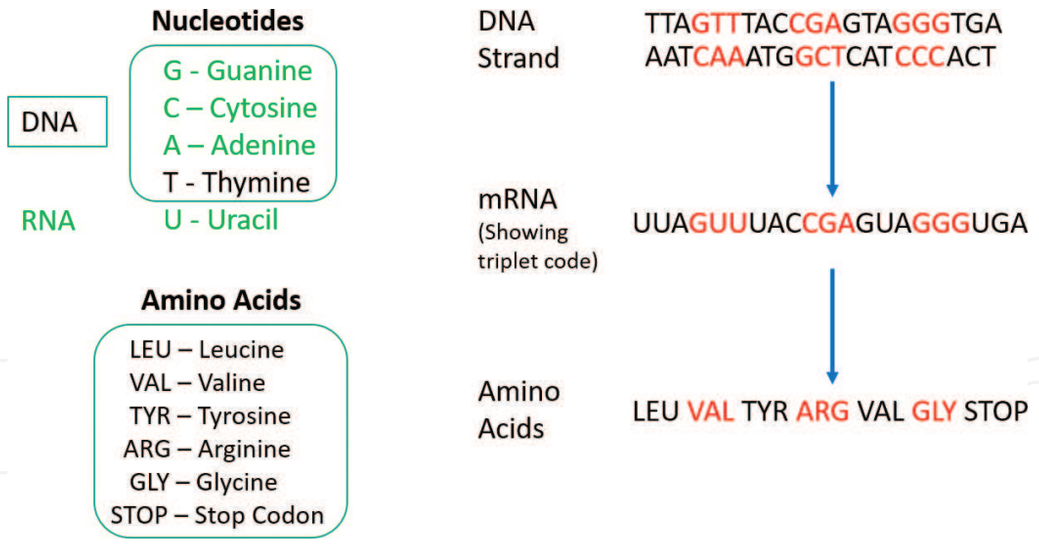



**Figure 1.**
*The molecular codes.*

gene in both prokaryotes and eukaryotes [11], then the potential semantic diversity of the code becomes evident.

To complicate matters further, there are other 'codes' that must be deciphered by cells. The nucleotides present in a portion of coding DNA, are converted by a process of transcription into messenger ribonucleic acid (mRNA). These molecules also comprise just four different nucleotides, namely guanine, cytosine, adenine, and uracil (**Figure 1**). Here, thymine is replaced by uracil (U) [12], with the maintenance of an impressive semantic range. Both DNA and RNA molecules are therefore composed of nucleotides and are referred to as nucleic acids.

Cells have evolved one additional group of codified molecules. The mRNA molecules are further translated into a string of amino acids based on the arrangement of triplet nucleotide sequences [13] in the mRNA molecule, referred to as the RNA codon. Cells therefore possess at least three distinct molecular codes, each with its own 'alphabet', that allows the transformation and transfer of information from DNA to RNA to protein (**Figure 1**).

## 2.2 A molecular language?

What exactly do these molecular codes represent? Do cells use a molecular language? An often-used test is Zipf's Law, which when applied to languages, states that a word's rank in terms of frequency is inversely proportional to its frequency. Therefore, the product of a word's rank and frequency equals a constant, as shown in Eq. (1) below [14].

$$R \times F = C \tag{1}$$

In addition, if the rank and frequency of all words in a language were determined and plotted on a logarithmic scale, the rank-frequency distribution would approximate a linear plot that obeys a power law (**Figure 2**), known as a Zipfian distribution [14].

But authors disagree on whether the molecular codes obey Zipf's Law, with some reporting favorable evidence [15, 16], while others refute such claims [17, 18]. In this regard, there appears to be important differences between coding and non-coding regions of the genome. It is the coding regions that appear to lack higher structure

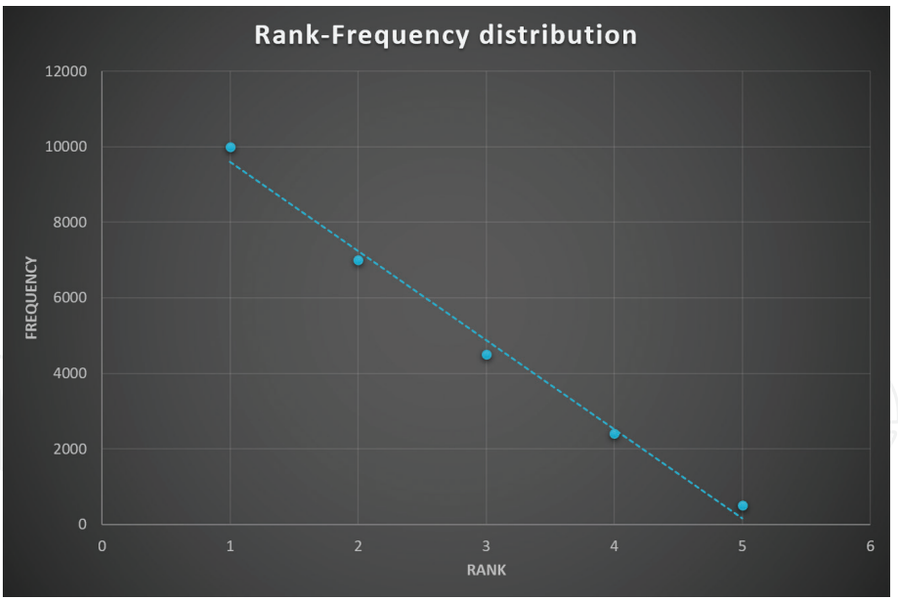



**Figure 2.**
*Zipfian rank-frequency distribution.*

and therefore fail Zipf's Law [16]. The codes simply stand on their own. In contrast, non-coding or 'junk' DNA does appear to possess some linguistic features, including compliance with Zipf's Law and demonstrating redundancy, features not expected in random texts or sequences [16, 19]. Still others argue that DNA does not demonstrate linguistic properties [20].

However, Zipf's Law applies to a diverse range of phenomena. For example, the rank-size plot for cities greater than 10 kilometers throughout the world, is remarkably Zipfian [21]. A similar distribution has been reported for global income distribution [22]. In fact, many phenomena obey the power law including number of citations, telephone calls received, relative income, earthquake magnitude, and the number of species in a genus, implying that a Zipfian distribution is not a definitive criterion of languages [17, 23, 24].

It is also worth noting that we still do not fully understand molecular codes. For example, of the approximately 3 billion base pairs that comprise the human genome, it is estimated that only 3% is coding DNA, that is nucleotides that code for proteins [25]. The remaining 97% is described as non-coding DNA and was often referred to as 'junk' [16, 26]. This is an unfortunate term as increasing evidence has accumulated that demonstrate that this 'junk' DNA may actually have important functions [27] implying it carries some sort of message [16]. Unlike the non-repetitive coding regions that transmit the conserved blueprints for protein architecture, the repetitive syntax of the non-coding regions governs organization, and coordination; a dualism reflected in natural languages [26].

In addition, parallels can be made between the genetic code and other codes including human speech. Ji outlined eight linguistic analogues between human language and 'cell language', including alphabet, lexicon, sentences, grammar, phonetics, semantics, first articulation and second articulation [25]. To this, Witzany adds pragmatics, recognizing context-dependent meaning found in both natural languages and codes [26]. Others suggest that nucleotide bases that represent the fundamental structure of DNA are grouped into triple codons that parallels the fundamental units of sound (phonetic features), which are grouped into phones [28].

When the molecular codes are finally fully deciphered, it is plausible that we will marvel at the extent of their vocabulary (e.g. non-coding DNA sequences), syntax, grammar (e.g. regulatory units), semantics and pragmatics (e.g. epigenetics). Perhaps only then will the elegance and sophistication of the molecular codes be fully appreciated.

However, despite the analogies, this chapter does not argue for equivalence. Molecular codes are obviously not natural languages, notwithstanding the challenge of defining the latter [29].

Acknowledging the difficulty, Wardhaugh suggests the possibility of different types of language, a situation that makes them hard to be subsumed under a single definition [29]. A pragmatic approach offered by Bell entailed using various criteria to distinguish between these different kinds of languages. These include standardization (process of codification), vitality (existence of community of speakers), historicity (provides a sense of identity), autonomy (distinct from other languages), reduction (existence of subordinate varieties), mixture (lack of purity of the variety), and de facto norms (of proper usage) [29, 30].

Direct comparison is obviously futile as it is unreasonable to expect molecular codes to exhibit the linguistic features of natural languages. Yet nucleic acid codes and codons, and amino acid sequences could be described based on some of Bell's criteria. They entail obvious codification and autonomy. As described above, there are also in-built de facto norms of use. Molecular codes could therefore be considered to represent an ancient mode of communication, a group of biological languages, conveying units of information that are sent and received by cells across the kingdoms of life [31, 32].

Further, our written and spoken codes, remarkably unique among the kingdoms of life, probably represent a relatively recent adaptation to the bio-social conditions that presented a fitness-advantage to reciprocal altruism in humans [33]. It seems intriguing that natural languages, whose development across species was restricted by evolutionary costs [33], still echo some of the blueprints embedded in the molecular codes. The question then is not only whether molecular codes are languages, but also what traces of these ancient codes, prototypes of communication, have bridged the apparent bio-social divide.

### 2.3 The multilingual cell

The codes and codons transmitted as nucleic acids, and amino acid sequences, must be understood not only by the source or donor cell, but also by other cells with which it communicates. The relationship between these molecular codes is popularly represented by what is known as the central dogma of molecular biology (**Figure 3**) described by Watson [34] (cf. the original concept published by Francis Crick [35], in 1958).

Functional sequences embedded in the DNA code are first transcribed into messenger RNA molecules (mRNA). The resulting nucleic acid sequences represent a complimentary but limited replica of the DNA molecules from which it originated; like a local dialect or subordinate variety [29]. The cell's machinery recognizes these mRNA molecules, which direct various cellular functions. For example, the mRNA code is reinterpreted as triple codons, another dialect, which directs the cell to add the corresponding amino acid to a growing peptide chain that will ultimately form a mature protein molecule. The latter is represented by yet another code, the amino acid sequence; a different molecular language.

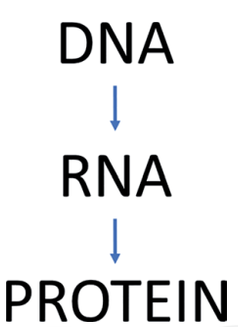



**Figure 3.**
*The central dogma of molecular biology, as described by Watson [34].*

Subsequent discoveries have modified and expanded Watson's portrayal of the central dogma. For example, the unidirectionality of information flow would be challenged [36, 37]. In addition, epigenetic markings are now known to determine context-relevant expression [26, 38]. Further, other types of RNA can direct cellular processes. These include micro-RNA (mi-RNA) molecules, which are involved in the regulation of gene expression [39, 40]. These regulators often determine which, among the vast number of genes, is transcribed. In other words, in a given cell, the local epigenetic and mi-RNA dialects could determine the semantic range of the genetic code.

In addition, there are other types of information that are relayed between cells. These take the form of lipids, carbohydrates and a diverse array of signaling molecules, each with its own set of molecular structures [41].

Cells must therefore understand various molecular codes in order to function effectively. Throughout the vast diversity of life forms, one mechanism has emerged as a highly conserved communication system, capable of protecting and relaying the multiple codes and other signals utilized by cells. This system is deployed by what are known as extracellular vesicles [41].

## 3. Vesicular communicasomes

Extracellular vesicles (ECVs) are produced by cells from all three domains of life: archaea, bacteria, and eukaryotes [42, 43]. Eukaryotic ECVs are classified in many ways, including their mode of biogenesis and size. Based on biogenesis, consensus appears to have emerged around the classification of these vesicles as exosomes, microvesicles or apoptotic bodies [41, 44–46]. However, some controversy remains regarding their size, with estimates ranging from as low as 10 nm, to over 5000 nm; with exosomes being the smallest, microvesicles intermediate and apoptotic bodies the largest [42–44, 46]. Gram-negative bacterial vesicles have been referred to as outer membrane vesicles (OMVs) [47] and those of gram positive bacteria and archaea, which both lack an outer membrane, as simply membrane vesicles (MVs) [47, 48]. Vesicles derived from the prokaryotes (bacteria and archaea) tend to be smaller, ranging from well below 100 nm to a few hundred nanometers [42, 47–51]. This nomenclature will be used throughout the remaining sections.

### 3.1 Early history of extracellular vesicles

Bacterial OMVs were described several decades ago, at least as early as 1966, when Knox et al. described the presence of blebs protruding from the outer membrane of *Escherichia coli* cells grown in lysine-limited culture, with subsequent

extracellular formation of "globules" [52]. A subsequent report, a decade later, included the description of outer membrane fragments and vesicles in *E. coli* culture during normal growth [53]. By 1990, Dorward and Garon confirmed vesiculation and vesicle DNA content in several bacteria, including two species of the gram-positive bacteria *Bacillus* [54]. Several years later this phenomenon, then understood to be common among gram negative bacteria was reviewed by Beveridge, who referred to them as outer membrane vesicles [55]. By then, it was also understood that OMVs were involved in bacterial virulence, and had potential medical applications including as drug delivery and vaccine agents [55]. Towards the turn of the century, vesiculation was observed by electron tomography of the ice-embedded archaea, *Sulfolobus* [56], a phenomenon soon shown to be widespread among the thermoacidophilic members of the *Sulfolobus* genus [48].

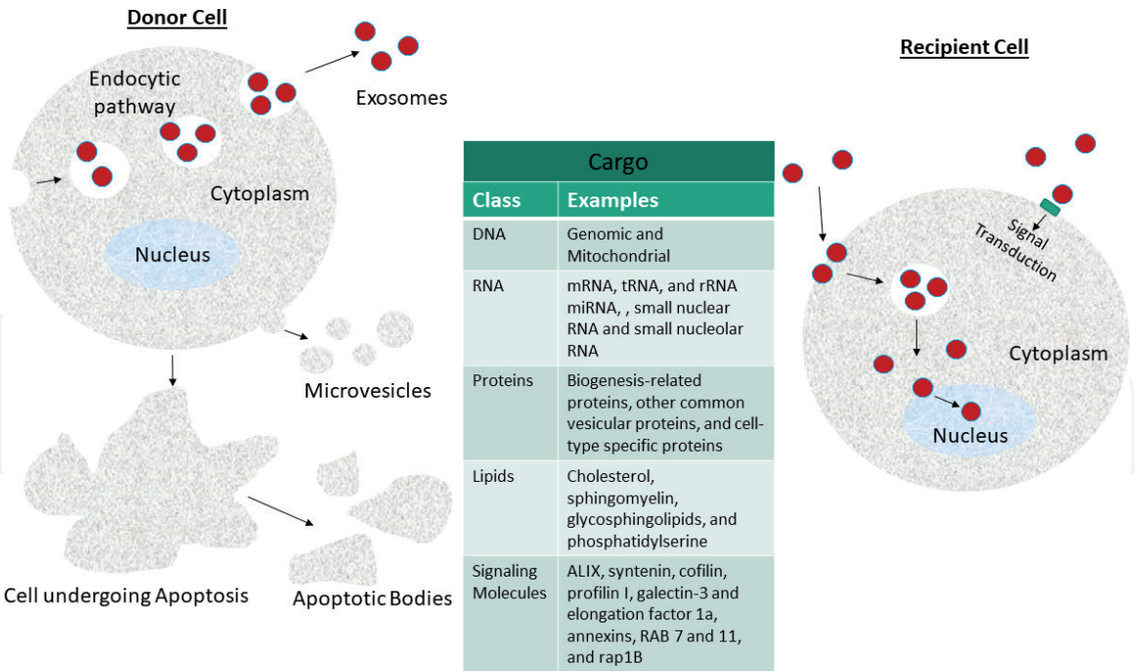
Eukaryotic vesicles may have been alluded to from as early as 1941, when MacFarlane et al. described the loss of coagulation attributable partly to either the deposit derived from high-speed centrifugation of human plasma or filtration through 0.46 μm membranes [57]. This procoagulant component appears to be the particulate fraction sedimentable at 31,000 g that was referred to as "the thromboplastic protein of blood" a few years later [58], and subsequently a vesicle-containing fraction called "platelet dust" [44, 59]. Another earlier function attributed to these vesicles was the selective removal of no-longer required surface membrane components during reticulocyte maturation. These vesicles were termed "exosomes" [60]. Eventually several other terms would enter the literature, including ectosomes, microvesicles, shedding vesicles, microparticles, apoptotic vesicles and apoptotic bodies [44].

Subsequent studies would reveal the ubiquitous secretion of ECVs across the domains of life as well as the plethora of functions related to both normal and pathological processes, as will be discussed later. But before delving into these aspects of vesicular biology, it is important to understand how vesicles are produced, and delivered between donor and recipient.

### 3.2 ECV biogenesis, release

It has been known for some time that exosomes are formed as part of the endosomal system or endocytic pathway (**Figure 4**). Early endosomes result from the inward budding of the plasma membrane. When they fuse with endocytic vesicles, they together with their membrane-derived nucleic acids, proteins and lipids are destined for recycling, degradation or secretion [41, 61–63]. Early endosomes not targeted for recycling, develop into late endosomes that accumulate increasing numbers of inner vesicles by subsequent inward budding of its limiting (outer) membrane, forming what are known as multivesicular bodies MVBs [61, 63, 64]. This process of vesiculation allows for the sorting of cytosolic nucleic acids, proteins and lipids into the inner vesicles [41].

The process of exosome biogenesis can be mediated by different groups of drivers. These include the endosomal sorting complexes required for transport (ESCRTs) I, II and III, which together induces cargo clustering, membrane bud formation and subsequent cleavage to form inner vesicles in yeast cells [65]. There is also an alternative ESCRT pathway in which syndecan, syntenin and ALIX play key roles in the MCF-7 human cell line [66]. In addition, an ESCRT-independent but ceramide (lipid)-dependent pathway has been reported in Oli-neu cells, a mouse oligodendroglial cell line [67]. Importantly, the mechanism of biogenesis appears to vary with cell type and with exosome content [41], implying that cells may recruit from a slate of internal machinery to produce various exosome phenotypes.




| Cargo | |
|---|---|
| **Class** | **Examples** |
| DNA | Genomic and Mitochondrial |
| RNA | mRNA, tRNA, and rRNA miRNA, , small nuclear RNA and small nucleolar RNA |
| Proteins | Biogenesis-related proteins, other common vesicular proteins, and cell-type specific proteins |
| Lipids | Cholesterol, sphingomyelin, glycosphingolipids, and phosphatidylserine |
| Signaling Molecules | ALIX, syntenin, cofilin, profilin I, galectin-3 and elongation factor 1a, annexins, RAB 7 and 11, and rap1B |

**Figure 4.**
*ECV biogenesis, uptake and cargo.*

MVBs not destined for recycling or degradation complete a membrane-to-membrane cycle by fusing with the plasma membrane to externalize the limiting membrane and release the enclosed vesicles, called exosomes [61]. Exosome release is also influenced by a range of mechanisms: stimulation by RAB GTPases in mouse Oli-neu cells [68]; SNARE proteins in the human chronic myeloid leukemia cell line, K562 [69], as well as diacylglycerol kinase α (DGKα) inhibition in human T-cells [70]. Here again various cell types utilize different mechanisms to trigger the release of exosomes, with distinct cargo [66, 69, 71]. The endocytic pathway therefore facilitates not just the recycling of materials but the selective packaging and release of specific molecular codes and signals.

Microvesicle biogenesis and release are somewhat merged processes as vesiculation involves outward budding of the plasma membrane (**Figure 4**). This involves initial redistribution of phosphatidylserine to the outer membrane leaflet and completed by ERK-induced phosphorylation and activation of the myosin light chain resulting in cytoskeletal contraction and membrane fission [63, 72]. Phosphatidylserine translocation is induced by increased intracellular $Ca^{2+}$ and $Ca^{2+}$-induced activation of the protease calpain [73], as seen for example with platelet microvesiculation [74]. However, as with exosomes, other effectors and mechanisms may be involved, including hypoxia, which induces microvesicle production in human breast cancer cells through hypoxia-inducible factors (HIF)-dependent RAB22A GTPase expression [75].

Apoptotic bodies, or apoptosomes, are formed during the process of apoptosis (**Figure 4**) that involves chromatin condensation, membrane blebbing and disintegration of cell contents into the defined membrane-enclosed vesicles [63]. In Jurkat cells (a hematopoietic cell line) vesiculation involves Caspase 3-induced cleavage of the serine/threonine kinase ROCK1, which is associated with myosin light chain phosphorylation [76], suggesting apoptosome formation also involves cytoskeletal rearrangement [63].

Like microvesicles, OMV production in gram negative bacteria may be initiated by rearrangement of membrane components, leading to curvature of the lipid bilayer [47]. Such rearrangement could involve deposition of peptidoglycan fragments into the periplasm producing an elevated turgor pressure; down-regulation

of outer membrane proteins that favor peptidoglycan interaction; or charge repulsion in regions with accumulation of negatively charged lipopolysaccharide (LPS) O-antigen [51]. Yet another proposed mechanism, potentially highly conserved among gram-negative bacteria, involves membrane curvature induced by accumulation of phospholipids in the outer leaflet of the outer membrane, which is further enhanced by subsequent accumulation in both leaflets, until the vesicle is finally pinched off [77].

Similarly, vesiculation in gram positive bacteria may involve protrusion of plasma membrane microdomains as well as peptidoglycan degradation [51]. Although less is known of archaeal vesicle formation, protein homologs of ESCRT-III subunits have been isolated from these membrane vesicles [48].

### 3.3 Vesicular uptake

Uptake of eukaryotic ECVs by recipient cells also occurs by several mechanisms. These include the interaction of the vesicle with the plasma membrane with release of content, or the internalization of the ECV through endocytosis (**Figure 4**). There are several different types of endocytosis recently reviewed by Abels and Breakefield, including clathrin-, caveolin-, and lipid raft-mediated endocytosis, macropinocytosis, and phagocytosis [41]. Internalized ECV exosomes must be released into the cytoplasm and this process is promoted by the low pH-environment within endosomes resulting in fusion of exosomal and endosomal membranes [78]. Interestingly, prokaryotic vesicle uptake is also mediated by similar processes, including macropinocytosis, various endocytosis-dependent processes and membrane fusion [79].

Apoptotic body uptake is mediated by specific interactions between altered apoptotic cell membrane components and receptors on phagocytes, which engulf and remove these vesicles [63]. These components include phosphatidylserine translocated to the outer membrane leaflet bound by Annexin V in Scott B lymphoblastoid cells [80], complement C3b deposition on Jurkat cells [81], surface molecules bound by thrombospondin, and exposed side chain sugars [82], all of which are recognized by phagocyte receptors [63, 82].

These mechanisms imply that ECV biogenesis, release and uptake are evolutionarily conserved processes that although demonstrate divergence across the domains of life, still exhibit remarkable similarities. This underscores their fundamental functional importance. Considering their cargo, their importance becomes even more evident.

### 3.4 Extracellular vesicle cargo

The content of specific ECVs vary based on several factors, including their mode of biogenesis, cell type of origin and the prevailing physiological state [41]. However, both eukaryotic (**Figure 4**) and prokaryotic vesicles have been shown to carry a wide range of biologically active molecular codes and signals.

Eukaryotic ECVs contain many types of nucleic acids. These include vesicle enclosed genomic DNA as derived from mouse cardiomyocytes [83], and mitochondrial DNA from rat astrocytes and human glioblastoma cells [84], cargo that could facilitate recipient evolution and enhanced functions, as will be discussed. Also found are various RNA species, including mRNA, tRNA, and rRNA, as well as various non-coding RNAs including miRNA, small nuclear RNA and small nucleolar RNA [39, 83, 85–88]. Among these, rRNA may dominate in apoptotic bodies [87], and small RNAs including miRNA seem to be the dominant RNA species in exosomes [87, 88]. DNA has also been shown associated with the external surface of bacterial OMVs as well as within intact vesicles [89].

It is difficult to draw conclusions on the protein content of different ECV types as the cell types and research methodology used varies among studies [41]. However, despite this variability, review of different reports gives an overview of the types of proteins normally found in vesicles. Proteins found in eukaryotic ECVs can be classified as biogenesis-related proteins, other common vesicular proteins, and cell-type specific proteins [41, 43]. Among the early proteomic analyses was that performed by Théry et al. on dendritic cell exosomes. They identified proteins involved in exosome biogenesis, release and function as well as intracellular membrane transport and signaling (ALIX, syntenin, cofilin, profilin I, galectin-3 and elongation factor 1a, annexins, RAB 7 and 11, and rap1B), many of which were cytosolic [90]. Parotid gland exosomes also contain several proteins involved in exosome biogenesis and release (ALIX, RAB proteins), as well as several cytosolic proteins involved in signaling and immune functions [91]. Bacterial vesicle proteomes have also been studied. OMVs from the gastric pathogen *H. pylori* were reported enriched in membrane proteins, porins, adhesins, immune-modulators, and virulence factors including vacuolating cytotoxin (VacA), cytotoxin-associated gene A (CagA), and neutrophil-activating protein (NapA) [49]. Membrane vesicles from the crenarchaea *Sulfolobus acidocaldarius*, *S. solfataricus* and *S. tokodaii*, were shown to contain proteins involved in vesicle biogenesis, including proteins homologs of ESCRT subunits as well as proteins associated with transcription and translation [48].

Being derived from membrane structures, the lipid composition of ECVs share many similarities with their cells of origin and reflects their biogenesis [41]. However, differences are clear. Exosomes derived from the prostate cancer cell line PC-3, contained several fold greater lipid:protein content and were highly enriched in cholesterol, sphingomyelin, glycosphingolipids, and phosphatidylserine [92]. In contrast, the OMVs of *Pseudomonas aeruginosa* contain large proportions of phosphatidylglycerol [93], while membrane vesicles from *Sulfolobus* species have been reported enriched in archaeal tetraether lipids [48].

### 3.5 Active sorting of cargo

The diverse cargo of ECVs suggests the involvement of some kind of sorting process in their biogenesis. This is in keeping with an effective communication system that requires targeted delivery of information; a deliberate separation of the signal, from the noise that would otherwise drown it. The immense array of molecular codes and signals that could be packaged into ECVs must be filtered so that meaningful information is ultimately delivered.

The evidence demonstrates that this is exactly what cells do. For example, there is relative enrichment of membrane and cytoplasmic compared with nuclear and mitochondrial proteins in eukaryotic ECVs [43], and preferential selection of specific proteins for inclusion in both prokaryotic and eukaryotic vesicles [43, 94]. During exosome biogenesis, both membrane proteins and lipids are selectively incorporated into the MVB limiting membrane and subsequently into the exosome bound inner vesicles [61]. Similarly, *H. pylori* OMVs are enriched in outer membrane and periplasmic proteins [49]. Although eukaryotic ECVs contain several membrane lipids, there is enrichment of a select group of lipids, including sphingomyelin, cholesterol, ganglioside GM3 and phosphatidylserine. In addition, the preferentially sorted mix of lipids varies between cell types [95]. Lipid content also varies between the outer membrane and outer membrane vesicles of gram-negative bacteria [93]. Selective packaging of ECV nucleic acid content has also been shown. Eukaryotic exosome analysis has identified enriched and depleted mRNAs and miRNAs compared with the donor cell, as well as mRNAs not detected in the donor cell [85, 86]. Similar differential sorting has been reported in prokaryotes,

as with *Pseudomonas aeruginosa* OMVs enriched in specific chromosomal regions involved in virulence, stress response, antibiotic resistance and metabolism [89].

Several methods may be involved in cargo sorting into eukaryotic ECVs. It has long been know that various proteins can be sorted into, or excluded from, cholesterol/sphingolipid-enriched lipid rafts [96]. Similarly, galectin-3 may be involved in the sorting of proteins into exosomes by stabilizing their cross linking to form high molecular weight clusters in the apical membrane that are sorted into the vesicles [97]. Various mechanisms have been proposed for miRNA sorting. These include an interaction between a four-nucleotide motif (GGAG) and the ribonucleoprotein, hnRNPA2B1; post-transcriptional 3′-uridylation; protein mediated pathways via neutral sphingomyelinase 2 (nSMase2) or Protein Argonaute-2 (AGO2); and elevated cellular levels of miRNA [41, 98]. Loading of mRNAs has been associated in human HEK-293 T cells with a particular 3′ untranslated region (UTR) containing a CUGCC core on a stem-loop structure as well as an miRNA-binding site, whose interaction enhances loading [99].

In bacterial and archaeal vesicles, various mechanisms may also be utilized to accomplish this [51]. Among these mechanisms specific proteins could be localized to certain microdomains based on their affinity to particular moieties, the overall charge or length of local lipopolysaccharide (LPS) molecules, or through recruitment by a sorting factor that simultaneously binds recruiting signals in the protein and specific sites on the LPS molecules [94].

Cells are therefore not simple automatons. Instead their messages are delivered by multiple molecular codes and signals; diverse, nuanced and presumably meaningful. If they were automatons, ECVs would be produced by repetitive packaging of identical cargo as observed on a factory assembly line. If they were, ECVs would be monosemic, devoid of physiological and pathological pleiotropism. Evidence for this final link in the communicative process, logical response to the transmitted information, will now be presented.

## 4. ECV-mediated communication

Now that the message has been packaged, transported and received, is it intelligible? As with any other form of effective communication, the transferred information delivered by ECVs must have meaning to the recipient. Otherwise, the signal will be understood as non-sense and no communication would have occurred. However, we know that this is not the case. ECVs do affect recipient cells and in specific ways. As shown in **Figures 4** and **5**, some may initiate signaling through interaction with recipient cell receptors. Others must enter the cell and be released into the cytoplasm or delivered to the nucleus [41].

### 4.1 Communicating with living codes

For ECVs to function as a communication system, they must be able to package and transport relevant information to recipient cell(s). This is exactly what they do. There is mounting evidence that the delivered cargo is functional.

ECVs transport molecules which are themselves living codes of information, in the form of nucleic acids and proteins. For example, DNA associated with mouse cardiomyocyte-derived vesicles has been shown to be distributed within fibroblast cytosol and nuclei, in conjunction with differential gene expression of more than 300 genes [83]. Similarly, new mouse proteins were recovered from recipient cells after the transfer of mouse exosomal RNA to human mast cells, suggesting that the delivered RNA was successfully translated in the presence of functional

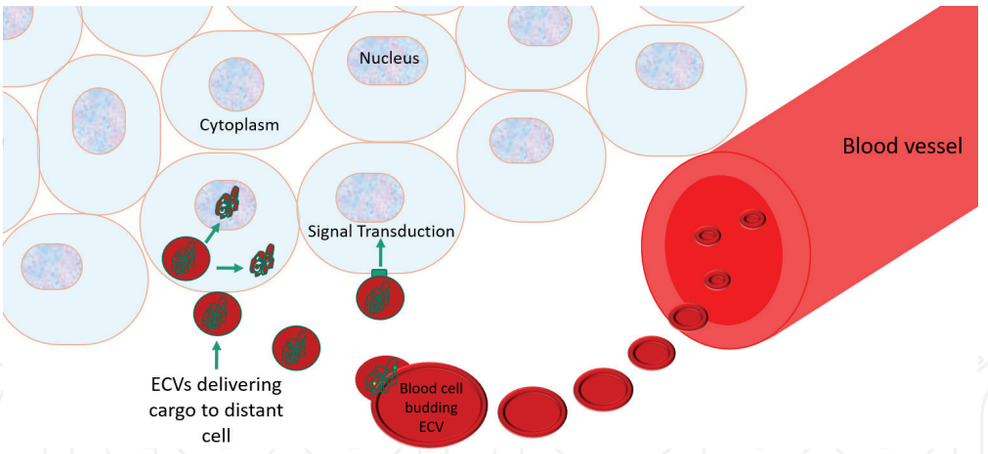



**Figure 5.**
*Intercellular communication mediated by extracellular vesicles.*

protein synthesis machinery, made available within the recipient cells [85]. Such translation of functional proteins has also been reported in healthy human brain microvascular endothelial (blood vessel wall) cells in response to delivery of cancer cell (glioblastoma)-derived microvesicles [86], implying that ECVs derived from abnormal cells can be utilized to direct the genetic machinery of normal cells.

Furthermore, recipient cells respond predictably to the delivery of ECV-delivered regulatory cargo. Dendritic cell-derived exosomes containing miRNAs, were shown to target and repress mRNAs in recipient dendritic cells [39]. Such responses may also be part of a pathological process. For example, glioblastoma-derived microvesicles can stimulate the proliferation of other glioma cells, as well as promoted angiogenic processes in normal endothelial cells [86]. Viral mi-RNA molecules derived from Epstein–Barr viruses (EBV) that have infected nasopharyngeal carcinoma (NPC) cells are packaged into NPC-derived exosomes [100]. EBV-infected B-cells secrete EBV mi-RNAs via exosomes, which are internalized by dendritic cells in co-culture and lead to suppression of known target genes, including immunoregulatory genes [40]. Similar suppression has been documented in murine endothelial cells treated with exosomes isolated from bone marrow-derived macrophages [98]. Horizontal transfer has also been demonstrated between rat fibroblasts and murine recipient cells, resulting in in-vitro loss of contact inhibition and a tumorigenic phenotype in vivo [101].

As with eukaryotic ECVS, bacterial vesicles have also been shown to package and transport biological codes to others cells. One of the most well studied bacteria, *Escherichia coli*, has been demonstrated to release OMVs carrying DNA, which are transferred to recipient bacteria [50]. Nuclear localization has been reported with *P aeruginosa* OMVs, delivering bacterial genetic codes to the cytoplasm and then nuclei of eukaryotic epithelial cells [89]. These vesicles can relay functional biological codes. *E. coli* delivers DNA to other *E. coli* cells as well as to other bacterial cells where it is expressed and results in appropriate biological response [50].

### 4.2 Communicating with signaling molecules

Multicellular organisms also utilize ECVs to deliver other signaling molecules for physiological processes as well as to facilitate pathogenetic mechanisms.

Vesicular transport is essential for sperm motility, a critical component of one of the most fundamental biological processes; reproduction. Normal fertility requires, among other factors, motile spermatozoa. Motility is dependent on $Ca^{2+}$ signaling [102] and involves $Ca^{2+}$ mobilization and entry [103]. These processes in turn require a slew of molecules, including various receptors and enzymes, which

are transferred to the neck of the sperm, delivered by fusion of prostate gland-derived extracellular vesicles called prostasomes [103, 104].

Among the most lethal ECV-mediated dysfunctions, cancers represent a significant cause of mortality worldwide [105]. Evidence suggests that one of the methods involved in the subversion of normal biology, to promote cancer growth and survival, is the delivery of molecules by ECVs. For example, it has been shown that cancer cells release the protein Survivin into the extracellular space, in the form of exosomes [106]. In addition, the extracellular form of Survivin is secreted by several types of cancer cells (including breast, cervical, prostate, pancreatic, bone and blood cancer cells), is transferrable to other cancer cells and induced increased proliferation and reduced apoptosis (cell death) of the recipient cell [107], features that enhance cancer progression.

OMVs also mediate host-pathogen interactions that could result in pathology. Vesicles isolated from *H. pylori*, contain the known virulence factors CagA, VacA, and NapA, which not only induced morphological changes in gastric cells, but promoted a pro-inflammatory environment including enhanced interleukin-8 (IL-8) secretion from gastric cells, colonic cells and duodenal explants, as well as neutrophil migration [49].

### 4.3 Interspecies transfer and evolution

Perhaps the most powerful impact one cell could have on another is through horizontal gene transfer. Not only does intercellular genetic transfer allow for an immediate response, there is the possibility that the donor could influence the recipient's progeny for generations, if not millennia, to come.

Despite several claims, genetic transfer from prokaryotes to eukaryotes is replete with challenges [108]. However, there is clear evidence of DNA transported into eukaryotic recipients from endosymbiotic (mitochondria and plastids) and other eukaryotic sources, which could introduce new genes into the genome or replace existing genes [32, 109]. It is now becoming clear that ECVs may also introduce new genetic material into recipient cells. Within a multicellular eukaryotic model, this is what appeared to happen when fibroblasts were transfected with cardiomyocyte-derived vesicles, resulting in altered gene expression within recipient cells [83].

On the contrary, horizontal gene transfer into prokaryotic cells is thought to be common, conferring evolutionary benefits, including acquisition of antibiotic resistance and enhanced virulence. Such transformations can be mediated by mobile genetic elements such as bacteriophages and plasmids [31, 110]. It is therefore not surprising that Yaron et al. had previously reported vesicle-mediated DNA transfer from the food-borne pathogen *Escherichia coli O157:H7*, to *Salmonella enterica serovar Enteritidis* and *E. coli JM109*, leading to several fold increased cytotoxicity of recipient cells, as well as ampicillin resistance in transformed *E. coli JM109* cells [50].

ECVs are therefore agents of interspecies genetic transfer. As such, they have the potential to serve as drivers of evolution.

## 5. Conclusion

Cells interact using various coded and non-coded molecules, which although not natural languages, could be considered types of biological language. It seems logical that these highly-conserved molecules pre-date the emergence of natural languages, whose evolutionary advantage arose relatively recently and only in limited circumstances. Ubiquitous molecular languages were, and will remain, fundamental to life because they direct the most basic of cellular functions throughout all life-forms. Natural

languages on the contrary probably developed under the limited circumstance when reciprocal altruism conferred a selective advantage [33]. Molecular languages are therefore an adaptable prototype, representing a highly conserved model of information.

Their significance is further underscored by the fact that cells from all three domains of life have evolved limited modes of transporting such crucial cargo. Despite the clearly diverse mechanisms involved in ECV biogenesis, packaging, release and uptake, the basic modes of ECV-mediated intra-cellular, inter-cellular and inter-species communication have been widely replicated. Extracellular vesicles are therefore also another adaptable prototype, representing a highly conserved model of communication.

This scenario probably reflects the enormous evolutionary pressures brought to bear over evolutionary time, as well as across various habitats, for cells to effectively communicate with each other. It also underscores a fundamental biological principle: structure determines function. Development of universal codes allowed for wide-spread interpretation of shared information [111]. Similarly, development of universal cellular transporters allowed for wide-spread accessibility to this information. However, selective packaging and targeting of these codes, which have evolved over time, facilitates an extensive context-relevant semantic range, and therefore selective and specific communication. In this regard, ECVs have proven fit for purpose.

As with any communication system, this prototype is versatile, diverse, nuanced and meaningful. This is exactly what one would expect from an effective communication pipeline that delivers targeted information; one that intuitively separates the signal, from the noise. In so doing, ECVs ensure that actionable biological information is ultimately delivered. It is this prototypal communication system that not only directs normal physiology and induces pathology when disrupted, but has the potential to influence the evolution of recipient cells.

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## Conflict of interest

The author has declared no conflict of interest.

## Author details

Paul A. Brown
Department of Basic Medical Sciences, University of the West Indies, Kingston, Jamaica

*Address all correspondence to: paul.brown02@uwimona.edu.jm

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