

REVIEW

The Biological importance of cells secreted Exosomes

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Abstract: Exosomes are the extracellular vesicles secreted normally by most of the cells, containing important bioactive molecules including lipids, carbohydrates, protein, DNA and RNA resulting in cell to cell communication and many other biological activities. In this review we have focused on different insight onto exosomes to cover its basic mechanism, biogenesis, biomolecules it carries and how they are altering secondary sites. In cancerous cells these tiny bodies are reported to be secreted aberrantly and through paracrine signalling contributes in metastasis. Each type of cancer cells exosomes is unique with types of load inside, thus behave with an individual pattern to transfer cancer load from origin to other sites. Because of its involvement in cancer metastasis and its role as biomarkers in early stage disease identification and also as suitable particles for drug delivery system, Exosomes research has been focal field since last two decades. Currently exosomes are the hot area of research and because of their biologically important structure and composition some studies have also been conducted to use them as early stage biomarkers in different diseases and also by a modification these could also be a biocompatible source in drug delivery. The current researches data, results and advancement in exosome studies are quit promising and are positive indication in resolving many clinical complexities in future but still further investigations are needed to evaluate the clinical importance and applications of exosomes in detail.

Keywords: Exosomes, composition of exosomes, importance in research, future insights.

INTRODUCTION

Exosomes are released by majority of the cell types and have found to be in range of 30 to 120 nm in size. These are released from the cells by fusion of late endosomes with cell membrane and thus enter into extracellular spaces to travel through blood and deliver it's inner contents to close or distant recipient cells and form multiple phenotypic alteration causing pathological or physiological changes (Van Niel, d'Angelo *et al.*, 2018). Exosome are used in diagnosis, prognosis, and in establishing treatment regimens has enormous potential to revolutionize patient care (Javeed and Mukhopadhyay, 2017). Many biological fluids including cerebrospinal fluid, ascites, amniotic fluid, urine, nasal secretions, pleura effusions, saliva, serum, breast milk, semen and plasma are found to be containing exosomes. These tiny particles have a major role in metastasis, invasion and carcinogenesis by involving in cell signalling and communication by interacting with receptor protein or lipid ligands or through pinocytosis or phagocytosis. The role in tumorigenesis is through the transfer of certain protein, miRNA and mRNAs from cancer tumour to other sites either by inactivating tumour suppressor genes or activating oncogenes (Samanta, Rajasingh *et al.*, 2018). Some recent studies have also found that exosomes produced by tumour can reside in other healthy sites and

start formation of pre-metastatic niche leading to metastasis. Use of exosomes as biomarkers has also been a hot topic of research and focus to identify a variety of early diagnostic biomarkers and prognostic biomarkers for various diseases through studying miRNA or some particular protein specifically expressed in a disease condition (Clark, Fondrie *et al.*, 2016). Another important feature of exosomes research also focuses on biological or chemical modification of exosomes to make it suitable drug delivery system, because of its certain favourable characteristics to load drug, deliver to a targeted site, unload accurately and degraded or released safely without any side effects. To study and use exosomes, many extraction methods including On-Chip Isolation, Membrane-Mediated Exosome Separation, Nanoplasmon-Enhanced Scattering (nPES), Integrated Double Filtration Microfluidic Device, Ultrafiltration Separation Technology, Differential Centrifugation/Ultracentrifugation, Density Gradient Centrifugation, Extraction Using Immuno-magnetic Beads, Extraction using Exoquick™ and Chromatography are used to extract exosomes with high quality and quantity (Yu, Zhu *et al.*, 2018). The aim of this review was to summarize some important features, current research and future of exosomes in medical applications.

Biogenesis of exosomes

Although exosomes were discovered in 1980's but have gained its significant recognition since last three decades

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through immense research. Currently most of the aspects are studied starting from its biogenesis to their clinical applications. After many cellular stages mature exosomes are released in extracellular spaces, these steps involve formation of intraluminal vesicles (ILVs) in multi-vesicular bodies (MVBs), MVBs delivery to plasma membrane and its fusion to plasma membrane in the presence of multiple carrier molecules but in many studies the role of these molecules is not evident due to methodological challenges (Hessvik and Llorente, 2018). This remain unclear either all MVBs or specific ones only can fuse with plasma membrane. The latter is supported by research showing B-lymphocytes produce two pools of MVBs. These can be identified based on cholesterol content. It was observed that only MVBs with higher cholesterol content could fuse with plasma membrane leading to exosome release. Interestingly, Epidermal growth factor and its receptors reach a subpopulation of MVBs which differ from morphologically similar vacuoles marked with BMP. The composition of exosome differed when the exosomes secreted from apical or basolateral side of a cell (which was polarized) (Chen, Takada *et al.*, 2016). Hence it also supports the idea of different subpopulations. Certain biomolecules are incorporated in the membrane during invagination while many components of cytosol are engulfed and packed inside ILVs. These are moved to lysosomes for degradation alternatively. The density of exosomes varies accordingly with the source of secretory cells. The density is 1.13g/mL of B lymphocyte derived exosome and 1.19g/mL of epithelial cells derived exosomes (Yellon and Davidson, 2014).

Components of exosomes

Exosomes are regarded as miniature versions of parental cells based on dependency of their architecture on the cell from which they are originated. The complexity and diversity of exosome can be comprehended via number of biomolecules identified in exosomes releasing from various types of cells. Fig. 1 modified source shows internal composition of exosomes. (Kim, Kang *et al.*, 2013, Mathivanan, Fahner *et al.*, 2012).

Proteins

Exosomes have found to have several proteins including tetraspanins (CD9, CD37, CD53, CD63, CD81, CD82), HSP (HSP27, HSP60 HSP90, HSP70), signalling proteins (GTPase HRas, furloss, Src homology 2 domain phosphatase, Syntenin-1, 14-3-3 Proteins, Ras-related protein, Transforming protein RhoA, GDP dissociation inhibitor, extracellular signal-regulated kinase), cell adhesion proteins (integrins, intercellular adhesion molecule 1, lactadherin), Growth factors and cytokine (TNF- α , Transforming growth factor- β , TNF Receptors), Death receptors (TNF-related apoptosis inducing ligand, FasL), iron transport (transferrin receptor), cytoskeletal components (Actins, Vimentin, Tubulins, Erzin, Myosin, Radixin, Moesin, Cofilin-1). In addition, it also has

Tsg101, Vps, Lysosomal-associated membrane protein, CD13, Alix, Ribosomal proteins, Rab proteins, major vault protein, PG regulatory-like protein, Complement factor 3 and ubiquitin protein (Yellon and Davidson, 2014). Table 1 shows some proteins of exosomes and their respective functions.

Nucleic acids

Exosomes also have a wide variety of RNAs some of them are shown in fig. 2. As a result of RNA sequencing analysis, MicroRNAs were found to be high amount in exosome RNA species derived from human plasma (Huang, Yuan *et al.*, 2013). These RNA species have their respective functions after being incorporated in recipient cells through exosomes as shown in table 2.

Lipids

Exosomes, besides proteins and nucleic acids also have lipids directly or indirectly participating in various biological activities. Common lipids present in exosomes are phosphatidylserine (PS), arachidonic acid, phosphatidic acid, cholesterol & sphingomyelin (SM). They also have fatty acids, prostaglandins, and leukotrienes which are specifically involved in provision of structural rigidity and stability. Many lipids in exosomes are found to be involved in immunosuppression (PGE2) (Subra, Grand *et al.*, 2010), inflammation (PGE2), increasing production of exosome (PA), sorting cargo in MVBs (ceramides) exosome secretion regulation (cholesterol) and also account for membrane curvature (AA, LPC) (Trajkovic, Hsu *et al.*, 2008).

Carbohydrates

Glycoproteins (with NeuAc α 2,3/6-containing structures or high amount of mannose) was noted in SKOV3 cells. Moreover T-cells derived MVs showed high mannose & sialic acid content (Krishnamoorthy, Bess Jr *et al.*, 2009). Endocytosis was increased by removal of NeuAc, as their removal reduced net negative charge on exosomes surface. Hence physicochemical alterations led to creation of new ligands for proteins (which bind to carbohydrates) at cellular surface. Other sugars involved in reduction of exosome uptake include D-galactose, α -D-mannose, the disaccharide β -lactose, α -L-fucose, and D-N-acetylglucosamine. Hence the sugars reduced endocytosis. Since protein oligomerization promote sorting in exosomes, glycans can oligomerize by interacting with lectins, glycans may therefore be involved in exosome sorting (Escrevente, Keller *et al.*, 2011).

Role of exosomes in biological mechanisms

In the start the idea of exosomes simply being clearance system of the cell was changed after studying these tiny particles in detail. The exosomes have multiple roles in biological mechanisms starting from cell to cell communication to regulation of gene in distant cells. Due

Table 1: Functions of some proteins in exosomes (Vlassov, Magdaleno *et al.*, 2012)

S No.	Proteins	Function
1.	Tetraspanins (CD9, CD63, CD81, CD82)	Involved in cell penetration, fusion and invasion.
2.	Heat shock proteins (HSP70, HSP90)	Involved in stress response
3.	MVB proteins (TSG101 and Alix)	Play important role in release of exosomes
4.	Annexins & Rab	Play important role in membrane transport & fusion

Table 2: RNA species in exosomes and their respective functions

RNA Species	Function	References
MicroRNAs (miR-214, miR-1, miR-320, miR-29a, miR-126)	Angiogenesis, tumorigenesis, exocytosis, hematopoiesis, cell-cell communication	(Waldenstrom and Ronquist, 2014)
Long RNAs (non-coding)	Cancer development, biological processes	(Hewson, Capraro <i>et al.</i> , 2016)
Circular RNAs	Cancer development, biological processes	(Hewson, Capraro <i>et al.</i> , 2016)

to its internal magical components especially miRNA and certain protein, they have a major role in performing certain important biological activities.

Role of Exosome in immune system

B-cells derived exosomes are involved in T cell stimulation and antigen presentation. Exosomes derived from antigen presenting cells (APCs) (e.g. Dendritic cells) express MHC (class I & II), co-stimulation molecules as well as adhesive molecules. These traits allow exosomes to activate T cells (CD4+ & CD8+) thereby inducing a strong immune response. Strong anti-tumour immune response is initiated by dendritic cells pulsed with tumour peptides, by release of immunogenic immune response. Exosomes can cause T cell activation indirectly (by transferring antigenic peptides to APCs) or directly (by antigen presentation) (Admyre, Bohle *et al.*, 2007). Exosomes derived from tumour can activate DCs by expression of tumour antigens, hence initiating a specific cytotoxic response. This response is stronger than the one initiated via lysed tumour cells or vaccines. Natural killer cells derived exosomes contain perforin and granzyme B as their cargo and can initiate antitumor activities (in vivo as well as in vitro). Mast cells derived exosomes are presented by DCs and initiate specific immune response. Exosomes are also involved in immune suppression. Tumour derived exosomes have immunosuppressive activity (e.g. NK cells are suppressed by tumour exosomes by modulation of NKG2D receptors). These exosomes can induce apoptosis of T-cells either by activating Fas, or expression of galectin 9. They can also affect dendritic cell maturation (Lugini, Cecchetti *et al.*, 2012).

Exosomes and neuro-degenerative diseases

Exosomes are not only crucial in development but also during regeneration of neurons. It was demonstrated that cortical and hippocampal neurons secrete exosomes. Their secretion was regulated via calcium influx and glutamatergic synapse activity. Oligodendrocytes derived exosomes are released in response to neuronal stress signals. They deliver mRNA, enzymes, proteins and are

endocytosed by neurons via endocytic pathway (Frühbeis, Fröhlich *et al.*, 2013). The generation and prognosis of many neurodegenerative diseases is due to exosome's cargo of misfolded proteins. They are involved in shipment of toxic agents from unhealthy cells to healthy neighbouring cells. Parkinson's disease spreads from cell to cell via transfer of mutated protein α -synuclein (from Lewy's bodies to extracellular milieu) through exosomes. In Alzheimer's disease tau proteins are culprit that spreads (from glial cells and affected nerve cells) via exosomes to healthy cells causing the disease. Tau pathology was observed in transgenic mice by aggregation of extracellular α -amyloid which stimulated neurodegeneration (Danzer, Kranich *et al.*, 2012).

Role of exosomes in cancer

For progression of cancer direct interaction is required between tumour cells and their environment. To ensure cell-cell communication, exosomes play critical role in information exchange. They suppress immune response thereby ensuring tumour progression and survival (by induction of a pro-tumour microenvironment). Exosomes participate in thrombosis, tumour cell proliferation, remodelling of extracellular matrix and promotion of angiogenesis. Exosomes being very stable promote pro-tumour environment for harbouring metastatic niches (Rana, Malinowska *et al.*, 2013). Exosome upon internalization deliver their cargo. Mutated proteins like KRAS and MET onco-proteins are delivered via exosomes. The uptake of these proteins is favoured by hypoxic TME (Tumour Micro Environment). Onco-miRNA in tumour cells can affect function of acceptor cells. Exosomes are MVs that can serve as biomarkers for disease diagnosis and prognosis in a non-invasive manner. For purpose of therapeutics, exosomes are preferable over EVs owing to their numerous properties. These include stability (in vivo and in vitro), ability to cross blood-brain barriers, distribution in an organism, bioavailability etc. These traits not only highlight their importance as vaccines for treatment of cancer but also for delivery of natural biologics (De Toro, Herschlik *et al.*, 2015).

Exosomes and cardiovascular diseases

While patients can control various behavioural risk factors of cardiovascular diseases, these still remain a leading cause of death worldwide. Many studies have found a direct link between MVs and cardiovascular diseases. Exosomes can have either anti-inflammatory, pro-angiogenesis & pro-coagulant effects as well as their opposite effects on vesicular walls. This feature is most probably due to cell to cell cargo (proteins, miRNA, mRNAs etc.) delivery. The association of various biomolecules with exosomes have a direct role in various physiological mechanisms. Total RNA extracted from cardiosomes (cardiomyocyte's exosomes) led to identification of 1520 mRNAs. Moreover, about one third were involved directly in various biological processes as gene expression (Waldenström, Genneback *et al.*, 2012). CPCs (cardiac progenitor cells) derived exosomes can protect cardio myocytes from stress (oxidative) in vivo and in vitro. This is done by inhibition of ischemia / reperfusion-induced apoptosis. The transcription of miR451 is due to GATA4. GATA4 is a transcription factor involved in cardiomyocytes survival, cardiac morphogenesis, and maintaining cardiac function of adult heart. It was demonstrated that TNF- α can be produced by cardiomyocytes when subjected to HIF-1 (hypoxia-inducible factor 1). This cytokine is involved in inflammation and remodelling of cardiac tissues, and is present in exosomes under hypoxic environments (Chen, Wang *et al.*, 2013).

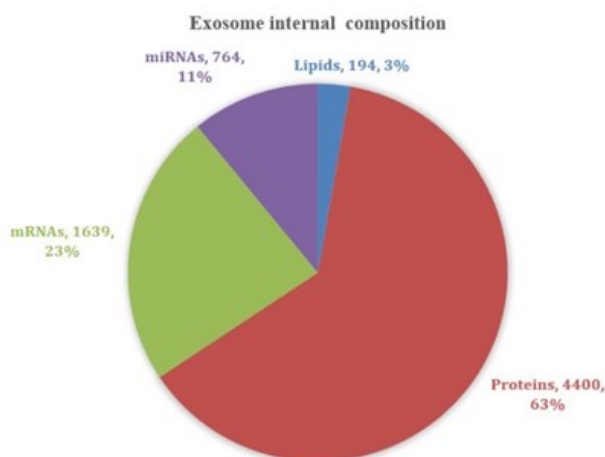


Fig. 1: Important biomolecules reported in exosomes

Different methods of exosome extraction

To study structure, function, mechanism, bio molecular composition and other different phenomena, exosomes must be extracted from any of the biological fluid in high quantity and quality depending upon the experiments. Till now a number of methods are optimized and used.

Centrifugation

Exosomes are isolated via sequential centrifugation in research studies. The methodology involves low speed

centrifugation (twice or thrice) to remove cellular debris and cells. The isolates are then subjected to ultrafiltration with 0.2 micrometre filter and then ultracentrifugation at 100,000g (60-120min) and then pellet is washed with 1x phosphate buffer saline (PBS) and again centrifuged at 100,000g (90 min) (Théry, Amigorena *et al.*, 2006). The method does have a drawback that it does not give 100% pure exosomes. This is because of the fact that vesicles similar in size to exosomes can be isolated with exosomes in centrifugation process. Since pellet obtained at 100,000g can be divided into several groups via floatation in iodixanol gradients and also by immune-isolation (via usage of beads coated with immunoglobulins targeting CD9, CD63 or CD81), hence the small vesicles isolated at 100,000g pellet are called small extra cellular vesicles (SEVs) instead of exosomes. Furthermore, if exosomes are present in serum with lipid droplets and lipo-particles, at ultracentrifugation, these particles will be isolated along with exosomes (Skotland, Sandvig *et al.*, 2017). The rotor capacity also limits the throughput of ultracentrifugation. The most commonly used method for exosome isolation is differential centrifugation which yields isolates enriched with exosomes. Comparatively the purest exosome population can be isolated from density gradient centrifugation. The drawback includes time consumption and low yields (Kowal, Arras *et al.*, 2016).

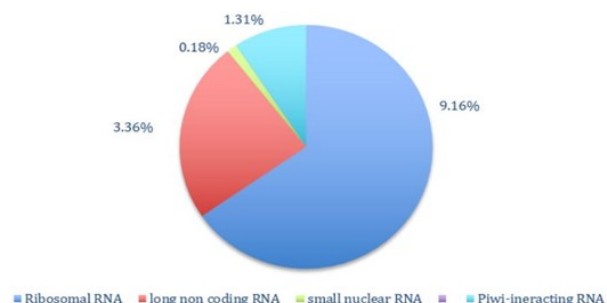


Fig. 2: Various RNA species and their respective concentration in exosomes.

Immuno-Isolation

Pure subpopulations of EVs (extracellular vesicles) can be obtained via immune-isolation which involves use of specific proteins. The process however requires optimization of protocol and careful selection of protein membranes. Using immune affinity exosome capture (from colon cancer cell lines) was reported to be more efficient as compared with density gradient centrifugation or ultracentrifugation (Kowal, Arras *et al.*, 2016).

Surface plasmon resonance

Exosome quantification can be done via collection of exosomes (using an immune-functionalized surface) followed by measurement of variation in refractive index. This technique has a promising potential for marker based isolation of exosomes (e.g.CD63 or proteins involved in ontogenesis) (Sina, Vaidyanathan *et al.*, 2016).

Nano sight for quantification of Exosomes

Exosomes separation and quantification is performed by using Nanosight machine through light diffraction mechanism to analyse concentration and size of nanoparticles. An alternate method is qNano Gold (Izon Science) which works on the principle of Tunable Resistive Pulse Sensing (TRPS). These types of techniques have the drawbacks of uncertainty for the presence of exosomes because these can identify all nanoparticles regardless exosomes e.g. plasma isolated exosomes may have IgG or albumin as contaminants which also stay in range of the exosomes manipulating the results (Lane, Korbie *et al.*, 2015).

Exosome as biomarker for prognosis and diagnosis

In the last few years, exosomes have been detected in numerous body fluids including blood, urine, Saliva, ascites, amniotic fluid, breast milk and semen. Since these exosomes carry specific biomolecules (derived from cell), they give the idea about physiological condition of donor cell and can therefore be used for detection of various diseases. The exosomes can be used as biomarkers because they vary in composition and cargo package in accordance with the donor cell. Various pathological conditions can be identified via exosomes e.g. Alcoholic liver disease (miR-122 and miR-155) (Yagi, Ohkubo *et al.*, 2017), ovarian cancer (miR-21, miR-141, miR-203, miR-205, miR-214, miR-200a, miR-200b, ADAM 10, CD24, L1CAM, EMMPRIN and claudin) breast cancer (miR-141 and miR195) prostate cancer (miR-16, miR-34b, miR-92a, miR-92b, miR-103, miR-107, miR-197, miR-328, miR-485-3p, miR-486-5p, miR-574-3p, miR-636, miR-640, miR-766 and miR-885-5p), nephrotic syndrome (Nephrilysin, aquaporin-2 and podocalyxin) (Rood, Deegens *et al.*, 2010), renal ischemia reperfusion (Aquaporin-1), acute kidney injury (Fetuin-a), bladder cancer (EGF receptor pathway proteins, retinoic acid-induced protein 3, α -subunit of GsGTP-binding protein and resistin) etc. While biological fluids are easy to procure and have high levels of exosomes, the exosomes as biomarkers has not widely been put to practice. (Sonoda, Yokota-Ikeda *et al.*, 2009)

Exosome as drug delivery system

Exosomes can be used as potential carriers for therapeutics having the ability to target specific tissues or cells, are excellent vehicles for drug delivery (Crenshaw, Sims *et al.*, 2018). They can cause horizontal gene transfer thereby leading to modified recipient cells. Moreover, exosomes can easily be obtained from patient's body fluids or tissues and therefore have splendid biocompatibility & bio delivery. This allows the exosomes to evade immune response (Natasha, Gundogan *et al.*, 2014). The problem of immunogenicity can hence be avoided and cargo (therapeutically drug) can be delivered without any toxicity or clearance. The idea of delivering drugs via exosomal vehicles across numerous

biological constraints, has gained popularity and are therefore an attractive centre piece for research. A variety of therapeutic agents (siRNAs, r-proteins, antagomirs, anti-inflammatory drugs etc.) may be delivered via exosomes. The exosome mediated delivery may be through various approaches 1) packaging the drug after isolation of exosome in ex vivo from donor cells, 2) transformation of cell with drug encoding DNA, this can be expressed and packaged in exosomes, 3) encapsulating donor cells in drug which will be packaged in exosomes during exosome biogenesis (Batrakova and Kim, 2015). In one of primary reports, exosomes derived from peripheral blood were used to silence MAPK gene in monocytes and lymphocytes. The exosomes cargo was exogenous siRNAs. Exosomes are also potential candidates for Huntington's therapy. In a study conducted on mice, the huntingtin mRNA was targeted via exosomes carrying siRNAs. Huntington RNA and protein were hence silenced (Didiot, Hall *et al.*, 2016). Exosomes derived from endothelial cells of brain were able to transport VEGA-siRNA across blood brain barrier in zebra fish and reduced the fluorescence labelled cancerous cells' intensity. A significant reduction in osteosarcoma cells migration was observed when exosomes with miR-143 was used. Dex are exosomes derived from dendritic cells are extensively being studied in recent years. Dex (pulsed with peptides of tumour), are being investigated as cancer vaccines which are cell free (Syn, Wang *et al.*, 2017). Dex have MHC (class I & II), costimulatory adhesion molecules which enhance their function in vivo. Research showed that Dex have the ability to induce cytolysis in tumour cells (Escudier, Dorval *et al.*, 2005). Dex remains a potential strategy for cancer treatment and requires further research.

CONCLUSION

In last few years, exosomes have been a centre of research due to their various biological features. These are secreted by various cell types and exist in all kinds of body fluids. They play a critical role in physiological and pathological processes. The ability of exosome to package a variety of drugs as cargo and specific delivery to target sites has made exosomes a potential candidate for therapeutics. The isolation of exosomes can be standardized to ensure feasibility. Clinical application of exosomes is still in its early stages. More research needs to be done to successfully combat cancer and other disorders via exosomes. Data based on research strongly suggests that exosomes have a significant role in medicine in future because these are vectors for wide range of molecules including proteins, nucleic acids, chemicals etc. Promising therapeutic effects were observed in mouse AD model for gene silencing via miRNAs encasing exosomes (Alvarez-Erviti, Seow *et al.*, 2011). Since exosomes sometimes contain disease biomarkers or potentially useful vectors for drug delivery, they are perfect for

therapeutic purposes (Hood and Wickline, 2012). They can allow early detection of disease as well as its monitoring, and carry minimal side effects if used as vectors. Since exosomes play a significant role in many mechanisms and pathways of the body as discussed in this review, they can be used as all in one approach to combat obstacles in therapeutics. From the current knowledge of the applications of exosomes as biomarkers, tools for drug delivery, tools to prevent the proliferation of tumors, as a treatment for arthritis and their use in regenerative medicine, strongly supports that exosomes have therapeutic value and can play a vital role in activating and treating many diseases. However, there are some limitations in the isolation and purification of exosomes, and their exact mechanism of action still needs to be validated. These challenges need to be addressed before exosomes can proceed to clinical application. In the future, detailed characterization is required to define the various sub-populations of exosomes. Available evidences supporting the use of exosomes as novel therapeutic agents, but these will need to be clinically validated.

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