Milestones in Research of Small Cellular Particles. Membrane Modeling and Theoretical Description in Connection with *In vitro* and *Ex vivo* processes

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Abstract:
Sub-micron sized small cellular particles (SCPs) (microparticles, microvesicles, exosomes, extracellular vesicles, extracellular particles) are recently gaining attention due to their potential role in all fields involving living organisms (e.g. medicine, food science, agriculture, ecology). Due to their small size and dynamic nature, their properties and biological roles are not yet understood up to a level to be widely used and remain potentially important. Employing the knowledge from different fields is highly warranted to progress towards better understanding of physiological and patophysiological processes based on SCPs. This contribution outlines four milestones in SCP research: M1: Model of the biological membrane, M2: Membrane budding and vesiculation, M3: Theoretical description of the membrane shape and M4: Transferrin release from the reticulocyte membrane in the form of membrane – enclosed SCPs. Also we outline the increasing interest of the scientific society for SCPs reflected in highly cited papers and present a synthesis of the theoretical and experimental view on membrane-enclosed SCPs within the fluid crystal mosaic model of the membrane.

Keywords: Extracellular vesicles; Extracellular particles; Extracellular vesicles; Small cellular particles; Microvesicles; Exosomes

1. Milestone 1: Model of biological membrane

In 1972, a paper was published by Singer and Nicolson (1972) following thorough study of biological membrane thermodynamics and proposing the Fluid mosaic model. According to this model, the membrane is composed of a lipid bilayer with embedded proteins that are integral to the membrane (**Figure 1A**). The proteins are arranged with the ionic and highly polar groups protruding from the membrane into the aqueous phase, and the nonpolar groups largely buried in the hydrophobic interior of the membrane. The proteins are more or less free to move laterally over the membrane analogously to a two-dimensional solution of integral proteins (or lipoproteins) in bilayer solvent. In the next 50 years the agreement of this model with experimental results established it as a base for the description of membrane features. However, already in the paper (Singer and Nicolson, 1972), it was suggested that direct interactions between the embedded entities may take place and may have also consequences for biological features involving the membrane. Lateral segregation of the protein molecules was observed in connection with the pinocytosis of the membrane – endovesiculation - which refers to the uptake of substances by a cell. This concept was later further developed and reported in Simons and Ikonen (1997) and Brown and London (1998). Simons and Ikonen (1997) presented a model based on the dynamic clustering of sphingolipids and cholesterol to form rafts (**Figure 1B**) that move laterally within the fluid bilayer and function as platforms for the attachment of proteins. Furthermore, lateral segregation into rafts was connected with membrane curvature within the entities called calveolae. It was suggested that spingolipid–cholesterol rafts may be an essential feature of all organellar membranes involved in biosynthetic and endocytic traffic. Brown and London (1998) further up-graded the model by considering order within the particular regions in the lipid bilayer.

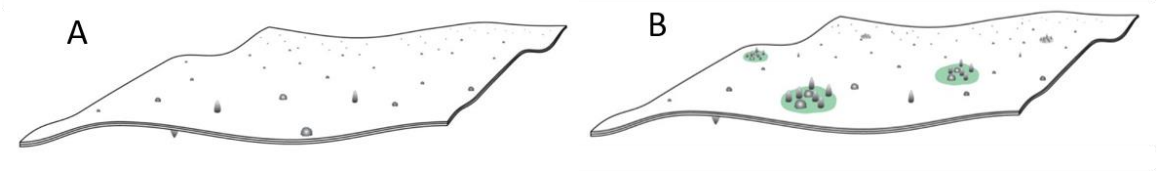

Figure 1. Illustration of the fluid mosaic model of the membrane in its original form (A) and by considering formation of membrane rafts (B). Adapted from Kralj-Iglič (2012).

2. Milestone 2: Membrane budding and vesiculation

Erythrocytes are convenient for the study of the properties of the cell membrane. Mature cells do not have nucleus nor cytoskeleton. Their shape is largely determined by the properties of the membrane (lipid bilayer with underlying membrane skeleton). The process of microvesiculation (**Figure 2A**) and the pinched-off vesicles (**Figure 2B**) were imaged and described (**Figure 3**) by Weed and Reed (1966).

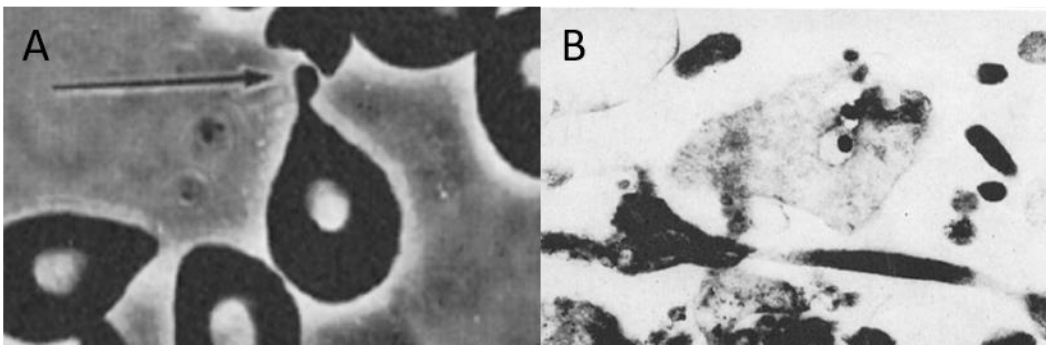

Figure 2. A: Budding of the erythrocyte. B: Electron micrograph of "fragments". Panel B has magnification 14000 X. Adapted from Weed and Reed (1966).

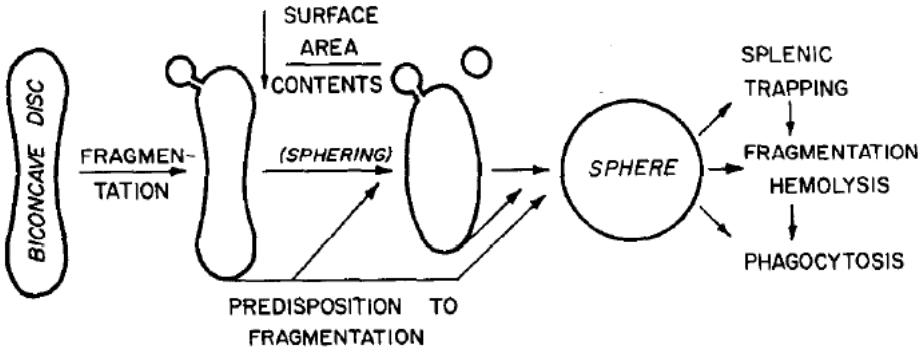

Figure 3. Scheme of erythrocyte membrane fragmentation as presented by Weed and Reed (1966).

In 1967, SCPs were identified as fragments shed from platelets (Wolf, 1967). Already then, their **potential** biological role was indicated (Wolf, 1967). Later it was observed that blood contains a mixture of young and old erythrocytes which differ in the size, density and surface charge (Lutz et al., 1977). It was suggested that microvesiculation is responsible for the loss of the erythrocyte membrane and therefore increase of the volume to area ratio is reflected in senescent rounding of erythrocytes (Lutz et al., 1977). In the following decades, erythrocyte shape change, hemolysis and vesiculation were thoroughly studied experimentally (Nelson et al., 1983, Isomaa, 1979, Isomaa et al., 1987, Hagerstrand et al., 1992). It was found that the erythrocyte membrane may bud inward or outward and if this process continues, it terminates in vesiculation (Isomaa et al., 1987, Hagerstrand et al., 1992). Procedures were developed to isolate and image microvesicles (Isomaa st al., 1987, Hagerstrand et al., 1992). It was found that the shape of the buds and of SCPs may vary depending on the added compounds that intercalate into the membrane (**Figure 4**).

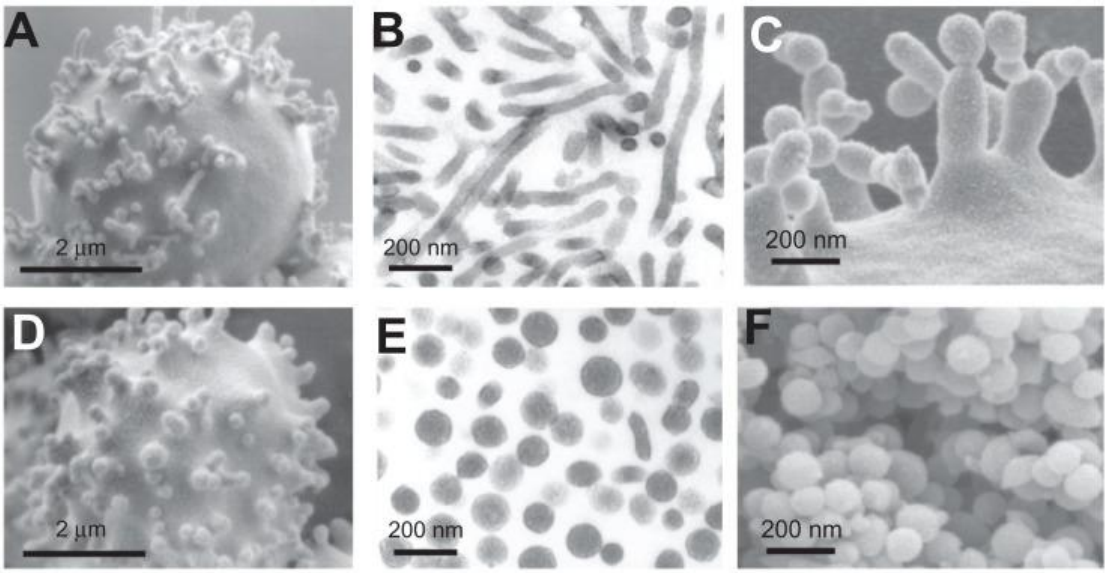

Figure 4. Budding of erythrocytes (panels A, C and D) and isolated microvesicles (panels B, E and F). The process was induced by adding amphiphilic molecules into the erythrocyte suspension (A,B: dodecyl maltoside; D,E,F: dodecyl zwittergent). A, C, D and F: scanning electron micrographs; B and E: transmission electron micrographs. Adapted from (Schara et al., 2009).

Budding and vesiculation of nano to micro – sized particles were detected and observed in different biological systems. **Figure 5** shows budding and vesiculation in a microalgae *Ochromonas danica*. Assessment and visualization of SCPs in different samples led to the conclusion that cells of all types are prone to shed their fragments in the surrounding solution (Yanez-Mo et al., 2015). Being freed from the mother cell, they can travel with surrounding liquid, reach nearby or distant cells and interact with them.

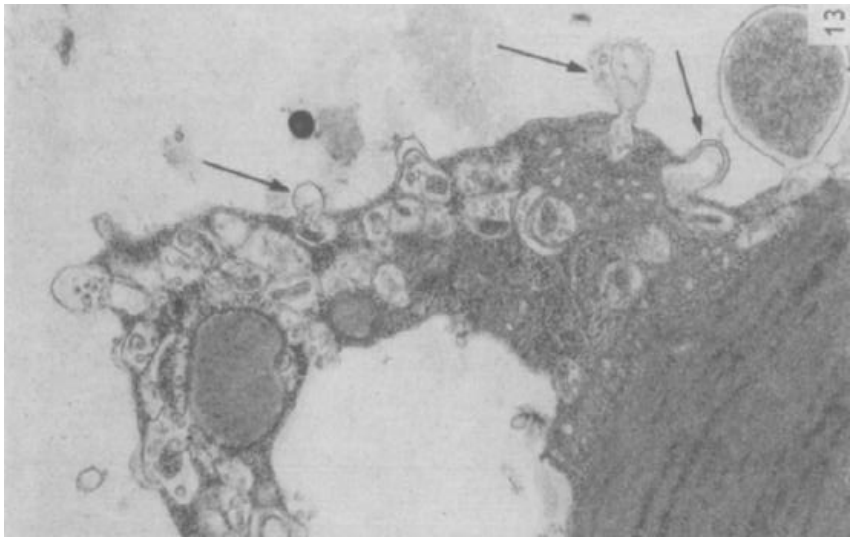

Figure 5. SCPs emerging from the surface of the cell membrane of a 9-day-old *Ochromonas danica* cell; see arrows. Adapted from (Aaronson et al., 1971).

3. Milestone 3: Theoretical description of the bilayer membrane shape

Erythrocyte shape was described theoretically (Canham, 1970) by considering the membrane as a thin, locally slightly curved elastic shell. The contour of the rotationally symmetric shape was approximated by Cassini ovals and the bending energy of the shell was minimized at chosen geometrical constraints by determining the parameters of the ansatz (Canham, 1970). Good agreement was obtained between calculated shapes and shapes observed under the optical microscope (Canham, 1970). Theoretically advanced expression for the membrane free energy including the assumption that the membrane has spontaneous curvature was presented by Helfrich (1973). The description was improved by stating the variational problem for minimization of the free energy by a set of Euler-Lagrange differential equations (Deuling and Helfrich, 1976). This enabled a rigorous solution of the set of differential equations for the axisymmetric shapes. The calculated shapes shown in **Figure 6** (Kralj-Iglič et al., 2022) were obtained by using this method.

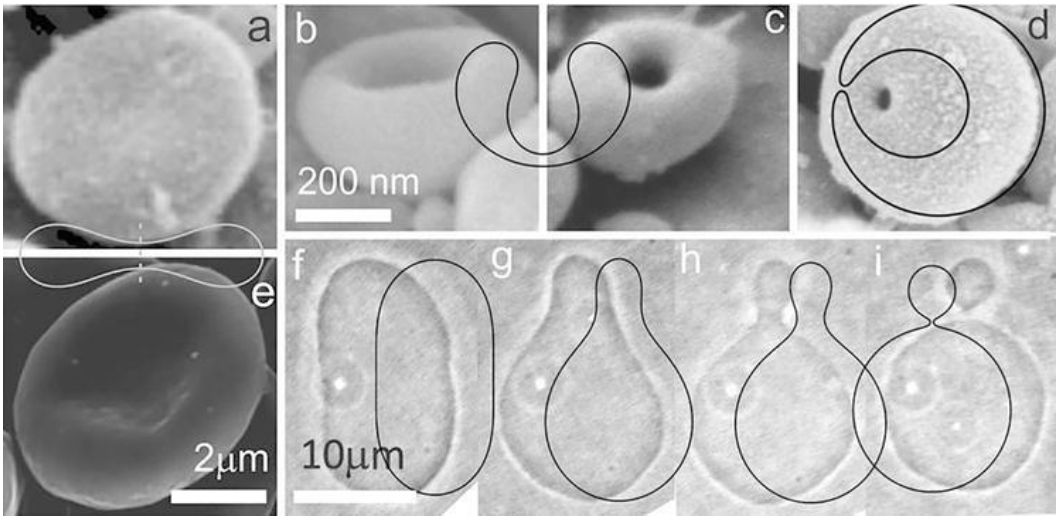

Figure 6. Observed and calculated membrane shapes corresponding to a sequence leading to formation of internal vesicle (endovesiculation) in SCPs found in isolates from blood and to a sequence leading to formation of external vesicle (exovesiculation) in a giant phospholipid vesicle. The theoretical shapes were taken as axisymmetric with respect to the vertical axis and were calculated by solving the variational problem presented as a system of the Euler-Lagrange differential equations. Note the relevance of the model over 100 fold size difference. From Kralj-Iglič et al., (2022).

Bilayer couple principle considering that the two membrane layers are in close contact but can slide over each other (Sheetz and Singer, 1974) was implemented to explain intercalation of compounds into a particular lipid layer (Helfrich, 1974, Evans, 1974) thereby changing average mean curvature of the membrane. The models were theoretically elaborated to yield the equilibrium membrane shape by minimization of the membrane free energy (reviewed by Seifert, 1997). Membrane budding and vesiculation were described by an increase (in the case of exovesiculation) or a decrease (in the case of endovesiculation) of the average mean curvature of the membrane. The pinching off of the bud was preceeded by the thinning of the neck connecting the bud and the mother membrane. When the bud is pinching off, the opening in the membrane is very small and can be sealed with little energy change by rearranging the adjacent molecules.

In considering the models of the membrane it was taken into account that besides lateral redistribution and segregation the anisotropic membrane constituents may undergo orientational ordering with respect to the axis perpendicular to the membrane. This introduced curvature deviator as an important parameter (Fischer, 1992; Kralj-Iglič et al., 1999). Changing of the constraints and/or parameters (e.g. relative volume (expressing volume to area ratio v, average mean curvature <h>, average curvature deviator <d>) yields different shapes of membrane-enclosed entities without internal structure. A (v, <h>, <d>) phase diagram featuring some shapes corresponding to the minimal free energy is presented in **Figure 7**. Regions are defined within limiting shapes composed of spheres, cylinders, tori and flat regions. Shapes composed of spheres lie in the <d>=0 region. Red, blue and green lines indicate transformations of shapes due to changes in v, <h> and <d>. Endo and exo vesiculation (**Figure 6**) are presented by the blue and red – marked sequences, respectively.

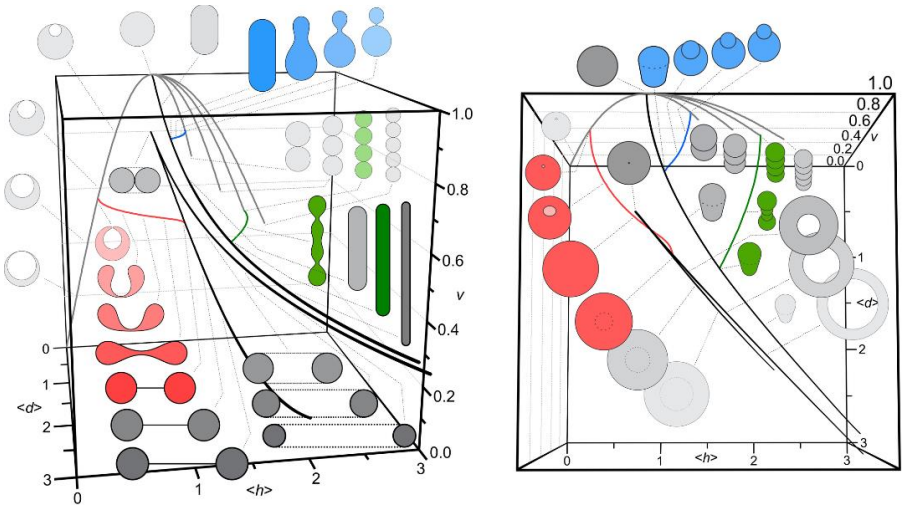

Figure 7. A (v, <h>, <d>) phase diagram of shapes calculated by minimization of the membrane free energy. Two aspects (side view (left) and top view (right)) are shown. From (Kralj-Iglič et al., 2020).

Taking into consideration orientational ordering of anisotropic membrane constituents indicated stability of thin (below cca 100 nm) tubular vesicles (Kralj-Iglič et al., 2002) and required revision of the model of the membrane (Kralj-Iglič, 2012). As these effects were similar to the ones observed in liquid crystals, the up-graded model which becomes relevant in nano-sized anisotropic membrane regions was called the Fluid crystal mosaic model (**Figure 8**).

Figure 8. Illustration of the fluid crystal mosaic model of the membrane. Adapted from Kralj-Iglič (2012).

Milestone 4: Transferrin release from the reticulocyte membrane in the form of membrane – enclosed SCPs.

While internal structures filled with SCPs were observed already in 1971 (Coons and Axtell, 1971) (**Figure 8A**), the importance of this feature was revealed in the study of transferrin release from maturing reticulocytes (Harding et al., 1983; Pan and Johnstone, 1983). It was suggested and supported by measurements and observations that transferrin was released in the form of small vesicles that are formed in invaginated compartments of reticulocytes (**Figure 8B**). These results were considered as a base for the general mechanism of formation of »exosomes« - vesicles that form within internal compartments of the cell and are eventually released into the surrounding solution.

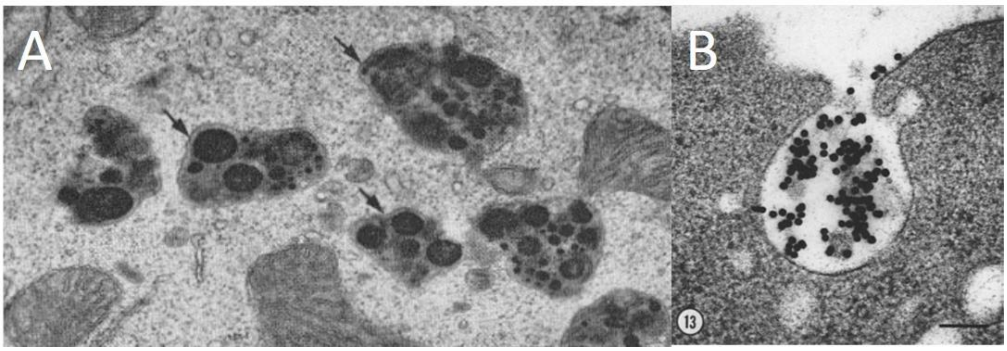

Figure 8. A: Cross-section of an excretory tube cell of the mesostigmatid mite *Macrocheles muscaedomesticae* showing a group of lysosomes (arrows). Each is bounded by a single membrane and contains many round bodies of varying degrees of size and electron density. X 20,000. Adapted from (Coons and Axtell, 1971). B: Release of SCPs labelled with gold (black dots) from the invagination of an unfixed reticulocyte. The cell was incubated for 30 min with AuTf, subjected to a 20-min chase with unconjugated transferrin, and then quick-frozen without prior fixation and freeze-substituted. Bar, 200 rim. X 61,000. Adapted from (Harding, 1983).

Notorious SCPs

Although the existence of SCPs has been acknowledged, their roles and underlying mechanisms are not yet completely understood and are a subject of extensive study. As SCPs were discovered in different samples by scientists from different fields, they were called different names. International Society for Extracellular Vesicles (ISEV) is coordinating effort to nominate SCPs and impose quality requirements on experimental procedures. For his purpose ISEV has issued several »position papers«. Some of these works have become highly cited. We have browsed Google Scholar database for papers using keywords »extracellular vesicles«, »microparticles«, »microvesicles« and »exosomes« and outlined papers cited more than 2000 times in **Table 1.** It can be seen that the majority of highly cited papers are reviews, however the one listed with the highest number of citations in **Table 1** is the ISEV position paper (Thery et al., 2018). Recently, the requirements were updated in a new publication MISEV2023 (Welsh et al., 2024) with contribution of over 1000 ISEV members. The goal of this paper is to provide »snapshot of available approaches and their advantages and limitations for production, separation and characterisation of EVs from multiple sources, including cell culture, body fluids and solid tissues«. As SCP-based mechanisms are very basic and relevant in all living systems, new SCP research areas are emerging. As the biological roles of SCPs are acknowledged, their better understanding is highly waranted. However, further efforts are needed to accomplish this goal and achieve breakthrough of SCP-based methods in everyday practice. Model approach and theoretical work is therefore an important element in SCP research. For comparison, **Table 2** shows a list of highly cited papers on membrane models and theoretical descriptions and also some milestone papers referred to in this work.

Table 1. Highly cited papers on cellular fragments found in first 20 pages of Google Scholar database by keywords »extracellular vesicles«, »microparticles«, »microvesicles« and »exosomes« subject to more than 2000 citations.

Paper	N	Expression browsed	Journal	Type of the paper
Thery et al., 2018	8043	Extracellular vesicles	J Extracellular Vesicles	ISEV Position paper
Raposo and Stoorvogel, 2013	8027	Exosomes/microvesicles	J Cell Biol	Review
Van Niel et al., 2018	5864	Extracellular vesicles	Nature Rev Mol Cel Biol	Review
Thery et al., 2006	5821	Exosomes	Curr Prot Cell Biol	Research
Colombo et al., 2014	5639	Exosomes/Extracellular Vesicles	Ann Rev Cell Develop Biol	Review
Thery et al., 2002	5624	Exosomes	Nature Reviews Immunology	Review
Kalluri and LeBleu, 2020	5503	Exosomes	Science	Review
Skog et al., 2008	5481	Microvesicles	Nature Cell Biology	Review
Yanez Mo et al., 2015	4887	Extracellular vesicles	J Extracellular Vesicles	Review
Johnstone et al., 1987	3217	Vesicles	J Biol Chem	Research
Tkach and Thery, 2016	3001	Extracellular vesicles	Cell	Review
El Andalousi et al., 2013	2985	Extracellular vesicles	Nature Reviews Drug Discovery	Research
Mathivanan et al., 2010	2728	Exosomes	J Proteomics	Review
Lotvall et al., 2014	2633	Extracellular vesicles	J Extracellular Vesicles	ISEV Position paper
Costa Silva et al., 2015	2548	Exosomes	Nature Cell Biology	Research
Mulcahy et al., 2014	2468	Extracellular vesicles	J Extracell Vesicles	Review
Pisitkun et al., 2004	2438	Exosomes	Proc Nat Acad Sci	Research
Gyorgy et al., 2011	2305	Extracellular vesicles	Cell Mol Life Sci	Review
Witwer et al., 2013	2251	Extracellular vesicles	J Extracellular Vesicles	ISEV position paper
Al Nedawi et al., 2008	2275	Microvesicles	Nature Cell Biology	
Doyle and Wang, 2019	2212	Extracellular vesicles	MDPI Cells	Review
Vlassov et al., 2012	2192	Exosomes	Biochim Biophys Acta	Review
Pan and Johnstone, 1983	2166	Vesicles	Cell	Research
Cocucci et al., 2009	2141	Exosomes		
Robbins and Morelli, 2014	2121	Extracellular vesicles	Nature Reviews Immunology	Review

N: number of citations on March 19, 2024.

Table 2. Selected milestone papers.

Paper	N	The scope	Journal	Type of the paper
Singer and Nicolson, 1972	13186	Membrane model	Science	Research
Simons and Ikonen, 1997	12015	Membrane model	Nature	Research
Helfrich, 1973	7186	Membrane shape	Z Naturforschung	Theory
Brown and London, 1998	3908	Membrane model	Ann Rev Cell Develop Biol	Research
Seifert U,1997	2015	Membrane shape	Advances in Physics	Review
Canham, 1970	2005	Red blood cell shape	J Theor Biol	Theory
Wolf, 1967	1999	Platelet dust	Br J Haematol	Research
Harding et al., 1983	1988	Vesicles and tubules	J Cell Biol	Research
Sheetz and Singer, 1974	1940	Membrane model	Proc Nat Acad Sci	Research
Weed and Reed, 1966	268	Cell fragments	Am J Med	Research

N: number of citations on March 19, 2024.

Conclusions

Biological roles of SCPs inspire great effort of the scientific community to fulfill the potential effects and uses of SCPs in health improvement of living organisms. As SCPs are tiny and dynamic entities, their harvesting, assessment and manipulation presents a challenge. Modeling and theoretical description are powerful tools which should be included in consideration of SCPs.

Acknowledgement

Due to increasing interest in SCPs and large number of published works we are aware that some important works may not have been included in the survey.

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Conflicts of Interest: The authors declare no conflict of interest.

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