

# 1 Full Artificial Exosomes: Towards New Theranostic Biomaterials

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## 10 Abstract:

11 Bio-nanotechnology routes have been recently developed to produce Full Artificial  
12 Exosomes: biomimetic particles aiming to overcome certain limitations regarding  
13 Extracellular Vesicles biology and manipulation. These particles could become true  
14 therapeutic biomaterials in the near future. Here, we outline the current preparation  
15 techniques, their explored and future possibilities and their present limits.

## 16 Keywords:

17 Extracellular Vesicles, Artificial Exosomes, Biomimetic Material, Bio-nanotechnology,  
18 Nanomedicine

## 19 Extracellular Vesicles: The revolution of cell biology and physiology (365)

20 The last decade represent a revolution in our knowledge about human body  
21 homeostasis based on cell communication. Advances in our comprehension of the  
22 development and expansion of several pathologies are greatly due to the  
23 understanding of Extracellular Vesicles (EVs) biological behaviour, with special focus on  
24 Exosomes [1].

25 The increasing amounts of data about composition, biogenesis, and roles of exosomes  
26 in physiological and several pathologies have opened new possibilities in diagnosis  
27 and therapy [2]. Exosomes have unique characteristics that emerge from their cellular  
28 origin. These confer them a high value as new biomarkers for diagnosis, stratification

29 and plannification/evaluation of treatment efficacy. They represent the access to a set  
30 of molecules with information about their origin cell and its status through all the  
31 lipids, proteins and nucleic acids travelling in both, the membrane and cargo. The  
32 opportunity to get all these valuable cellular info and the fact that it comes also from  
33 hard-to reach tissues, has promoted the term *liquid-biopsy*, a new frontier in the  
34 clinical field.

35 On the other hand, exosomes combine the advantages of both nanocarriers, (particles  
36 for the efficient delivery of molecules), and therapy agents. Nowadays, they are  
37 considered as the most promising drug delivery systems, especially for gene therapy in  
38 different disorders (such as genetic deficiencies or anti-tumour progression) [3]. This  
39 attention is noticed in several papers published during the last 5 years, regarding the  
40 modification of targeting moieties and/or the encapsulation of endogenous and  
41 exogenous material during exosomes pre- or post-isolation from cell cultures. These  
42 represent the development of so-called Exosome-based Semi-Synthetic Nanovesicles,  
43 a subtype of artificial exosomes, which englobe all exosomes with modification for  
44 intended purposes. They have been even tested for autologous therapy.

45 Besides the great explosion of techniques for semi-synthetic exosomes development,  
46 the main drawbacks concerning their clinical applications are the production, isolation,  
47 modification, and purification at large-scale clinical grade. This need is the driving force  
48 at the search for a new perspective: the design and manufacture of full synthetic  
49 exosomes mimetic particles based on Bio-nanotechnology. The following sections are  
50 an overview of these techniques, presented as the two trends in nanofabrication: the  
51 Top-Down and Bottom-Up approaches (see **figure 1**).

## 52 **Top-Down methodologies: Bioengineering Cells as membrane fragments precursors** 53 **(378)**

54 Top-Down methodologies relies on the production of nanosized material by  
55 fragmentation of bigger and more complex units to smaller products. In this case,  
56 production of artificial exosomes starts from cultured cells. Different methodologies  
57 based on this approach have been developed, mainly for drug delivery, but also for cell

58 proliferation enhancement and for the generation of exosome mimetic models applied  
59 to bio-distribution analysis (**table 1**).

60 Extrusion over polycarbonate membrane filters is a common practice to reduce and  
61 homogenize size distribution of colloidal systems. Applying this technique to cultured  
62 cells, NVs for the treatment of tumours by targeted encapsulated chemotherapeutics  
63 have been developed with a simple commercial liposome extruder and diminishing  
64 pore size filters [4,5]. Moreover, a scaled-up version was developed with a device  
65 designed to be used with conventional lab centrifuges [6]. Mass production of NVs  
66 with this device was applied in a study for cell proliferation enhancement [7].

67 The use of microfluidics devices is also reported for artificial exosomes preparation. A  
68 simple pressurization over an array of parallel hydrophilic microchannels-based  
69 device was described for the production of NVs aimed to endogenous RNA delivery to  
70 targeted cell cultures [8]. The fabrication of microchannels on micro-blades (fabricated  
71 in silicon nitride) resulted on a device able to slice living cells during their flowing  
72 through the channels [9]. The incorporation of exogenous material to the cells  
73 suspension enhanced their encapsulation by plasma membrane fragments during re-  
74 assembling, and described a method for *in vitro* exogenous material delivery.

75 All these methods are suitable for the production of higher amounts of effective  
76 particles in comparison to natural exosome release yield (over 250 fold times larger).  
77 Sizing and biochemical profiling of NVs had high similarities with exosomes. Their  
78 ability to exhibit receptors and co-receptors, essential to target and produce effective  
79 interactions with receptor cells populations, is of special relevance. And, of course,  
80 their natural origin from cells confers them immunotolerance.

81 Beside these positive characteristics, these methods have some drawbacks. Cargo  
82 sorting lacks of selectivity due to passive encapsulation of surrounding medium during  
83 self-assembly of membrane fragments. Another relevant issue regarding the  
84 production of these mimetic particles is the need of final purification steps identical  
85 to those used for exosome isolation, which requires trained personal and are time-  
86 consuming.

87 **Bottom-Up techniques: mimicking plasma membrane through the preparation of**  
88 **artificial bilayers (410)**

89 Bottom-Up techniques, on the other hand, create complex structures of higher order  
90 by the manipulation of physical and chemical properties of some molecules  
91 (supramolecular chemistry). These methods are well known in the cosmetic and  
92 pharmaceutical industry by the preparation of liposomes, particles formed by a  
93 bilayered structure of lipids that resemble the plasma membrane. This connection is  
94 the starting point to design and create artificial exosomes: the preparation of a  
95 synthetic bilayer that is then functionalized with selected proteins to mimic desired  
96 exosomal functions.

97 From the diverse methods for the preparation of liposomes, one of the more  
98 employed in the development of mimetic NVs is the Thin Film Hydration Method  
99 (TFHM). TFHM is a two-steps process where a dried film of lipids is hydrated by an  
100 aqueous media with the compounds to be encapsulated. Artificial exosomes have  
101 been produced with a classical liposome formulation [10] and a lipid composition  
102 simulating the exosomal one [11]. By chemistry-based bio-conjugation procedures,  
103 MHC Class I/peptide complexes and ligand involved in T-Cell receptor interactions and  
104 activation have been attached to vesicles for immunotherapy (*ex vivo* and *in vivo* cell  
105 expansion). Also the incorporation of APO2L/TRIAL for induction of apoptosis and  
106 down-regulation of T-Cell activation in autoimmune diseases, such as antigen-induced  
107 arthritis, was reported [11]. These NVs were evaluated in the treatment of  
108 hematologic tumour cells [12].

109 More recently, a different method based on micro-emulsification and micelle  
110 assembling was described for the encapsulation of BSA as a model to simulate artificial  
111 antigen-presentation to Dendritic Cells [13]. To specifically target DCs, a monoclonal  
112 antibody against DEC205 (a highly expressed receptor on the surface of DCs that  
113 facilitate endocytosis) was selected.

114 The main advantage of this strategy relies on the production of a high pharmaceutical  
115 grade product, since the final composition is fixed by the selected formulation.  
116 However, publications on this topic are still scarce. Besides, since these are methods

117 adapted from the conventional routes of liposome production of liposomes, expensive  
118 high purity lipids are required (especially if functionalization with proteins is going to  
119 be carried out). The attachment of multiple molecules to the NVs is also challenging,  
120 since conjugation procedures requires stable and specific conditions [12].

121 On the other hand, encapsulation of nucleic acids is a challenge process that deals with  
122 a very unstable type of molecules, and seems that the best encapsulation efficiencies  
123 are obtained with cationic lipids, more immunogenic than regular counterparts [13].

#### 124 **Concluding remarks and future perspectives (259)**

125 The incipient recent success in basic and clinical studies by using full artificial exosomes  
126 has created the basis of the future nanomedicine. The multidisciplinary approach with  
127 contributions from molecular biology, engineering, biotechnology and chemistry will  
128 be essential to overcome the limits present in up-today methods. Further research  
129 work is necessary to improve the methods of production, but the solution seems to  
130 pass through the combination of techniques designed with both approaches: semi-  
131 and full synthetic artificial exosomes.

132 Techniques for the modification of cells prior to exosome isolation, could be perfectly  
133 coupled to any Top-Down method in order to tailor artificial exosomes with  
134 complementary elements to those natural presented in selected donor cells. Possibly,  
135 these could also be used to enhance the physical stability of generated products, a not  
136 fully studied property.

137 Regarding Bottom-Up techniques, microfluidics, a really versatile platform for particle  
138 production and drugs encapsulation, represent a very promising approach. This  
139 enables rapid testing of multiples formulation thanks to the quickness in product  
140 generation, but also with the consuming of significantly less amount of chemicals. In  
141 that direction, alternatives to lipids may be explored. Currently, our research group is  
142 testing the possibilities of niosomes (vesicles formulated with Non-ionic surfactants) as  
143 an alternative to lipid-based particles in the development of artificial exosomes. The  
144 main advantages of these vesicles are their wide spectra of starting compounds, more  
145 economical, better physical and chemical stability, and high grade of biocompatibility .  
146 As new productions routes are improved, novel nanovesicles closer to real exosomes

147 will be available, making possible personal nanomedicine and theranostics agents  
148 adapted to particular needs.

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177 **Table 1.** Full artificial exosomes published works based on Top-Down and Bottom-Up Bio-nanotechnology.

<b>Works based on Top-Down approach</b>				
<b>Mechanism of membrane fragmentation</b>	<b>Precursor Cell lines</b>	<b>Type and examples of material incorporated</b>	<b>Application</b>	<b>Reference</b>
Manual extrusion over polycarbonate membrane filters with a device for liposome preparation	Human Monocytes (U937) and Murine mouse Macrophages (Raw 264.7)	Exogenous, chemotherapeutic drugs (Doxorubicin, 5-FU, gemcitabine and carboplatin)	Targeted delivery of chemotherapeutics to an <i>in vitro</i> model (TNF $\alpha$ - treated HUVEC) and <i>in vivo</i> induced malignant tumours (CT26 mouse colon adenocarcinoma cells)	[4]
	Murine mouse Macrophages (Raw 264.7)	Exogenous, Radiolabelling agent <sup>99m</sup> Tc-HMPAO	<i>In vivo</i> bio-distribution of exosomes and artificial counterparts	[5]
Centrifugal-induced extrusion over membrane filters in a device designed to be used in lab centrifuges	Murine mouse embryonic stem cell line-D3	Endogenous, Precursor cells characteristic RNA (mOct 3/4 and mNanog)	Gene delivery to NIH-3T3 fibroblast cells	[6]
	Murine mouse embryonic stem cell line-D3	No intention to encapsulate any specific compounds	Enhance <i>in vitro</i> cell proliferation for regenerative medicine (Mice skin fibroblasts)	[7]
Pressurization over hydrophilic microchannels array on a microfluidic device	Murine mouse embryonic stem cell line-D3	Endogenous, Precursor cells characteristic RNA (mOct 3/4 and mNanog)	Gene delivery to NIH-3T3 fibroblast cells	[8]
Living cells slicing with silicon nitride blades in a microfluidic device	Murine mouse embryonic stem cell line-D3	Exogenous, Polystyrene beads as representative exogenous material	Material delivery to Mousse embryonic fibroblasts	[9]
<b>Works based on Bottom-Up approach</b>				
<b>Type of formulation</b>	<b>NVs preparation strategy</b>	<b>Proteins for NVs functionalization</b>	<b>Application</b>	<b>Reference</b>
Classical liposome, PC:Chol	Thin Film Hydration Method (TFHM) and maleimide based bio-conjugation strategy	MHC Class I peptide complexes and FAB regions against T-Cell receptors for adhesion, early and late activation, and survival.	<i>Ex vivo</i> and <i>In vivo</i> T-Cell expansion for immunotherapies	[10]
Mimicking exosomes lipid composition, PC:Chol:SM	TFHM and Ni <sup>2+</sup> /His-Tag protein coordination as bio-conjugation strategy	APO2L/TRIAL-His <sub>10</sub> recombinant proteins	Down-regulation of T-Cell activation in an autoimmune disease animal model (antigen-induced arthritis)  Immunotherapy for apoptosis induction in hematologic tumours	[11-12]
Innovative liposomes, PC:CpEL:DOPE	Micro-emulsion and micelle assembling method for vesicle formation	Monoclonal antibody against DEC205 Dendritic Cell antigen	Probe of concept of artificial antigen presentation to Dendritic Cells	[13]

178 <sup>99m</sup>Tc-HMPAO: <sup>99m</sup>Tc-Hexamethylpropyleneamineoxime; **Chol**: Cholesterol; **CpEL**: Chemopor EL; **DOPE**: Dioleoyl-Phosphoethanolamine; **HUVEC**: Human Umbilical Vein Endothelial Cells; **MHC**:  
179 Major Histocompatibility Complex; **NVs**: Artificial exosomes, here referred as the general term Nanovesicles; **PC**: Phosphatidylcholine; **SM**: Sphingomyelin; **TFHM**: Thin Film Hydration Method;



181 **Figure captions:**

182 **Fig.1.** Advantages (green) and disadvantages (red) of the two approaches for bio-nanotechnological development of full artificial exosomes as  
183 theranostic agents.

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