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A comprehensive guide to extract information from extracellular vesicles: a tutorial review towards novel analytical developments

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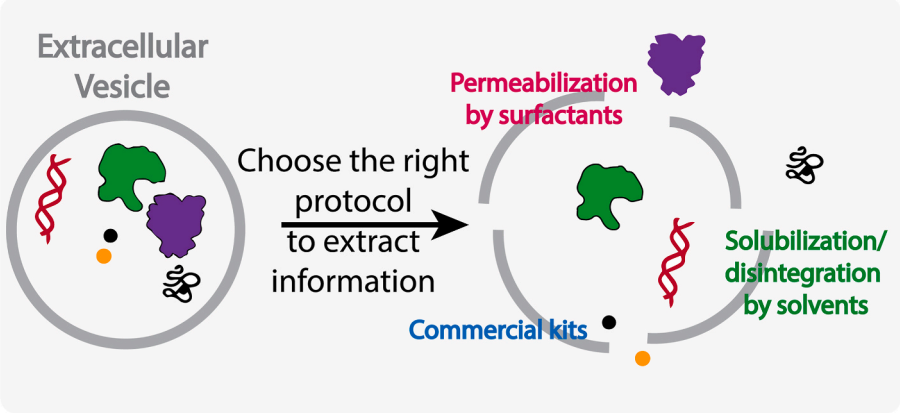
HIGHLIGHTS

- A guide to quickly understand the optimal extraction of information from EVs.
- Nucleic acids, proteins, lipids, enzymes cannot be extracted using a unique procedure.
- Depending on the information to be extracted from EVs, various solutions are provided.
- All the extraction methods included are characterized by pros and cons.
- A comprehensive table summarizes the right protocol at a glance for non-specialists.

GRAPHICAL ABSTRACT



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ABSTRACT

In the medical field, extracellular vesicles (EVs) are gaining importance as they act as cells mediators. These are phospholipid bilayer vesicles and contain crucial biochemical information about their mother cells being carrier of different biomolecules such as small molecules, proteins, lipids, and nucleic acids. After release into the extracellular matrix, they enter the systemic circulation and can be found in all human biofluids. Since EVs reflect the state of the cell of origin, there is exponential attention as potential source of new circulating biomarkers for liquid biopsy. The use of EVs in clinical practice faces several challenges that need to be addressed: these include the standardization of lysis protocols, the availability of low-cost reagents and the development of analytical tools capable of detecting biomarkers. The process of lysis is a crucial step that can impact all subsequent analyses, towards the development of novel analytical strategies. To aid researchers to support the evolution of measurement science technology, this tutorial review evaluates and discuss the most commonly protocols used to characterize the contents of EVs, including their advantages and disadvantages in terms of experimental procedures, time and equipment. The purpose of this tutorial review is to offer practical guide to researchers which are intended to develop novel analytical approaches. Some of the most significant applications

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are considered, highlighting their main characteristics divided per mechanism of action. Finally, comprehensive tables which provide an overview at a glance are provided to readers.

1. Introduction

Extracellular vesicles (EVs) represent a family of membrane-enclosed vesicles ranging in size from 30 to 5000 nm, that are secreted into the extracellular space by all living cells [1,2]. Classifying this heterogeneous family has been a difficult task since their discovery given their possible overlap and coexistence. For this reason, the scientific community currently divides them mainly into three sub-group [3,4]: exosomes (Exo), microvesicles (MV) and apoptotic bodies (AB) based mainly on the biogenesis/secretion mechanism and on their specific cargos or membrane markers that characterize them [5–9]. ABs are the largest EVs, approximately 100–5000 nm, which are formed as a result of programmed cell death and carry information related to this process [10]. The MVs have a characteristic size of about 100–1000 nm and are formed by budding from the plasma membrane, they are often associated with cell signaling processes and immune responses [11]. Exo, on the other hand, are specifically defined by their diameter (~30–150 nm) and are produced by late endosomes within multivesicular bodies (MVB) which, fusing with the plasma membrane, release them into the extracellular space in the form of small vesicles where they can be taken up by target cells [12]. Exo are distinguished by the presence of typical markers, e.g., Alix, TSG101, HSC70, HSP90β and tetraspanins (CD81, CD9, CD63). Since their discovery in the late 1980s [13,14], EVs and in particular exosomes have generated a great interest and a growing number of publications have highlighted their important role in cell-to-cell transmission [15–18].

They possess and transport valuable molecular content representative of the cell of origin in terms of lipids, proteins [19–21], and genetic material such as DNA [22,23], RNA [24,25], mRNA [26,27] and a variety of smaller non-coding RNAs (miRNA) [28,29]. These bioactive molecules enrich all human biological fluids [30] and can represent indicators of health status. A graphic representation of EVs biogenesis, exosomal content and their collection from human body is reported in Fig. 1.

The synergy between their specific molecular composition and bioavailability makes them particularly attractive for clinical applications. Plenty of studies underline their role as biomarkers of many different diseases not only in physiological but also in pathological

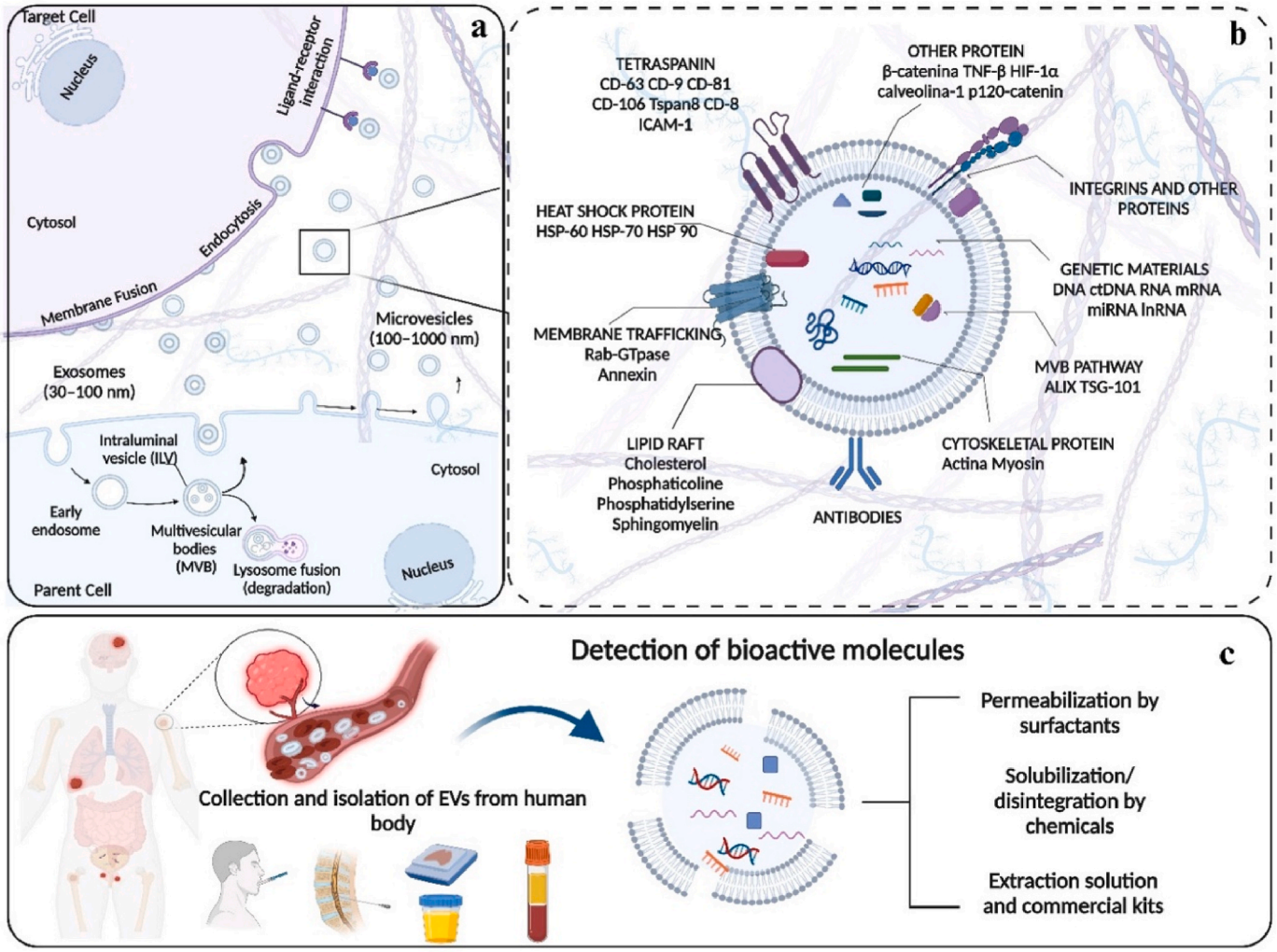

Fig. 1. Extracellular Vesicles a) biogenesis and vesicular trafficking between cell-cell transmission by mechanism of membrane fusion, endocytosis and ligand -receptor interaction; b) Typical biological marker of exosomes contents. c) EVs enrich all tissues and fluids of the human body, isolation and collection enable the detection of bioactive molecules using the different approaches discussed below for bilayer opening.

process [31–33]. Several studies consider EVs responsible for the spread and propagation of tumorigenesis [34], defense and immunological response [35,36], and also the influence of inflammation [37–39], neurodegenerative disorders [40–43] and cardiovascular diseases [44,45]. Consequently, their identification and determination hold great promise for the development of liquid biopsy techniques for early diagnosis and prognosis [46,47].

1.1. EVs isolation techniques

The great biological impact and diagnostic potential of EVs have driven many researchers to improve the techniques and method of extraction, with the aim to obtain a specific and pure vesicular population from the biological matrix and to achieve reproducibility of isolation [48]. The choice of separation method and subsequent concentration of the isolated vesicular particles depends on factors that may vary from one study to another: the one-size-fits-all approach does not work.

The most commonly used techniques are ultracentrifugation (UC), polymer-based separation, size-exclusion chromatography and biological immunoaffinity techniques.

UC is the most widely used technique and it has considered as the "gold standard" for the extraction and separation of exosomes. Using differential centrifugal force applied at up to 1,000,000×*g*, suspended particles are separated sequentially according to their physical properties (density and viscosity). Since this technique involves the co-purification of soluble lipoproteins [49] and proteins [50,51] of a similar size, a variant density gradient centrifugation (dg-UC) has been developed to obtain exosomes in a specific size range compared to whole exosomes isolated by differential centrifugation [52] and is usually used in combination with ultracentrifugation to improve the purity of exosomes. However, even if UC is effective for removing lipoproteins, extravesicular protein complexes, aggregates and other contaminants, is not suitable for isolating exosomes from clinical samples because it requires large sample volumes, is time-consuming and expensive equipment.

Polymer-based separation, such as polyethylene glycol (PEG) with high molecular weight (>1000 kDa), causes precipitation of EVs by reducing their hydration and altering their solubility/availability.

Currently, several commercial kits such as ExoQuick exploit this mechanism and are compatible with vesicular isolation from body fluids such as serum, plasma, ascites, urine and cerebrospinal fluid [53]. With these methods, the purity of the exosomes obtained is approximately 10^7-10^9 particles per microgram.

Polymer precipitation-based isolation methods reduce laboratory time and are easy to use, providing high EV efficiency with well-preserved structure. Despite these advantages, this method suffers from the interference of co-precipitated proteins due to the non-specific interaction between polymer and protein, which is however often solved by integrating other separation methods [54].

A variant of chromatography, namely size exclusion chromatography (SEC), is largely used to isolate EVs based on the separation of molecules that differ in their hydrodynamic radius. Today, commercially available exosome SEC purification columns, such as qEV separation columns, are capable of isolating EVs from diverse biological fluids such as blood, plasma and urine [55]. This isolation method is able to isolate exosomes with complete structure, uniform size and unaltered biological characteristics. However, the presence of other particles of similar size leads to reduced purity [56], so SEC is often used in conjunction with other isolation methods.

The main drawbacks associated with this approach are the potential contamination with lipoproteins and protein aggregation. Another important strategy usually employed to isolate exosomes is immunoaffinity capture, based on the specific separation of surface proteins expressed in exosomes. Among these proteins we can consider CD63, CD81, CD9, Alix, and EpCAM which are specifically found on the surface of the exosome [57], contributing to high-purity isolation. The selectivity of isolation is also improved by the combination with magnetic beads [58], chromatography [59] and microfluidics [60]. The immunoaffinity capture approach has high advantages especially in isolation purity compared to other methods, but commercially available antibodies are limited and very expensive, thus discouraging this strategy.

1.2. Advancement in point-of-care platforms

The goal is to convert the significance of cellular communication into measurable signals in point-of-care (POC) settings. Compared to other biomarkers in biofluids such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and microRNAs [61,62], EVs have numerous advantages [63]. They have higher concentrations and provide more information as represent a wider range of biomarkers and molecules, which are specific to the cells of origin and are found either within EVs or in the lipid bilayer. The emerging demand for POC technologies arises from the need for a practical device, designed to be easy to use and quick to respond. In this, the creation of biosensors can meet this demand due to their simple design which brings ease of application, automation, rapid and sensitive analysis [64]. The biosensors currently tested exploit the combination with nanomaterials and specific functionalization to search for one or more selective targets, presenting better reliability, specificity and limits of detection (LOD) compared to conventional techniques such as ELISA and polymerase chain reaction (PCR) [65]. Different approach technologies have been tested, including biosensors based on the surface plasmon resonance effect (SPR) [66–68], electrochemical sensors [69–71], and immunoaffinity-based sensors [72–74]. The SPR phenomenon is based on the excitation of electrons in polarized light at the interface of a dielectric and conductive metallic surface, generally silver or gold, which uses total internal reflection systems to monitor changes in the refractive index of the analyte. SPR biosensors have proven to be exploitable and versatile for EV analysis, as first demonstrated by Ref. [75] approach with nPLEX where the authors instead of total internal reflection, adapted the system to measure optical transmission through periodic nanoholes on a metal surface. Here, by functionalizing with specific antibodies it was possible to discriminate the expression of CD-24 and EpCAM in exosomes isolated from ovarian cancer cells with a LOD of 3×10^3 EV/mL. Once again exploiting the SPR Park et al. [76] targeted internal proteins (AKT1) after lysis of immunocaptured EVs by gold functionalization offering signal amplification via plasmonic coupling achieving a LOD of 10^4 particles/ml. Electrochemical sensors are based on measuring electric current generated by redox reactions or substances. Modern electrochemical biosensors are portable platforms [77,78] that, if appropriately functionalized, show great versatility and high sensitivity in the biomolecular detection of EVs. As demonstrated by Ref. [79] an integrated electrochemical aptasensor -based detachable microfluidic device for detection and genomic characterization of breast cancer-derived exosomes. Their platform leverages aptamer immobilization specific to epithelial cell adhesion molecules on the gold electrode and integrates microfluidics into a 3D-printed magnetic housing. This platform demonstrated ultrahigh sensitivity (17 exosomes μL^{-1}) over a wide dynamic range (1×10^2 to 1×10^9) exosomes uL^{-1}. Immunoaffinity-based approaches are employed for the isolation and quantification of exosomes expressing specific surface markers (CD-63, CD-81 CD-9). The specificity of the antibody and the degree of nonspecific binding of exosomes to the surface influence the purity of the exosomal subpopulation. ExoPRIME is an example of a platform created by Ref. [80] based on the use of CD-63 probe beads that provide a rapid, non-invasive and efficient alternative for the isolation and analysis of exosomes both from conditioned astrocyte media (CAM) which give suspension of enriched exosomes. The probe captured a sufficient number of vesicles for subsequent genomic lysis and determination, genomic and proteomic analysis. The isolated RNA efficiency was 0.54 ng $probe^{-1}$ and 0.30 ng $probe^{-1}$ for EXO and CAM samples,

Table 1
Different types of chemical reagents with advantage and disadvantage.

Chemical reagents	Type	Advantage	Disadvantage	Time of incubation [a]	Volume required of EVs samples [b]
Triton-X	Non-ionic sufractant	Mild lysis reagent; Solubilize lipids; Less damage to proteins and enzymes;	High concentration can lead denaturation of proteins; Low permealization of membrane;	Short time	Low volume
SDS	Ionic sufractant	High affinity for proteins and denaturation; Able to provide negative charges of protein;	Strong lysis reagent; Not suitable for delicate proteins (e.g., phosphorylated ones)	Long time	Low volume
NP-40	Non-ionic sufractant	Mild lysis reagent; Suitable for membranal protein and for total lysate;	High concentration can lead denaturation of proteins; Low temperature incubation required;	Short time	Low volume
Saponin	Non-ionic natural sufractant	Suitable for sensitive protein extraction; No damage to proteins and enzymes;	Poor lysis reagent; No complete permealization of membrane;	Short time	Low volume
Tween	Non-ionic sufractant	Mild lysis agent; Less damage to proteins and enzymes;	Low denaturation action;	Short time	Low volume
Methanol	Alchool	High solubilization of lipids and protein;	Strong lysis reagent; Damage structure of EVs; Destroy part of internal contents of EVs	Very short time	High volume
4-hexylphenyl azosulphonate (Azo)	Photocleaving sufractant	Controlled lysis reagent; Low invasive treatment; Hight efficiency of lysis;	Need the UV for activation; Require digestion of proteins;	Long time	High volume
Ammonium and urea buffer	Chaotropics	High ability for fragment proteins and peptides;	Strong lysis reagent; Damage structure of EVs; Destroy part of internal contents of EVs	Very long time	High volume
TRIzol	Phenol and guanidine isocyanate mixutre	High denaturation activity and stabilization of genetic material;	Strong lysis reagent; Not suitable for protein analysis;	Long time	High volume
Water	Distilled water	High avaiability in laboratory;	Poor lysis reagent; No complete rupture of membrane; Damage structure and same contents of EVs	Short time	High volume
Extraction solution and commercial kit	RIPA and commercial kit	Complete permeabilization of membrane; Quick and easy procedure; High efficiency of extraction compounds;	Different reagent needed for RIPA formulation; cost-expensive of commercial kit	Short time	Low volume required for RIPA and commercial kit

[a] The authors indicated the "long" and "short" time for time of incubation action of reagents respectively in time range >30min or <30min. They used "very long" for time >60 min and "very short" for time < of 10 min.
[b] Authors indicate the "high" and "low" volume for EVs, respectively to > 1 mL or < 1 mL.

respectively and their reported protein loading capacity was 940 ng probe^{-1} and 728 ng probe^{-1} for EXO and CAM samples, respectively. The evolution of technology and the ever-increasing demand for low-cost methods make paper biosensors cutting-edge [81–83] as demonstrated by Chen et al. [84] with their aptameric biosensor based on energy transfer through luminescence resonance. By functionalizing the paper cellulose via the CD-63 aptamer, the EVs reached the paper sensor together with a second fragment of CD-63 aptamer (hybridized with gold nanoparticles), causing a quenching of the luminescence due to the distance between the paper and the nanoparticles' gold. This platform showed a LOD of 1.1 × 10^6 EVs per ml, which is very sensitive for low-cost and portable EV detection.

1.3. Approaches for EVs contents

Among the applications of the different techniques it is important to underline the characterization of EVs and in particular the detection of the internal contents requires the use of lysis protocols aimed at solubilizing/permeabilizing/destroying the lipid bilayer membrane to identify and differentiate the markers resident inside or outside of their membrane [18]. It goes without saying that this process is crucial from a clinical point of view and can be achieved through different approaches.

Extraction methods must be designed based on the nature of the target sought (e.g. membrane or internal protein) and the type of experimental procedure to be used (e.g. Western blotting or mass spectrometry). This review is dedicated to discussing these two aspects together. Diverging from other contemporary reviews that offer extensive insights into isolation methods [12,30,48,51,57,85,86], characterization [30,52,87–90], analysis techniques [7,91–95] or specific content of EVs [24,96,97], this review focuses on the fundamental chemical lysis approaches applicable across diverse analytical methods.

In particular, in this review, we report on the main procedures and findings investigating specific markers within EVs as biomarkers in cancer.

The authors would like to inform readers that they can consult the recent guidelines published by International Society of Extracellular Vesicles (MISEV 2023) [98] for aspects concerning the isolation, characterization and analysis of EVs.

The most commonly used protocols for this procedure involve the use of surfactants, blends of them, homemade or commercially available lysis buffers. The lysis of EVs is a critical step [99] as it can compromise the integrity of the biomarkers sought and can lead to contamination or detection of interfering substances, creating false positives or negatives. To a correct execution is essential to obtain precise, specific, and useful information in order to exploit the full potential of the biological molecules within them. It is therefore essential to have a robust and reproducible protocol for the success of many diagnostic and therapeutic applications. Furthermore, it is important to be able to use as few reagents as possible to avoid contamination, but above all to obtain a robust result in the shortest possible time in order to be analyze it quickly. Also important is establishing the approach for extraction and lysis strategies that can be used and exploited in cutting-edge analytical methods implementations that pave the way for personalized medicine.

This review focuses on exploring the chemical lysis reagents used to reveal the internal content of EVs, highlighting the advantages and limitations of the methods examined. A range of information is discussed on reagents, different approaches and techniques used to obtain valuable information on EVs, as well as their strengths and weaknesses in terms of experimental procedures closely related to the identified markers.

In this article the authors followed the nomenclature in EVs field used by the corresponding cited authors.

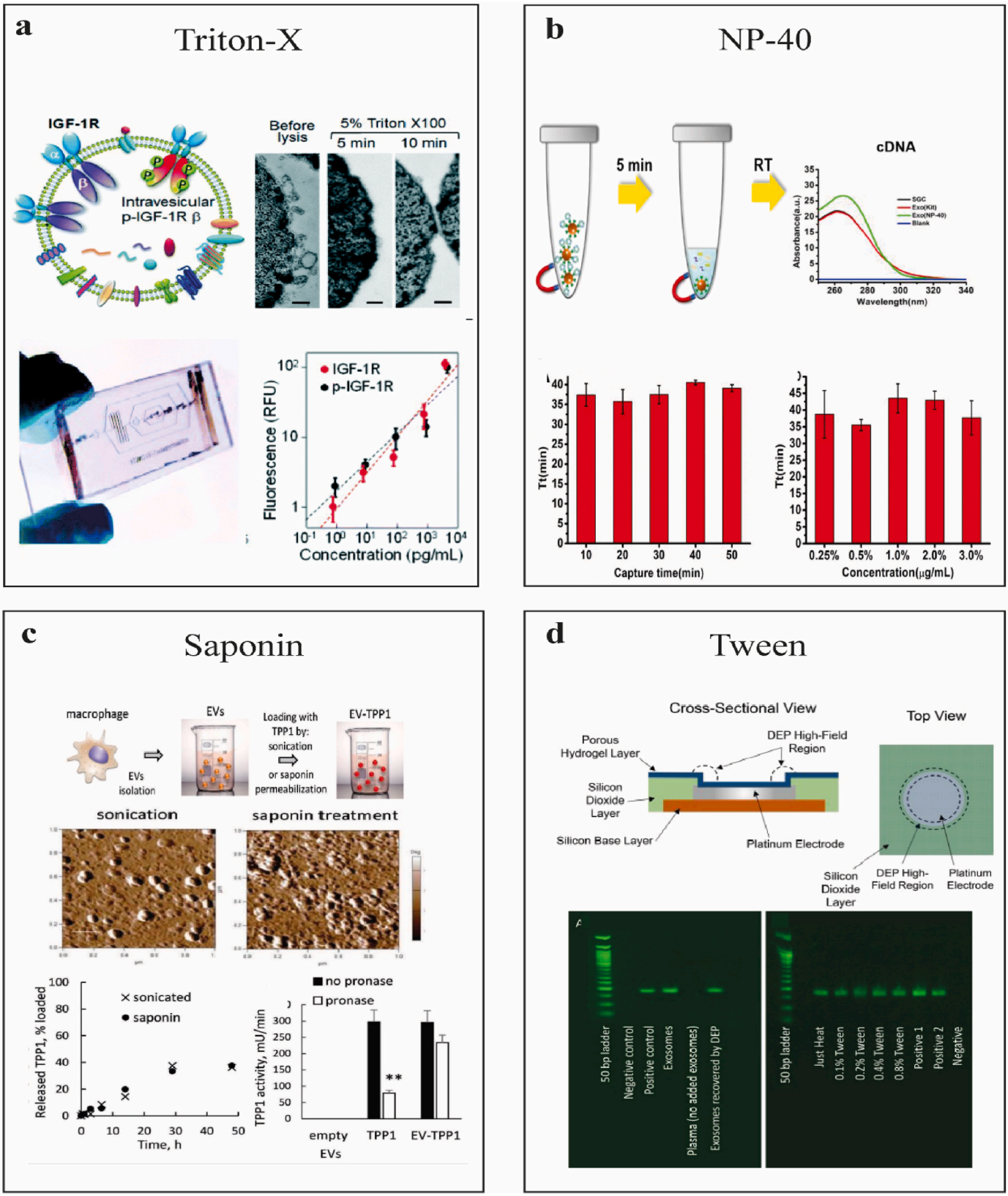

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Fig. 2. Permeabilization by surfactants. a) Schematic representation of target protein IGF-1R intra and extravesicular with chemical lysis of exosomes by using Triton X-100 as a surfactant reported by TEM acquisition images. The scale bar is 100 nm. In the panel below is reported the integrated microfluidic platform used exosome for analysis directly from human plasma with calibration measurements of on-chip capture and detection of IGF-1R and p-IGF-1R. Reproduced from Ref. [75] with permission from the Royal Society of Chemistry b) Representation of simple and rapid exosomal RNA extraction using NP-40 lysis buffer with UV–Vis absorbance spectrum of reverse transcription products (cDNA). Optimization experiment in term of different capture times for exosomes enrichment using immunomagnetic beads and different concentrations of NP-40 lysis buffer used for exosomal RNA extraction for LAMP assay. Reproduced from Ref. [84] c) Characterization of EV-TPP1-l obtained by TPP1 loading into native EVs by either sonication or saponin permeabilization: morphology by AFM (scale bar 200 nm), TPP1 release, and TPP1 stability in the presence of pronase protease from Streptomyces Greseus for sonicated EV-TPP1 versus free TPP1. Reproduced from Ref. [88] with permission from the Advanced Healthcare Materials d) Schematic of the microelectrode array chip showing the cross-sectional and top views of a single electrode. Below there are the results of endpoint RT-PCR and gel PCR analysis performed on RNA from glioblastoma exosomes and EVs isolated from plasma using the ACE device, using primers specific for mutated EGFRvIII mRNA. In the left the PCR results show that the specific 181 bp amplicon for the mutated EGFRvIII mRNA was produced for the positive control, exosomes and EVs isolated from plasma. The negative control was a plasma sample without addition of glioblastoma exosomes and did not show the presence of mutated EGFRvIII mRNA. In the right the RT-PCR results are shown as a function of heat and different concentrations of Tween 20 surfactant to release EGFRvIII mRNA from exosomes and EVs isolated from DEP. PCR results show that the specific 181 bp amplicon for EGFRvIII mRNA was produced for positive controls, Tween 20 treatments (0.8–0.1%), and heat treatment. Reproduced from Ref. [92] Copyright © 2017 American Chemical Society.

2. Types of chemical lysis

In order to evaluate which is the most suitable procedure to obtain the EVs lysis, all the strategies have been organized within different sections, depending on the nature and mechanism of lysis, including detergents as triton-x, sodium dodecyl sulfate (SDS), nonyl phenoxypolyethoxylethanol (NP)-40, saponin and tween, solvents as methanol, water, TRIzol, buffer and compounds based on nitrogen and also extraction solution and commercial kit.

In the paragraphs and sub-paragraph are collected for different approaches aim to provide the readers the most suitable reagents, depending on their need, to conduct the lysis of exosomes/EVs by considering the best compromise with respect to the species-to-be-extracted, the efficacy and the whole protocol. The difference between type of chemical reagents with advantage or disadvantage are summarized in Table 1.

2.1. Permeabilization by surfactants

Since the EV membrane consists of a bilipid layer made up of both hydrophobic and hydrophilic molecules, detergents also called surfactants are able to interrupt lipid-lipid, lipid-protein and protein-protein interactions and are therefore extremely useful for opening of the membrane.

Most of the research in the literature assigns the task of permeabilizing the double layer of the EV to detergents, obtaining the release of lumen biomarkers; a collection of results obtained with this approach is shown in Fig. 2. Detergents are divided based on their ionic and non-ionic charge which results in the difference in lysis capacity. Among these, the preferred ones are the non-ionic which are milder because they preserve the proteins and enzymes more. A very notable detergents exploited in much paper are represent by Triton-X 100 [100–106], SDS [107–110], NP-40 [111–114], Saponin [115–118] and Tween-20 [119–121]. These surfactants are commonly used in chemical and biochemical laboratories to permeabilized and manipulate biomolecules such as proteins, lipids and nucleic acids. The differences in term of sensitivity among these reagents on sub-populations of EVs isolated can be found in this informative study [122].

However, there are some fundamental differences between them in terms of chemical composition and properties that determine the advantages and disadvantages of their use.

2.1.1. Triton-X

Triton-X 100 is a non-ionic surfactant derived from ethylene oxide. It is a mixture of isomers that vary based on the chain length of the ethoxylated alkylphenol. It is widely exploited for cell lysis and for the solubilization of cell membranes [123] as it has a high capacity to solubilize lipids, but can cause the denaturation of proteins if used in high concentrations.

Studies conducted by Palanisamy et al. [105], He et al. [102] and Kowal et al. [103] collectively demonstrate the versatility of Triton-X in the context of exosome and EV research and both strengths and limitations can be highlighted based on their protocols. A common strength observed in these studies is the effective use of Triton-X as a lysis reagent for exosomes even at room temperature (RT). This is particularly evident in the investigation by Palanisamy et al. [105] on the content of the transcriptome of salivary exosomes, in which Triton-X at 1% concentration was the only reagent used for lysis and allowed the identification of 509 mRNAs contained within exosome by microarray analysis. In this study the incubation time of their lysis protocol, as the extracted material was evaluated after 30 min. In the context of portable devices, and not only, triton-X reveals adaptability to different experimental setups with microscale applications, as recently demonstrated in literature, focusing on a microfluidic platform for exosome detection [102]. Its ability to effectively lyse exosomes at room temperature was tested at 5 and 10 min of action with a concentration of 5%. The effectiveness of the surfactant effect is shown in enlarged detail on a vesicle observed at TEM shown in Fig. 2a where it is reported that with an incubation time of only 5 min the bilayer appears permealized.

This treatment was instrumental in creating a fast (less than 2 h) and sensitive microfluidic platform for the isolation and analysis of exosomal proteins with an LOD of 0.281 pg mL^{-1} for the membrane protein IGF-1R and 0.383 pg mL^{-1} for the intravesicular p-IGF-1R protein (Fig. 2a). This device has been shown to be effective in discriminating cancerous from non-cancerous phenotypes by surface exosomal and intravesicular biomarkers directly from plasma samples. Furthermore, Kowal et al. [103] exploited the capabilities of Triton-X at a concentration of 0.05% within a lysis buffer to differentiate the internal content between different EV populations by applying Western blot (WB) and proteomic analysis. This study required a laborious procedure since, unlike the previous ones, the samples were incubated on ice for 20 min and then analyzed the supernatant after centrifugation at 18,500×*g* for 15 min. This can introduce variability and complexity into experimental procedures, and meticulous optimization is essential to ensure reproducibility.

2.1.2. SDS

Sodium dodecyl sulfate (SDS), also referred to as sodium lauryl sulfate, is an anionic ionic surfactant. This ionic surfactant is often used in a lysis buffer with basic substances (pH 11.5–12.5) to work synergistically in lysing the cell membrane by using the OH^- ion to break glycerol fatty acid-ester bonds and subsequently making the membrane permeable and allowing SDS to solubilize proteins. SDS is able to denature the structure of proteins and provide them with a negative charge which is proportional to their mass. This is why it is commonly used in gel electrophoresis (SDS-PAGE). Additionally, many studies utilize it as a component of protein extraction buffers or as a lysing reagent [124]. Flanagan et al. [107] conducted a study on enzymatic activity in EVs using SDS to disrupt the membranes and analyze the contents. They used a 1% concentration of SDS and performed the lysis protocol at RT. The

study successfully identified enzymatic activity within EVs, including N-acetylgalactosamine-6-sulfate sulfatase (GALNS) in umbilical mesenchymal cells, demonstrating the effectiveness of SDS. Gao et al. [109] used SDS to lyse exosomes previously captured on TiO_2 microspheres. Their protocol used 4% SDS within a 0.1 M Tris-HCl lysis mixture (pH 7.6), supplemented with a cocktail of protease inhibitors (PI) by incubation on ice for 20 min. Even if the isolation procedure was validated with the use of WB and LC-MS/MS, the ice incubation step appeared as disadvantageous.

Jeppesen et al. [108] used SDS together with Na_2CO_3 at basic pH to fractionate exosomes into their membrane and lumen components delving into their proteomic content. This procedure required operational complexity in terms of time and necessary instrumentation such as incubation on ice for 1 h, sonication and ultracentrifugation for 2 h at a controlled temperature of 4 °C for material recovery. Despite this, the proteomic analysis of this fractionation allowed the authors to identify several proteins linked to the epithelial-mesenchymal transition (EMT) present respectively in the membrane and in the lumen of the exosomes of metastatic cells.

2.1.3. NP-40

NP-40 is a non-ionic surfactant. Its chemical structure is similar to Triton-X and is often used as a detergent for cell lysis, protein solubilization, and sample preparation for biochemical analysis [125]. However, like Triton-X, NP-40 can denature proteins when used in high concentrations. It is generally used together with other reagents composing a lysis buffer. Caby et al. [112] employed 1% NP-40 in a lysis buffer to analyze proteins in exosomes from human plasma and human cells (HMC-1). Their lysis protocol involved a 20-min incubation at 4 °C before WB and immunofluorescence analysis. The procedure showed effective extraction of exosomal proteins, particularly in human plasma samples. Similar Chitadze et al. [114] used NP-40 in the same lysis buffer to study immune factors and specifically major histocompatibility complex class I (MHC-I) factors in EVs. The lysis procedure used was different in that an incubation time of 25 min was used and then centrifuged at 14,000 rpm for 10 min at 4 °C to recover the inside and

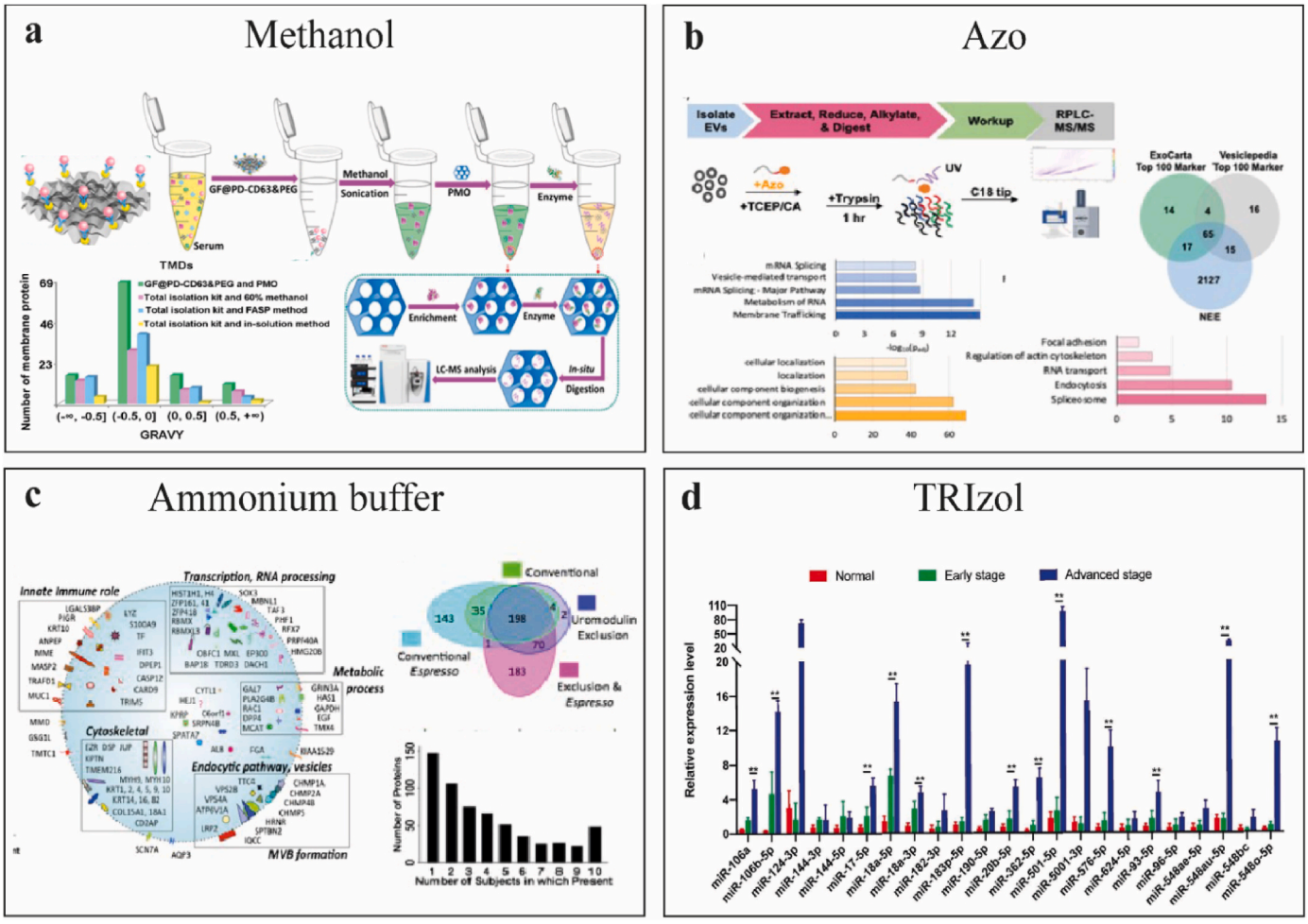

Fig. 3. Solubilization/disintegration by solvents. a) Schematic illustration of the efficient analysis process for exosomes isolation and protein profiling by using the integrated nanomaterial-based platform. Comparison of the distribution of number of TMDs and GRAVY values of the identified membrane proteins from serum exosomes by the integrated GF/PMO platform, 60% methanol, FASP, and in-solution digestion methods. Reproduced from Ref. [128] Copyright © 2018 American Chemical Society b) Depiction of the MS-based proteomics workflow for EVs analysis where proteins were extracted with photocleavable, Azo then reduced with TCEP, alkylated with 2-chloroacetamide (CA), digested with trypsin, and analyzed by liquid chromatography in reversed phase spectrometry after surfactant removal. Comparison of the proteins identified in this study with the top 100 proteins available in the ExoCarta and Vesiclepedia databases. Reproduced from Ref. [129] Copyright © 2022 American Chemical Society c) The human urinary exosomal proteome a representation of vesicles containing principal protein groupings included protein with innate immune role. A comparison of conventional methods only 237 proteins would have been evident. Graph reported in bottom right represent a most proteins identified (y axis) vs number of subjects (x asis). Reproduced from Ref. [130] with permission from the Journal of the American Society of Nephrology 25(9):2017–2027, September 2014. d) Screening of miRNA in sEVs of enriched plasma from metastatic breast cancer (BC) patient, different concentration level of miRNA expression between advanced stag > early stage > normal group. Reproduced by Copyright © from Ref. [131]Creative Commons CC licenses 4.0 https://creativecommons.org/licenses/by/4.0/.

outside membrane content. Despite the laborious procedure, the authors successfully extracted genetic material from EVs allowing the study of factors related to the immune system. Lin et al. [111] developed a "lab in a test tube" system that integrates enrichment, lysis, and LAMP for the detection of exosomal nucleic acids (lncRNAs). A schematic representation of their approach is shown in Fig. 2b where the strengths of this procedure are demonstrated through the rapid lysis (5 min), the low concentration of surfactant (0.5%) used and the high extraction efficiency. This method was demonstrated by detecting two gastric cancer exosomal lncRNAs with a detection limit of 10 ng/μL.

2.1.4. Saponin

Saponin is a natural surfactant that can be extracted from plants such as Quillaja saponaria or Yucca schidigera. It is a complex mixture of triterpene glycosides. Saponin is often used as an emulsifying and solubilizing agent for lipids, proteins, and nucleic acids. It is particularly useful for lysing delicate cells [126], such as red blood cells, as it is less denaturing than other surfactants such as Triton-X or SDS. Gray et al. [117] used a saponin-based methodological protocol to label EVs for calcein identification. Specifically, they tested multiple concentrations of saponin and incubation times of 10 or 20 min to conduct lysis at RT. Although high permeabilization of the lipid bilayer was achieved at a concentration of 1 mg/mL after 20 min of incubation, the disadvantage was the need for subsequent high-speed centrifugation (16,100×*g*) to remove residual agents, adding complexity to the protocol. Graner et al. [118] in their biochemical and immunological study on the treatment of tumor exosomes in the murine brain, they used a lysis protocol involving the presence of 1% saponin to lyse tumor exosomes isolated from patient serum. Briefly, isolated exosome pellets were resuspended in Laemmli buffer with 2-mercaptoethanol and 1% saponin and subsequently boiled for 15 min and then analyzed by the WB method. The results showed that brain tumor exosomes have unique characteristics such as the expression of the mutated tumor antigen EGFRvIII and the immunosuppressive cytokine TGF-β. Administration of such exosomes in syngeneic animals produced immune responses that led the authors to conclude that exosomes released from brain tumor cells have immunomodulatory properties and that, potentially escaping the blood-brain barrier, lead to systemic problems and distal signaling with the immune system.

Haney et al. [115] used saponin at a low concentration of 0.4 mg/mL to understand cellular process degradation and delivery of recycling to the lysosome with EVs. To test the incorporation of tripeptidyl peptidase I (TPP1) into macrophage-derived EVs, the authors evaluated two approaches for EV membrane disruption/permeabilization: sonication of EVs at room temperature in a water bath to 30 min or treatment with saponin (Fig. 2c). With the latter approach, saponin can selectively remove membrane-bound cholesterol of EVs, creating holes/pores in the lipid bilayers of EVs and thus promoting TPP1 loading. Catalytically active TPP1 was efficiently incorporated into EVs in both procedures, as shown in Fig. 2c. But interestingly, saponin permeabilization leading to a "gentler" approach resulted in 1.5 times lower loading efficiency than the sonication method.

2.1.5. Tween

Like saponin, Tween-20 or polysorbate 20 is a natural, non-ionic surfactant that comes from castor oil or sesame seed oils. Tween-20 is often used as a surfactant for the solubilization of hydrophobic proteins, the formation/stabilization of emulsions and the stabilization of suspensions and has a lower denaturing action on proteins than Triton-X and SDS. Several studies apply Tween-20 [127] alone or in a lysis buffer to analyze the contents of EVs. Ibsen et al. [119] in their proposal of a dielectrophoresis-based alternating current electrokinetic (ACE) microarray chip device (Fig. 2d) for the rapid isolation and recovery of glioblastoma exosomes from undiluted human plasma samples with a rapid procedure in less than 30 min. Using Tween-20 as the sole lysis reagent, glioblastoma exosomal RNA was extracted by testing low Tween concentrations (0.1–0.8%). As shown in Fig. 2d, the presence of mutated EGFRvIII mRNA in exosomes and EVs isolated from plasma using the ACE device was confirmed by RT-PCR followed by end-point PCR. The results of their RT-PCR analysis showed interference-free mRNA characterization. Delcayre et al. [121] used Tween-20 in the development of an Exosome Display application for the induction and analysis of the human leukocyte antigen (HLA)/peptide complex. Their lysis protocol involved a 0.05% concentration of Tween-20 within a lysis buffer requires the use of a cold lysis buffer and incubation on ice for 30 min and then clarification of the lysate by centrifugation at 10,000×*g* for 10 min at 4 °C. Although the very low concentration of surfactant reduces interference, their procedure requires instrumentation and cold temperature. Ciravolo et al. [120]employed Tween-20 to lyse exosomes in dot-blot analysis. They evaluated exosomal overexpression of interfering HER-2 in breast cancer cell lines with trastuzumab-based therapy. Their method used Tween as a reagent for lysing EVs with a low concentration of 0.5% surfactant at RT but with a long incubation time (1 h). The authors found that in advanced breast cancer, exosomes containing trastuzumab-bound HER2 were more prevalent than in early-stage breast cancer, suggesting potential implications for therapy.

2.2. Solubilization/disintegration by solvents

Although the use of surfactants saves more or less time, the use of various analytical techniques, such as spectrophotometry and occasionally liquid chromatography coupled mass spectrometry, hinders the use of surfactants due to the potential for contamination they could lead introduce. To overcome this problem, different chemicals with different characteristics are used to identify biological material in EVs. The main principal approach and their results are reported in Fig. 3.

The reagents most commonly used for this purpose are discussed and summarized below.

2.2.1. Methanol

In the context of membrane protein extraction and lipid extraction, methanol is a very useful solvent as it can break hydrophobic interactions between protein molecules and lipid components of membranes [132]. Methanol is often used in the application of mass spectrometry techniques or spectrophotometric techniques. In the study conducted [133] for enzymatic spectral determination of curcumin within exosomes, 100% methanol was used with sonication to break the integrity of the EVs and exosomal membrane. The lysis method employed therefore required centrifugation at 10,000 rpm for 5 min at 4 °C, and supernatants were collected for absorbance analysis. This approach made it possible to determine the absorption of curcumin without interference, certifying its internalization.

Luo et al. [134] in their study use methanol to analyze the exosome metabolome from the serum of pancreatic cancer patients before and after chemotherapy. For this purpose, they use a lysis procedure based on the use of 50% methanol with freeze-thaw cycles in liquid nitrogen for 1 min and thawing in an ice bath for 1 min five times. With this fast procedure they managed to extract the metabolome and analyze it with differential chemical isotope labeling techniques to increase the separation LC-MS successfully achieving the recognition of 1950 metabolites per sample they demonstrated the application to detect significant changes of some metabolites before and after chemotherapy in exosomes isolated from the serum of cancer patients.

Fang et al. [128] showed a novel nanomaterial-based approach for exosome separation followed by lysis and proteomic content analysis in order to validate the proposed exosome isolation method. This method exploits affinity pull-down for the isolation and enrichment of membrane proteins using nanomaterials such as graphene foam and amphiphilic periodic mesoporous organosilica (PMO). Their approach proposed the isolation of exosomes, their lysis and enrichment to perform exosomal protein profiling analyzes in situ with minimal sample loss (Fig. 3a). In their procedure the isolated exosomes were

dissolved in methanol and sonicated for 1 h, the proteins were frozen dry and redissolved with ammonium bicarbonate buffer, reduced (DTT), alkylated (IAA) and digested with trypsin.

Fig. 3a shows the comparison of the distribution of number of transmembrane domains (TMD) and average hydrophobicity (GRAVY) values of membrane proteins identified from serum exosomes comparing the proposed GF/PMO integrated platform with 60% of methanol, filter-assisted sample preparation (FASP), and in-solution digestion methods. As reported in Fig. 3a, with the method proposed by the authors, more membrane proteins with more than two TMDs were identified (a total of 35 proteins, approximately 31% of the total membrane proteins found), compared to just one protein of this type discovered by the conventional method. The method identified 9 times more hydrophobic proteins (GRAVY $\geq$0) and 3.4 times more hydrophilic proteins (GRAVY $\leq$0) than the traditional method. These results demonstrate the great potential of the proposed GF/PMO integrated platform for the analysis of exosomal proteins.

Although the entire procedure exceeded 5 h of sample preparation, the LC-MS/MS results identified a total of 334 proteins with 111 membrane proteins in their GF/PMO platform, in contrast to 151 proteins and 28 proteins of membrane found when exosomes were isolated with isolation kits.

2.2.2. Photocleaving surfactant

Among the surfactants used in the literature, (Azo) has recently been used in EV lysis as it represents an example of a photocleaving surfactant, used as a controlled cell lysis agent. A photocleavable surfactant is a compound that can be cleaved or activated by irradiation with light at a specific wavelength. In the case of Azo, ultraviolet (UV) light can be used to activate the compound and induce its degradation, generating reactive radicals that can rupture cell membranes. The idea is that surfactant is added to cell samples and then exposed to targeted UV light, which activates the compound and causes selective destruction of cell membranes [135]. This allows you to control the timing and location of cell lysis, avoiding the need for more aggressive or invasive chemical treatments.

Buck et al. [129]employ 0.1% Azo in a One-Pot approach for the proteomic analysis of exosomes. Their procedure required azo extraction of proteins and subsequent addition of reducing and alkylating agents (TCEP and chloroacetamide) and rapid digestion with azo-aided trypsin (1 h). The resulting peptides were irradiated with a high-power UV lamp for 5 min to degrade azo and subsequent centrifugation and desalting prior to analysis. This procedure shows a shorter approach compared to other protocols used for the proteomic analysis of EVs since in 2.5 h of preparation it was possible to analyze and quantify exosomal proteins with LC-TIMS-MS/MS effectively with high reproducibility for deep coverage of the exosome proteome. Also Gupta et al. [136] use Azo for mass spectrometry applications on exosomes for mechanistic insights into EV biogenesis during necroptosis. Their procedure is similar to that used by Buck with some differences (see Table 1), such as the lower concentration of Azo used (0.05%) and the shorter time spent in the lysis procedure which required 1 h and 30 min. Fig. 3b shows a schematic representation of the MS-based proteomics workflow for extracellular vesicle analysis and shows the comparison of the proteins identified in this study with the top 100 proteins available in the ExoCarta and Vesiclepedia databases. Thanks to this analysis it was identified for the first time that necroptosis is associated with the release of small EVs that contain RIPK3 and MLKL as well as being able to identify many other unique proteins.

2.2.3. Ammonium and urea buffer

Chaotropic agents like as ammonium or urea buffers are a heterogeneous class of organic compounds that possess the ability to break hydrophobic bonds and hydrogen bonds of nucleic acids and proteins resulting in denaturation of substrates. Their use finds ample space in the determination of a specific protein or peptide fragment such as amino acids since thanks to their action the substrate is fractionated several times until all the individual units that constitute it are obtained. Although these substances could damage or destroy part of EVs and exosomal contents, different research based on mass spectrometry have been employed urea or ammonium buffer in their lysis protocol recorded a good lysis method. An example in the Burke et al. [137] work their used an optimized 8 M urea lysis buffer, which contained 50 mM ammonium bicarbonate with 50 μM deubiquitinase inhibitor (PR-619) to study ubiquitinated proteins in exosomes secreted by myeloid-derived suppressor cells (MDSCs). Their lysis protocol occurs at RT but involves different procedures such as a series of centrifugations of 30 min each at 14,000 with a 3 kDa cut-off filter and the entire process exceeds 1 h in duration. Tandem mass spectrometry coupled with immunoprecipitation reveals successfully isolation and lysis of exosomes by identifying 50 ubiquitinated proteins and determining their conjugation positions.

Hiemstra et al. [130] used the ammonium acetate-acetone precipitation method to lyse exosomes to study urinary exosomes as innate immune effectors within the urinary tract. To determine the role of exosomal structural integrity they used a procedure of incubation of exosomes with 100% ammonium acetate in methanol overnight at 4 °C, followed by centrifugation and pellet washing steps. Fig. 3c shows the human urinary exosomal proteome compared to conventional methods. 601 proteins of cellular origin were identified from exosomal pellets by tandem MS where a significant percentage was made up of cytoplasmic and membrane proteins or constituents of the endocytic pathway or vesicles. A graphical representation of the major proteins is shown in Fig. 3c with the groupings consistent with previous reports on exosomes but also including those exosomes with an innate immune role.

Although this approach took almost a full day to complete, proteomic analysis of the analyzed lysate revealed that urinary exosomes are significantly enriched in innate immune proteins, including antimicrobial proteins and peptides, bacterial and viral receptors. Jayabalan et al. [138] aimed to understand the association between circulating exosomes and maternal metabolic changes in gestational diabetes mellitus (GDM). Their lysis method involved pretreatment with a lysis buffer that contained SDS, sonication, and heating, then treating the exosomes with 8 M urea buffer, centrifuged with a 30 kDa molecular cut-off filter, and digested overnight with trypsin. Although this procedure required the use of numerous reagents and laborious procedures, this comprehensive approach identified 78 proteins that were statistically significant in the expression of exosomal proteins in GDM.

2.2.4. TRIzol

Unlike protein extraction, the internal genetic material of EVs is generally carried out through the use of TRIzol [139] which represents the most used reagent for the extraction of nucleic acids such as DNA and RNA, so the uses of this reagent represent a standard protocol to extract genetic materials from EVs particles. TRIzol is a mixture of phenol and guanidine isocyanate that breaks down cell membranes creates a highly denaturing environment and stabilizes nucleic acids during extraction. The use involves carrying out different phases such as lysis in the presence of TRIzol, centrifugation, and separation with the addition of chloroform to separate the organic phase (containing lipids and denatured proteins), an intermediate phase containing the DNA and the aqueous phase containing RNA. The DNA or RNA present in these extraction steps is precipitated with ethanol or isopropanol, washed to remove any contaminants, and solubilized in an appropriate buffer for a variety of genetic analyses, including PCR, RT-PCR, DNA sequencing, expression genes, and much more. The procedure for using TRIzol is standard and does not require optimization, therefore researchers using it carefully follow established protocols to ensure effective extraction and purification of genetic material [140,141]. Thanks to the use of TRIzol, many studies have recognized various tumor biomarkers for diagnostic and prognostic purposes [131,142–145]. As in the study by Liu et al. [143] they identified miR-21-5p overexpression in patients with breast cancer, liver cancer, lung cancer, cervical cancer, and

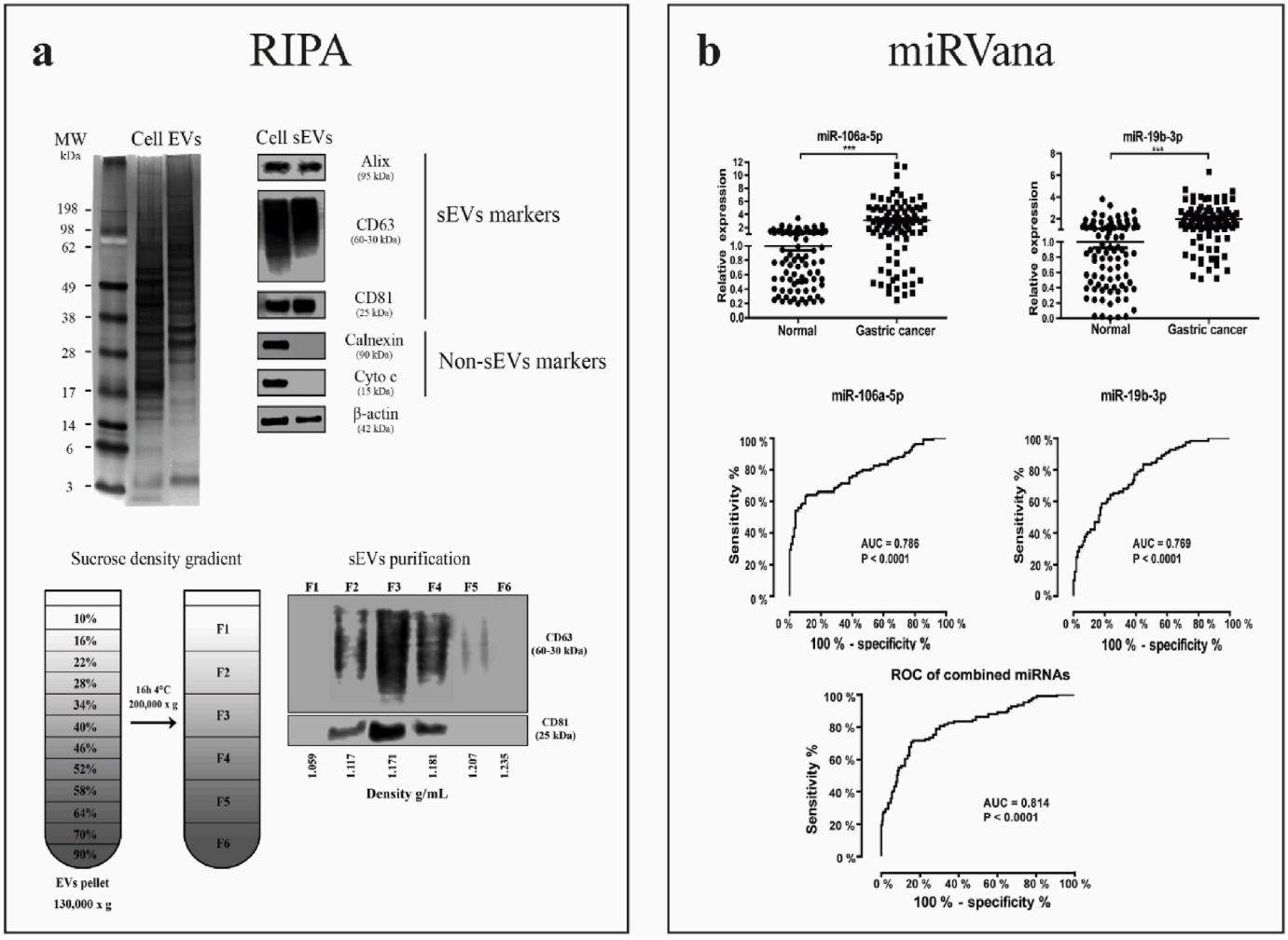

Fig. 4. Formulation and commercial kit. a) Representative image of silver nitrate-stained polyacrylamide gel after Western blot separation of 40 μg of total protein from cells or sEV pool lysates (pools included samples from four individual donors). Positive sEV markers (Alix, CD63 and CD81) and non-EV markers (Calnexin and cytochrome, β-actin was used as a loading control. The separation of sEVs based on the sucrose density gradient is shown below. Six fractions were collected from top to bottom of the gradient for further WB and confocal experiments. WB was performed with 15 μg of protein loading and the relative band intensity was calculated by ImageJ on the sEVs recovered in fractions F1–F6. Reproduced by Copyright © from Ref. [151] Creative Commons CC licenses 4.\0 https://creativecommons.org/licenses/by/4.0/b) Serum exosomal miRNA expression signature for gastric cancer (GC) diagnosis in the training phase. Expression of miR-106a-5p and miR-19b-3p in GC serum exosomes (n = 90) and normal samples (n = 90). ROC analysis for individual miRNAs. Combined performance of the ROC analysis for the two miRNA panel. Reproduced from Ref. [126] Copyright © 2017 Biochemical and Biophysical Research Communications.

ovarian cancer using microarray and RT-PCR analyses. The identification of miR-636, miR-21, miR-16, miR-142-3p, and miR-451 as in the study by Shin et al. [142] which discriminated their up- and down-regulation by discriminating between metastatic and non-metastatic states in prostate cancer patients. And again in the study by Xu et al. [131] TRIzol was used to elucidate the underlying mechanisms typical of sEVs and their role in the development of breast cancer metastasis, demonstrating that sEVs can induce metastasis through miR-106b-5p/PTEN/AKT/PD-L1 and miR-18a-5p/PIAS3/STAT3/PD-L1 in tumor-associated macrophages. Fig. 3d shows miRNA screening in enriched plasma sEVs from metastatic BC patients. To find the relationship between miRNA and metastasis, 5 healthy volunteers, 5 patients with early BC (Stage I) and 5 patients with advanced BC (Stage IV) were selected for plasma sEV-miRNA sequencing. As reported in Fig. 3d–a total of 42 miRNA expressions were found differentially upregulated in each group based on the multiple difference of 2, and they were higher in the metastasis group > early group > normal group. Enrichment analysis showed that differential sEV-miRNAs were significantly associated with BC, also confirming the tumor specificity of secreted sEV-miRNAs (Fig. 3d). Furthermore, the analysis showed that the differential sEV-miRNAs were significantly correlated with the immune and inflammatory system, and the target gene enrichment analysis showed that the differential sEV-miRNAs were significantly correlated with the immune molecules such as FOXP3 and E2F1. These results indicated that these sEV-miRNAs represent important regulatory factors connecting tumor and immune cells in the TME.

Although the extraction process involves several steps and requires other reagents, which leads to experimental difficulties, TRIzol represents a very reliable reagent for the extraction of genetic material from biological samples and has a wide range of applications in molecular and cellular biology. TRIzol was employed to elucidate the underlying mechanisms typical of sEVs and their role in the development of breast cancer metastasis, demonstrating that sEVs can induce metastasis through miR-106b-5p/PTEN/AKT/PD-L1 and miR-18a-5p/PIAS3/STAT3/PD-L1 in tumor-associated macrophages.

Although the extraction process involves several steps and need other reagents, which leads to experimental difficulties, TRIzol represents a very reliable reagent for the extraction of genetic material from biological samples and has a wide range of applications in molecular and cellular biology.

2.2.5. Water

A very minor approach to opening the membrane of EVs relies on the osmotic pressure exerted by the use of water. Although this procedure can affect EV membranes, it is usually not sufficient to completely rupture exosomal membranes, but its use has been explored. As in the study by Que et al. [146] in which exosomal proteins and miRNAs are separated, designed to study the potential value of miRNA-depleted exosomes for antitumor activation of cytokine-induced dendritic cells/killer cells (DCs/CIKs) against pancreatic cancer. Here the authors resuspend isolated exosomes in 1 mL of double-distilled H_2O shake vigorously for 1 min and use electron microscopy to validate breakage of exosome with this fast procedure. The exosome lysate is then treated to deplete free miRNAs in the 100 kDa cut-off lysate followed by two centrifugations at 4000 g for 30 min. The lysis procedure used was confirmed by RNA electrophoresis and proteome analysis. In the study by Kolonics et al. [147] employ hypotonic water lysis to conduct a comparative investigation of three distinct types of EVs produced by neutrophilic granulocytes with the goal of understanding how these vesicles modulate various cell and blood-related functions. Since zymosan residues resulting from cell activation are an intrinsic and inseparable part of EV fractions, after the isolation process they were sedimented at 15,700 g, 10 min, 4 °C, resuspended in distilled water, vortexed for 10 min and again sedimented and resuspended in Hypotonic Buffer HBSS. In this way they achieved that the relevant EV fractions were destroyed due to hypotonic lysis and mechanical destruction while keeping the zymosan particles resistant to this procedure.

2.3. Extraction solution and commercial kits

A more commonly used approach in the literature to lyse EVs in order to characterize proteins inside or outside the EV membrane or to determine genetic material is related to the use of extraction solutions such as RIPA buffer or the use of Commercial kits that support extraction in an easy-to-use manner. RIPA buffer, Radio-Immunoprecipitation Assay, is a formulation consisting of several components that work together to lyse the cells and stabilize the extracted proteins [148].

The uses of RIPA formulation is able to extract total or membrane/internal proteins released from lysis of EVs by recovery of membranal protein in the pellets and internal protein in the supernatant after incubation.

The typical composition of an RIPA buffer may include components such as Triton X-100 or NP-40, small percentages of SDS, NaCl, sodium orthovanadate (Na_3VO_4), glycerol, EDTA, Tris-HCl buffer and protease inhibitors (PI). Most researchers can purchase or create a RIPA solution to determine the protein content inside EVs typically by using the WB technique [66,149,150]. This preparation it possible to extend for other lysate preparation as for example the lysate preparation for RNA or DNA separation. Although this formulation is compounds more or less followed by the addition of this reagents into this proportions: 150 mM NaCl, 1.0% of NP-40 or Triton X-100, 50 mM Tris (pH 8.0), 0.5% of sodium deoxycholate and orthovanadate and 0.1% SDS this formulation is possible to change or adjust the concentration of the reagents at second of needed. The improving of this formulation can required the additional reagent or procedure to obtain the results as expected. Generally, researchers use this lysis procedure under sonication to achieve complete membrane disruption and usually perform centrifugation to clarify the contents and separate the membrane contents from the lumen contents. RIPA offers the great advantage of obtaining a total protein lysate of the EVs it is possible to use RIPA to perform the entire protein extraction procedure and ensure that the target protein is in the membrane or outside the membrane of the EVs/exosomes. The work of Arteaga-Blanco et al. [151] used RIPA buffer to determine the presence of small EVs released from primary human macrophages. Their protocol involved incubating the samples (50 μL) with RIPA buffer (30 μL) with PI and on ice for 10 min followed by three sonication (60 Hz frequency) in a water bath and vortex to ensure protein homogenization and lysis of the membrane. Fig. 4a shows Western blot application on the produced lysates where the authors observed that EVs showed lower protein content than mother cells and that endosomal sorting complex required for transport (ESCRT) proteins were present in the vesicles ad tetraspanin proteins, such as Alix, CD-63, and CD-81, as well as in the whole cell lysate reported in Fig. 4a lower panel.

However, the absence of markers for cytochrome *c* (mitochondria) and calnexin (endoplasmic reticulum) in the vesicle lysates indicates that the EV preparations were pure and not contaminated with cellular components. Furthermore, to confirm the presence of EVs of endosomal origin, the authors test different sucrose density gradient ultracentrifugation (S-DGUC) collection fractions. Fractions numbered F1 to F6 were lysed with the same protocol and subjected to blotting analysis for CD-63 and CD-81 markers as shown in the figure. Here this lysis procedure was satisfactory for the authors who managed to fractionate the EVs populations showing that CD-63 colocalized with CD-81 in fractions F2 to F4 with densities between 1117 and 1181 g/mL.

Among the study of typical protein of EVs population also Sun et al. [152] used a RIPA buffer for lysis procedure determining the presence of CD-63, TSG101, β-actin, and also other proteins such as RASA1, E-cadherin, vimentin and GAPDH in exosome derived from colon cancer (CRC) cell lines. This study, starting with the identification of the RASA1 protein, furthered the understanding of migration, invasion and exosome-dependent invasion in the epithelial-to-mesenchymal transition (EMT) of CRC cells.

Similar to work by Arteaga-Blanco et al., Pachlel et al. [153] identified a lot of proteins as CD-9, CD-81, TSG-101, GM-130, CD-90, CD-105, CD-14, CD-34, CD-45, CD-73, and HLA-II in EVs released from mesenchymal stromal cells by lysing with RIPA buffer incubation for 15 min at 4 °C and centrifugation to recovered and analyzed their distinct content (lumen and membrane).

Like the versatility of RIPA, a common approach used by researchers to characterize protein and/or nucleic acid content is to use commercial kits. These kit or most of them are able to perform both characterization/identification of markers in EVs as protein (like membranal protein to attribute the exosomes vesicles extraction) and genetic material (DNA, RNA or specific miRNA).

To date, various commercially available kits are compatible with different analytical techniques, and generally equipped with everything necessary to carry out the lysis e.g., lysis buffer with/or genetic material extraction solution. The use of commercial kits constitutes a valid support for carrying out a practical, rapid and productive lysis by following the steps suggested by the manufacturer. Since there are several commercial kits, only the most commonly used kits will be discussed in this review.

An example of these kits is Exo-check which contains all the components necessary to lyse and label EVs in a rapid and practical way providing qualitative analysis using dot arrays [154]. This method was utilized by Hsu et al. [155] to confirm the existence of protein marker as CD-63, CD-81, ALIX, FLOT1, ICAM1, EpCam, ANXA5 and TSG-101 in exosomes from healthy individuals, as well as those with lung and breast cancer.

In the extraction of genetic material, miRVana kit is the most widely used due to its selective extraction, high yield and practical procedures [108,156,157].

This kit is designed for the extraction of RNA, DNA and miRNA from biological samples such as cells, tissues or body fluids achieving quality and purity in the extraction [158,159]. It has wide compatibility with major analysis techniques such as RT-PCR, WB and NGS making it widely exploited.

An example of the application is reported in the work [160]who used the miRVana kit to determine a panel of four miRNAs (−19b-3p, -17-5p, −30a-5p, and −106a-5p) for gastric cancer. In Fig. 4b a quantification of miRNA overexpression identified by the analysis of exosomes released from the serum of a cohort of 20 healthy controls and 20 individuals with gastric cancer in the initial screening phase. The authors report

Table 2
All the features associated to the techniques applied to EVs lysis and information extraction.

Target	Protocol	Sample	EVs isolation method	Analytical Method	Reference
Triton-X and triton-X buffer based					
CD-55, CD-59	Lysis buffer contening 1% Triton X-100, 10 mmol/L Tris/HCl pH 7.4, 1 mmol/L EGTA, 5 mmol/L EDTA, and P.I. for 15 min at 4 °C with intermittent stirring;	RBC	UC	Flow cytometry and Western Blotting	[100]
CD-55,CD-59,CD-46	A solution of 5% Triton X-100 in complement fixation diluent for 5 min and with use of lysis buffer containing 2% NP40, 1 mM PMSF, 1 μg/mL pepstatin and leupeptin, 10 mM EDTA for 30 min;	CM and human serum	UC and Dyna-beads	Flow cytometry and Western Blotting	[101]
EpCAM, α-IGF-1R, p-IGF-1R and CA125, CD-9, CD-81, and CD-63	Lysis with use of 5% Triton X-100 for 5 min;	Human plasma from NSCLC (OVCA), and HD	UC and immunomagnetic beads microfluidic platform	Microfluidic chemifluorescence analysis, Western blotting and ELISA	[102]
CD-9, CD-63, CD-8, Syntenin-1 TSG101, ADAM10, EHD4, Actinin-4, Lamp2, Annexin II, Annexin XI, MHC II, Mitofilin, GP96 HSC-70 and proteomic analysis	Lysis with 50 mM Tris, pH 7.5, 0.3 M NaCl, 0.5% Triton X-100, 0.1% sodium azide with a mixture of antiproteases. Incubation for 20 min on ice, then centrifuged at 18,500×*g* for 15 min.	Monocyte-derived from blood samples of HD and HEK293T, RPE-1, HeLa-CIITA, MDA-MB-231, SHIN, IGROV-1, and OV2008	Sucrose and Iodixanol density gradient UC	Wester blotting and LC-MS/MS	[103]
Ox40L, CD-81, perforin, MIP-1β	Exosomes lysed with incubation of 0.3% Triton-X for 10 min	Human plasma	SEC	Flow cytometry	[104]
RNA and Proteomic Analysis	1% Triton X were incubated in exosomes solution at RT for 30 min with and without RNase A and DNase at a final concentration of 100 units/ml. mRNA was extracted with use of RNeasy Mini Kit	Human Saliva and human keratinocytes (OKF6tert1)	UC	Microarray analysis RT-PCR, 2-DIGE and mass spectrometry analysis	[105]
hsa-miRNA-148a-3p	Exosomes were incubated for 30 min at 37 °C with 10 μg/mL of RNase A with and without 1% of Triton-X. Total RNA was subsequently purified using QIAzol Lysis Reagent by the chloroform/phenol method	Bovine milk and human liver cancer cells (HepG2) and colorectal adenocarcinoma cells (Caco-2)	UC with SEC	Microarray analysis and RT-PCR	[106]
SDS and SDS-buffer based					
GALNS, actina, CD-90, CD-73, and CD-105, CD-14, CD-20, CD-34, and CD-45	Lysis with 1% SDS solution	Umbilical mesenchymal stem cells from HD	UC	Western blot and Flow cytometry	[107]
CD-63, Alix, CD-9, Calpain 1, Syntenin, VDAC1, Calreticulin, GPDH, β-actin and proteomics	SDS sample buffer in ice-cold 100 mM Na_2CO_3 pH 11 for fractionation lumen and membrane. Exosome suspensions were sonicated 2 × 5 s with intervals of cooling on ice and incubated on ice for 1 h to break open the exosome	Human bladder carcinoma cell lines T24, SLT4 and FL3	UC	Western blot and LC-MS/MS	[108]
TSG-101, CD-9 and proteomics	20 min on ice with lysis buffer (4% SDS, 0.1 M Tris–HCl, pH 7.6)	human serum samples	UC and kit	Western blot and LC-MS/MS	[109]
CD-63, TSG-101, albumin, calnexin and PKM2, and β-actin	50 mm Tris-HCl pH 6.8, 2.2% SDS, 5.5% glycerol and 1 mm PMSF)	SW-480 and HCT-116, HEK-293T and human serum sample	UC and Total Exosome Isolation Kit	Western blot and Flow cytometry	[110]
NP-40 buffer based					
RNA	NP-40 lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 0.5% (v/v) NP-40) on ice for 5 min	SGC 7901,Lovo, GES-1, MCF-7, HepG2 and HeLa cells lines	Magnetic beads	LAMP	[111]
CD-63, CD-9, CD-81, class II MHC, CD-86, CD-107b and CD-41a	Lysis buffer [20 mM Tris–HCl (pH 7.4); 140 mM NaCl; 2 mM EDTA; 50 mM NaF; 1% NP-40; 0.5% Na deoxycholate; 100 μM Na_3VO_4; 2 μg/mL antipain, pepstatin and leupeptin; 1% aprotinin and 1 mM phenylmethylsulfonylfluoride for 20 min at 4 °C.	Human plasma and Human leukemia mast cell line HMC-1 and PBMCs cell line	UC	Western blot and Immunofluorescence analysis	[112]
IL-6, CCL2, TNFa,NF-kB a, GCSF and CD-63, GAPDH	Exosomes were resuspended in NP-40 lysis buffer (20 mM Tris, 150 mM NaCl, 1% NP-40, 0.1 mM EDTA) containing PI cocktail	MDA-MB-231, MCF7 and MCF10A cell lines, Raw 264.7 cells, MyD882/2, TLR2/42/2 and TLR22/2 bone marrow	UC	Western blot, Flow cytometry, RT-PCR	[113]
ADAM10/17, CD-9 and MICA protein	1% NP40 lysis buffer in 20 mM Tris-HCl (pH 7.4) containing 150 mmoL/l NaCl and 5 mM EDTA with protease and PI aprotinin, leupeptin, phenylmethanesulfonylfluoride (PMSF), sodium pyrophosphate, Na_3VO_4 and NaF for 25 min	MDA-MB-231, PC-3 Panc89, PancTu-I, NKL and C1R-MICA*004 cell lines	ExoQuick Exosome Precipitation Solution	Western blot and Flow cytometry	[114]
Saponin					

(*continued on next page*)

Table 2 (*continued*)

Target	Protocol	Sample	EVs isolation method	Analytical Method	Reference
pDNA	Saponin with sonication in water bath	IC21 macrophages	UC	RT-qPCR	[115]
Flotillin-1, Alix, Calnexin, GAPDH and a-synuclein	0.1% (w/v) saponin	N2a cell line	UC	Western blot	[116]
ALIX,2H12,PDI,SPA-890, SPA-600, α-1 antitrypsin, GAPDH, CD9, MM2.57, TGFβ, actin, EGFR, EGFRvIII, GPNMB	Laemmli sample buffer with 2-mercaptoe-thanol and 1% saponin	human serum from glioblastoma multiforme	UC	Western blot and Flow cytometry	[118]
Calcein	Saponin at different concentration 1 μg/mL, 10 μg/mL, 100 μg/mL and 1 mg/mL for 20 min	RBCs, human plasma and CM from HAECs cell line	Centrifugation	Flow cytometry	[117]
Tween-20 and Tween-20 buffer-based					
CD-63, TSG-101, EGFRvIII	Tween-20 was used for EV lysis	U87 cell line and plasma samples from HD and cancer patients	ACE chip	Alternating current electrokinetic (ACE) microarray chip devices and RT-PCR	[119]
HER-2	0.5% Tween-20	SKBR3 cell line and sera from HD and tumor patients	UC	Western blot and flow cytometry	[120]
CCR7, CXCR4 or HLA-A2, Lactadherin C1C2	Lysis Buffer (MLBII) consisting of 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole and 0.5% Tween 20 and a cocktail of PI for 30 min on ice	D2SC-1 cell line	UC	Western blot and ELISA	[121]
4-hexylphenylazosulfonate (Azo)					
Proteomic analysis	0.1% Azo in 25 mM ammonium bicarbonate thermoshakered at 37 °C and 600 rpm for 10 min and sonicated in bath for 10min. Treated with 25 mM TCEP and 50 mM 2-chloroacetamide at 37 °C and 600 rpm on a thermoshaker for 30 min (pH 8.5). Digested with 50:1 (w/w) protein: trypsin for 1 h at 37 °C. 10 min of UV lap and then centrifuged at 21,000×*g* for 15 min	Fibroblast cell line	UC	LC–MS/MS	[129]
RIPK3 and proteomics analysis	0.05% Azo in 25 mM ammonium bicarbonate pH 7. Treated with 100 mM TCEP and 500 mM of 2-chloroacetamide at RT for 30 min. Digested with 0.3 μg/μl Trypsin solution at 37 °C for 1 h. 5 min of UV lamp and then vortexed and centrifuged at 20,000×*g* for 2 min.	Mouse embryonic fibroblasts and HT-29 cell line	Exoquick and qEV SEC	LC–MS/MS and Western blott	[136]
Ammonium and urea buffer					
Ubiquitin, K-48, K-63 and proteomics analysis	Exosomes were lysed in an optimized lysis buffer of 8 M urea in 50 mM ammonium bicarbonate with 50 μM of deubiquitinase inhibitor PR-619 and 1% of a PI cocktail	myeloid-derived suppressor cells	UC	LC-MS/MS and Western blot	[137]
TSG-101, CD-14, CD-59 protein and peptdite analysis	Exosomes were chemically lysed by sequential addition of ammonium acetate in methanol and acetone.	10 HD urine samples	UC	LC-MS/MS and Western blot	[130]
MUC1-M2, flotillin-2	Buffer solution costituited of 7 M urea, 2 M thiourea; 10 mM Tris, 4%w/v CHAPS, 50 mM DTT, PI cocktail, pharmalytes (pH 3–10)	MCF-7 cell line	UC	2-D Electrophoresis	[169]
Proteomic analysis	Pretreatment with 8% SDS, 100 mM Tris, pH 7.6, and 0.2 mM DTT, followed by sonication and heating to 95 °C. The cooled samples were then treated with 8 M urea buffer in 100 mM Tris, pH 8.5 followed by two centrifuges with a molecular cut-off of 30 K at 10,000×*g* for 15 min.	Maternal plasma semples from 61 womens	UC and SEC	LC-MS/MS	[138]
Methanol					
Acetylcholinesterase enzymatic activity	Exosomes lysed in 100% methanol and sonicated to disrupt exosome membrane integrity	PANC-1 MIA and PaCa-2 cell lines	UC	UV–vis	[133]
Metabolites analysis	The exosomes were then lysed and extracted with 50% MeOH and freeze–thaw cycles (five times).	Human serum samples from pancreatic cancer patients: before and after chemotherapy	UC	LC-MS/MS	[134]
Proteomic analysis	Exosomes were dissolved in pure methanol and sonicated on ice for 1 h and centrifuged at 8000g for 5 min. Freeze-dried samples redissolved in the ammonium bicarbonate buffer (25 mM, pH 8.0), reduced by 50 mM DTT at 37 °C for 2 h, and then alkylated with 55 mM IAA for another 1 h at RT in dark.	Serum sample	Total Exosome Isolation and macroporous graphene foam (GF) and the amphiphilic periodic mesoporous organosilica (PMO)	LC-MS/MS	[128]

(*continued on next page*)

Table 2 (*continued*)

Target	Protocol	Sample	EVs isolation method	Analytical Method	Reference
	Then, methanol was added, and sonicated on ice for 15 min.				
Water					
IL-8, E-selectin and VCAM-1	Hypotonic lysis and mechanical disruption using distilled H_2O and vortexed for 10 min, then sedimented again (15,700 g, 10 min, 4 °C), and resuspended in HBSS	Blood samples from HD	UC	ELISA, UHPLC with nanoelectrospray-MS	[147]
TNF-α, Perforin and proteomic analysis	The pelleted exosomes were resuspended in 1 mL of low osmotic pressure double-distilled H_2O and shaken vigorously for 1 min miRNA were extracted with miRNeasy mini kit	PANC-1 cell line	UC	Western blot and LC-MS/MS	[146]
TRIzol					
miRNA-636, miRNA-21, miRNA-16, miRNA-142-3p, and miRNA-451	Total RNA was isolated from the exosome's pellets using TRIzol	Patient plasma, serum, buffy coat, and urine from metastatic Prostate cancer (N = 224) samples from localized and metastatic patient)	ExoQuick-TC	Microarray analysis and RT-qPCR	[142]
miRNA-21-5p	Total RNA exosomes using the TRIzol™ Reagent	Serum samples from 30 patients with breast, liver, ung, cervical cancer, and ovarian cancers	UC	Microarray analysis and RT-PCR	[74]
miRNA-205	Exosomal miRNA was extracted using TRIzol reagent	Human brest cancer cells and MCF-7/TAMR-1 cell line	ExoQuick	Microarray and RT-qPCR	[144]
miRNA-106b-5p/PTEN/AKT/PD-L1 and miRNA-18a-5p/PIAS3/STAT3/PD-L1	Total RNA from sEVs was extracted using a Trizol ls Reagent	Plasma samples from HD and patients with early Breast Cancer (Stage I) and patients with advanced cancer. MDA-MB-231 and THP-1 cells and BALB/c mice	UC	RT-qPCR and NGS analysis	[131]
miRNA-122, miRNA-125b, miRNA-145, miRNA-192, miRNA-194, miRNA-29a, miRNA-17-5p, and miRNA-106a	Total serum exosome RNA was extracted by TRIzol	Human serum from 85 patients HCC and 30 healty controls	Total Exosome Isolation Kit	RT-PCR	[145]
Extraction solution and commercial kits					
CD-63, TSG101, β-actin, RASA1, Ras, E-cadherin, vimentin and GAPDH	Exosomes were lysed in RIPA buffer supplemented with a protease inhibitor	Colonrectal cancer cell lines SW480 and SW620	UC	Western Blot	[152]
CD-9, CD-63, Tim-4	RIPA lysis buffer containing 1 mM PMSF at 4 °C for 45 min.	HepG2 cells and human serum from 3 liver cancer patients and 3 HD	Magnetic beads immobilized on electrode surface and ExoEasy kit	ExoPCD-chip, Flow cytometry, ELISA and Western Blot	[170]
CD-9, CD-81,TSG-101, GM-130; CD-90,CD-105, CD-14, CD-34, CD-45, CD-73, HLA-II	EVs pellet were lised with RIPA buffer for 15 min at 4 °C, centrifuged at 12,000 g at 4 °C for 10 min.	Mesenchymal stromal cells	UC	Western Blot and Flow cytometry	[153]
mRNA and miRNA	Microvesicles diluite into RNase A at 100 μg/mL and incubated for 15 min at 37 °C. Total RNA was then purified using the MirVana RNA isolation kit	Primary cells obtained from 3 glioblastoma tumors and serum patients	UC	RT-PCR	[156]
miRNA-21, miRNA-141, miRNA200a, miRNA-200c, miRNA-200b, miRNA-203, miRNA-205 and miRNA-214	MirVana microRNA Array Labeling Kit	Sera derived from women diagnosed with serous papillary adenocarcinoma of the ovary (n = 70 at from beginning at different stages and age-matched HD with no evidence of ovarian disease)	Magnetic activated cell sorting (MACS)	Bioanalyzer Agilent 2100 with use microarrays containing probes for 467 human mature miRNAs.	[157]
miRNAs	Lysis buffer consist of 10 mM triethanolamine, 250 mM sucrose, proteinase inhibitors and deionized water at pH 7.6; miRNA were extracted with use of miRNeasy lysis buffer (Qiagen, Valencia, CA, USA)	Human saliva	UC	RT-PCR	[171]
mRNA	MirVana AmbionWT Expression Kit	Human bladder carcinoma cell lines T24, SLT4 and FL3	UC	Affymetrix GeneChip miroarrays	[108]
miRNA-18a, miRNA-221, miRNA-222 and miRNA-224	Exosomal RNA extraction kit	60 patients positive to hepatitis B surface antigen (n = 60) divided into different groups	ExoQuick Exosome Precipitation Solution	RT-PCR	[172]
miRNA-4448, miRNA-2392, miRNA-2467, and miRNA-4800	Exosomal miRNA was extracted using the miRNeasy Mini Kit	Triple-negative breast cancer patiens (n = 24)	ExoQuick	Microarray analysis and RT-qPCR	[173]

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Table 2 (*continued*)

Target	Protocol	Sample	EVs isolation method	Analytical Method	Reference
miRNA-21	Total RNA was isolated from exosomes by using miRNeasy Kit.For RNase treatment, purified exosomes were incubated with 20 μg/mL of protease-free RNase A for 30 min at 37 °C.	Cell lines MCF-7, HeLa and HEK-293; human serum from HD and breast cancer	UC with ultrafiltration ad eluition with exoEasy Maxi Kit	Platform of double-accelerated DNA cascade amplifier nanostructure in situ and RT-qPCR	[174]
miRNA-21, miRNA-210 miRNA-21-5p, miRNA-21-3p, miRNA-191-5p	Total RNA purification kit	A549, BEAS-2B, MDAMB-231, and MCF-10A cell lines and human samples	Total exosome isolation	A platform microRNA one-stop Exo-PROS biosensor and IMS-PCR	[155]
miRNA-21, miRNA-155	Total miRNA was extracted from the exosomes using miRNA extract reagent (Sangon)	Human plasma from 5 HD and 8 lung cancer patients	UC	metal–organic framework paper-based electrochemical biosensor and RT-qPCR	[175]
CD-63, CD-81, ALIX, FLOT1, ICAM1, EpCam, ANXA5, and TSG-101; EGFR and LG3BP	Exo-Check Exosome Antibody Arrays buffer	A549, BEAS-2B, MDAMB-231, and MCF-10A cell lines and human samples from HD, lung cancer patients, and breast cancer	Exoquick exosome isolation kit	Exo-check and ELISA	[155]

List of table abbreviations: RBC Red blood Cells; UC Ultracentrifugation; EDTA Ethylenediaminetetraacetic acid; PMSF phenylmethylsulfonyl fluoride; P.I. proteinase inhibitors; NSCLC non-small-cell lung cancer; HC Healthy donors; SEC Size exclusion chromatography; Tris Tris(hydroxymethyl)aminomethane hydrochloride; LAMP Loop-mediated Isothermal Amplification; CM Culture media; TCEP tris(2-carboxyethyl)phosphine; CHAPS 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate; DTT Dithiothreitol; HBSS Hanks' Balanced Salt Solution.

overexpression of miR-19b and miR-106a in individuals with gastric cancer compared to healthy controls ($P < 0.0001$) with ROC curves that yielded AUC values of 0.786 for miR-106a-5p and 0.769 for miR −19b-3p, managing to discriminate with an AUC equal to 0.814 in discriminating gastric cancer patients from healthy ones. These findings suggest that serum exosomal miRNA-19b-3p and −106a-5p may represent new potential biomarkers for the detection of gastric cancer. And still other studies [161–163] use it to determine and identify specific miRNAs such as in Kobayashi et al. [164] work in identifying let-7 and miRNA-200 family miRNAs in exosomes released from ovarian cancer cells and in their exosomes. In this context the important work that aimed to profiling miRNAs by next-generation sequencing [165] used exoRNeasy in comparison with miRCURY to RNA extraction with these commercial kit. In this clinical approach the researchers conduct the study with four patients with sepsis and five patients in septic shock with the sex-matched 10 healthy volunteers. A combination of different methods of isolations the authors the authors conclude that miRCURY was more accurate showing a 3.5–5 times higher percentage of mapped miRNAs, respectively. In agreement with this result other important works confirms the high recovery of miRNA obtained by using of mIRCURY kit [166–168]. Although the use of commercial kits offers quick procedures and less chance of operational errors, it should be noted how the big disadvantage is the high cost.

To provide a practical guide to the main chemical lysis methods reported in the literature, the various classes of reagents used with the relevant procedures and the targets detected have been collected in Table 2.

3. Conclusion and perspectives

Given the promising potential of extracellular vesicles in medicine, the practical and rapid determination of their content poses a significant challenge from isolation to extraction bioinformation content.

In this review, we provide an overview of the chemical reagents mainly used for the lysis of the lipid bilayer of EVs, collected and described to compare the different approaches in terms of time, experimental procedure, and especially according to the analytical technique used.

As discussed, the reagents used are often chosen based on the lysate analysis technique. To this end, we have seen detergents such as triton-x, SDS and NP-40 as protagonists in western blotting techniques, and chaotropic reagents, methanol and azo compounds as protagonists in mass spectrometry. There are several reference approaches for the extraction of proteins present outside and/or inside the membrane of EVs. Depending on the target and technique used, researchers define their goal and attempt to validate protocols. Certainly, the approach using the RIPA solution allows a total protein lysate contained in EVs to be obtained the position of a sought-after protein to be assigned if there is any doubt as to whether it is in the membrane or within the membrane.

Unfortunately, there is currently no standard procedure for protein extraction.

The situation is different for the extraction of genetic material for RT-qPCR analysis, which instead has a procedural standard based on the application of TRIzol which, however has the disadvantage of being a laborious and delicate procedure requiring the intervention of specialized personnel.

The choice to use the ease and speed offered by commercial kit procedures provides analysts with everything they need to extract and analyze specific content such as genetic material. This choice, however, has the downside of the high purchase cost of these kits, despite the fact that they offer excellent yields and standardization of procedures, as demonstrated by research conducted on clinical patient cohorts.

Clearly, identifying a single protocol or reagent capable of extracting both protein markers, including sensitive ones such as phosphorylated proteins, and genetic material proves challenging. The selection of specific approaches often involves compromises on certain markers.

Precise and targeted experiments are essential to evaluate reagents capable of fully characterizing both protein and genetic material, while avoiding contamination or compromise of the content. This currently stands as the foremost challenge in this crucial procedure: identifying a reagent or, ideally, a combination of two reagents capable of achieving this goal.

This review was conceived with the aim of collecting, within a single document, all the most commonly used chemical reagents for EVs lysis according to the specific analysis and extraction technique of the definite target(s). We have written this tutorial to help researchers address this crucial question and guide them through the procedural approach, providing them with advantages and disadvantages of different approaches in terms of time, feasibility, and instrumentation required by focusing the analysis on a specific research content, e.g. proteins or nucleic acids.

This review specifically intends to encourage the development of innovative analytical methods, such as point-of-care platforms designed to measure the contents of EVs. These advancements hold significant promise for early diagnosis, particularly in cases of cancer.

Although, as already mentioned, the main limitation in collecting information on EVs is the requirement to lyse and extract the target(s),

and there is currently no standard procedure that has been widely accepted, the collection of the different approaches reported here could lead to the goal of creating and/or implementing modern, state-of-the-art platforms in analytical science.

CRediT authorship contribution statement

Sabrina Romanò: Writing – original draft, Methodology. **Valeria Nele:** Writing – original draft, Data curation. **Virginia Campani:** Writing – original draft, Data curation. **Giuseppe De Rosa:** Writing – original draft, Supervision. **Stefano Cinti:** Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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