Editorial

Blu-ray beyond music and movies—novel approach to diagnostics measuring specific extracellular vesicles

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Comment on: Kabe Y, Suematsu M, Sakamoto S, *et al.* Development of a Highly Sensitive Device for Counting the Number of Disease-Specific Exosomes in Human Sera. Clin Chem 2018. [Epub ahead of print].

Received: 17 September 2018; Accepted: 30 September 2018; Published: 11 October 2018.

doi: 10.21037/jlpm.2018.09.12

View this article at: http://dx.doi.org/10.21037/jlpm.2018.09.12

Cancer diagnoses are mainly based on clinical information, scope or imaging techniques and biopsies. In blood samples, only a couple of general markers as hemoglobin and C-reactive protein (CRP) and a few more specific tumor markers with rather poor predictive values are currently available. Discovery of new and better markers for cancer in blood to be used as so-called "liquid biopsies" is therefore desirable. It has recently been demonstrated that blood contains numerous extracellular vesicles (EVs), which are small membrane-enclosed "sacs" (1) (as further described below). EVs hold great potential as circulating cancer markers, since they incorporate elements from their parent cells which may be cancer cells (2), but it is, however, technically challenging to detect and characterise EVs. In a recent paper by Kabe *et al.* in *Clinical Chemistry* (3) a new promising technique for detecting smaller EVs was presented which may improve their potential as diagnostic markers. In the following, we will first shortly describe EVs and methods for measuring these and then specifically describe this new technology and the results presented in the paper (3).

All body fluids contain a large number of EVs, which may be released from most cells (1,2,4). Because EVs have similar physicochemical properties to cells and the bulk of EVs are smaller than 1 µm, they are below the detection limit of conventional methods such as light microscopy, and, therefore, their presence and significance have remained elusive until recently. With the advancement of the field and the development of novel, highly sensitive methods in the past decade, their characterization can be accomplished with various techniques, and an increasing number of papers on this topic are published.

Although no internationally accepted nomenclature exists, EVs are usually divided into exosomes, microvesicles (MVs) and apoptotic bodies (1). Exosomes are the smallest vesicles with sizes suggested to be below 150 nm created by inward budding of endosomes, and released into the extracellular space by fusion of the endosome to the plasma membrane. MVs are larger than exosomes with sizes ranging from 100–1,000 nm and are created by outward budding of the plasma membrane and subsequent constriction and detachment from it. The rarer apoptotic bodies are produced during apoptosis and can achieve sizes of up to 3 µm, thereby being the largest subtype of vesicles found in biofluids. Regardless of biogenesis, EVs may contain proteins, various species of RNA, DNA, lipids, and metabolites that being inside the vesicles are protected from proteolytic, lipolytic and nucleolytic enzymes present in biofluids and, further, non-soluble proteins can be transported as membrane bound proteins and exert their biological function in surrounding or distant tissues. EVs proposedly have numerous functions as intercellular and intraorganism communication shuttles in health and disease by various mechanisms: e.g., through receptor-ligand interactions, in which EVs bind to the surface of a cell activating intracellular signaling pathways; or by transporting and delivering a cargo to recipient cells as e.g., proteins, metabolites, or various types of RNA including micro RNAs (miRNA) regulating mRNA translation (1,2,4).

Thus, EVs are involved in several physiological and pathophysiological processes and since cancer cells release

EVs, it has been speculated that they might yield great potential as biomarkers for cancer diseases if specific markers, i.e., proteins on the surface of the EVs, are present. If so, blood samples could be used as liquid biopsies in which the detected EVs could contribute to diagnosis and perhaps prognostication.

However, due to their physicochemical properties, detection and characterization of EVs remain challenging. Nonetheless, novel techniques for counting vesicles and determining their sizes have been developed, including nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS) (5). However, as these methods only make use of the physical properties of EVs, it becomes impossible to discriminate between EVs and other particles with similar sizes or refractive indices including lipoproteins, protein aggregates, and salt crystals. Therefore, results have heavily relied on whether or not EVs were isolated from biofluids by differential ultracentrifugation, size exclusion chromatography (SEC), or affinity-based purification. All isolation procedures have pros and cons, thereby also affecting the results of quantification and characterisation, and no consensus exists on purification protocols (6,7). Another commonly used method for quantification and characterisation of EVs is flow cytometry (FCM). Conventional and newer flow cytometers have a lower detection limit of 200–500 nm depending on the model and methodology and are therefore incapable of detecting smaller EVs. Nevertheless, FCM has been considered by many to be the gold standard for detection and characterisation of EVs directly in biofluids as it is possible to label EVs with fluorescent antibodies specific for their parental cells and characterise large amounts of particles within a short span of time. In recent years, ultrasensitive high-resolution flow cytometers have been developed specifically for analysis of small particles. However, the performance of these platforms has not yet been fully validated. Other specific tests have been published as ELISA test (8) or EV array (9) but they are primarily semiquantitative (relative amounts).

Thus, the present methodology has limitations in detection of EVs, and the new method described by Kabe and coworkers termed ExoCounter (3) seems to be a promising and innovative method. In the paper, the principle of the method, which is novel, is presented, and the authors describe an extraordinarily ability of the method to detect and quantify specific EVs (consistently called exosomes throughout the paper) based on their expression of surface proteins, and perform a benchmark with several different EV characterization methods on cell culture supernatant from several cell lines, and serum from healthy and diseased individuals.

The method is schematically described in Figure 1 in (3). In principle, this method revolves around modified Blu-ray technology coupled to a pulse-sensing circuit, in which changes in backscatter of a laser beam illuminating the Blu-ray disc can be detected. Antibodies specific to a desired surface marker are bound to a specially designed Blu-ray disc (with a modified surface structure) capturing exosomes in the samples. Detection is achieved by adding a secondary antibody bound to a magnetic nanobead, which can be detected optically by the laser in the Blu-ray equipment. The surface area of the Blu-ray discs consists of small grooves with a diameter of 160 nm at the bottom and 260 nm at the top, and the regions between grooves have a width of 60 nm. This means that there is space in the grooves for exosomes and the magnetic nanobeads (stated in the paper to be 200 nm), but binding between the grooves is unlikely. On top of the disc, a specific plate containing 16 wells for the samples can be attached. In the experiments, each well was coated with antibodies against CD9, a protein enriched in the membrane of exosomes. Therefore, exosomes in samples added to the wells will bind to the anti-CD9 antibodies and be immobilized on the disc. Next, antibodies against specific surface markers on exosomes conjugated to the magnetic nanobeads (called FG beads) are added to the wells under a magnetic field (which makes the reaction very quick) and immobilized on the bound exosomes. Finally, after washing, the presence of the specific FG beads can be detected by the optical system in the Blu-ray device and counted as pulses by the pulse sensing circuit. Scanning electron microscopy was used to visualize binding of exosomes and FG beads in the grooves of the Blu-ray disc and confirms the pulse interpretations, and the method appears to be both specific and reliable.

Several attempts were made in the paper to validate ExoCounter. First, the authors investigated the ability of ExoCounter to detect exosomes from various biofluids by using FG beads conjugated to anti-CD63 antibodies, another common protein enriched on the surface of exosomes. The dose-response relationship of this method was studied by using increasing amounts of exosomes from culture supernatant expressed in protein weight or increasing volumes of serum. Here, the authors demonstrated linearity with an impressive R^2 of more than 0.99 for purified exosomes from cell culture supernatant, while a slightly lower R^2 of 0.96 was observed for serum.

Based on the dose-response experiment, the authors calculated that the coefficient of variation for exosome concentrations was less than 10.2% for four measurements at nine different concentrations, which is appropriate for a method of this nature. The recovery of exosomes on the disc was determined to be 89.8% on average by subsequently incubating the non-pulldown fractions of the purified exosomes and counting the number of residual exosomes, and the recovery rate seemed stable for all of the investigated exosome concentrations ranging from 1.16 ng to 1.0 µg protein. Using unspecific antibodies as controls on either the surface of the disc or conjugated to FG beads, a very low level of background noise was observed and pulses similar to those of FG beads were very rare. A minor concern with the present methodology is that it is uncertain how the method would perform on samples with extremely high concentrations of EVs present such as it may occur in disease states. Specifically, it is uncertain whether ExoCounter would suffer from "swarm detection" of EVs, where either multiple small EVs would only bind a single FG bead due to their close proximity resulting in only a single pulse, or large EVs could be counted as multiple exosomes by binding multiple beads. While Kabe and coworkers were very thorough in their validation of the method, it could be argued that a dose-response experiment with differing concentrations (serial dilutions) would be in order instead of absolute amounts or differing volumes to address this question. Nonetheless, ExoCounter seems to hold great promise for quantification of exosomes, as it appears to have a large dynamic range, appropriate reproducibility, excellent recovery and high specificity towards exosomes.

Next, the authors compared ExoCounter to several other methods commonly used to quantify and characterize EVs. By comparing the number of counts to NTA measurements, they found a much lower number than NTA, suggesting that NTA fails to discriminate between exosomes and other particles with similar physical properties present in the biofluid. However, another explanation could be that not all exosomes contain CD9 and especially CD63, and therefore, the ExoCounter probably do not detect all exosomes present in the biofluid. The authors additionally compared the ability of ExoCounter to detect exosomes in culture media and human serum with a commercial ELISA test using anti-CD9 antibodies and found that the lower detection limit for ExoCounter was 800 fold lower than the commercial ELISA test. Furthermore, comparing ExoCounter to a bead-based FCM quantification method using anti-CD9 and anti-CD63 antibodies (a so-called on-bead ELISA) on a Gallios flow cytometer (Beckman Coulter, High Wycombe, United Kingdom) revealed that the lower detection limit for ExoCounter was 400 fold lower than the FCM-based method. Thus, ExoCounter certainly seems to have an impressive ability to detect exosomes compared to the other methods it was compared with. Although the authors have attempted to include both quantitative and semi-quantitative methods in their comparison of methodologies, these methods either suffer from being indiscriminant or having poor sensitivity, which are well known issues in the EV community. Therefore, it could be argued that the comparison is slightly unfair, and it would have been more appropriate to compare the method with other methodologies, which are more sensitive and provide a means of direct quantitation and discrimination of exosomes from other particles with similar physical properties. These methods could include quantitative transmission electron microscopy (10), or high-resolution FCM characterization of single particles (11). A drawback of ExoCounter is that it only detects one marker in a sample whereas FCM detects several.

Finally, the system was tested to detect disease-specific exosomes expressing CD147, a marker of colorectal cancer, CEA, a broader cancer marker, and HER2, a marker for breast and ovarian cancers. As expected, these markers were present on exosomes in media from various cultured cancer cells. However, CEA positive exosomes were not found in any sera from healthy controls or cancer patients, CD147 positive exosomes were present in both controls and cancer patients with no difference, while HER2 positive exosomes were found in a significantly increased amount in patients with breast or ovarian cancer compared to controls. While this seemed promising, there was a clear overlap between patients and controls positive for HER2, and the predictive values for cancer based on this method may not be very high. It should be noted that patient materials were from a biobank, and that the ability to predict must be tested on consecutive clinical samples. In addition, this problem might pertain to issues with antibody specificity or affinity, and selection of antibodies could contribute to all of the abovementioned issues.

One problem using EVs in cancer diagnostics is to find the relevant EV-proteins as markers, i.e., markers specific for cancer cells (12,13). Although many papers have been published, we lack really unique markers for cancer cells. Melo *et al.* (14) examined exosomes from various cell lines using mass spectrometry and bioinformatics analyses and

found a cell surface protein, glypican-1 (GPC1), exclusively present on cancer exosomes. Analysis of sera from healthy controls showed a low level of circulating exosomes with GPC1 whereas patients with pancreas cancer had a higher level resulting in a ROC curve with an AUC of amazing 1.0 for diagnosis of the cancer. Arbelaiz *et al.* (15) investigated proteome of EVs from patients with cholangiocarcinoma using mass spectrometry. They found several proteins differently expressed in EVs from patients and controls with an AUC of up to 90 % for the diagnosis, which was comparable to the existing tumor marker CA19-9. Although promising, the study reported no specific markers. Li *et al.* (16) recently presented an updated list of biomarkers on exosomes associated with cancer diagnosis. Sandfeld-Paulsen *et al.* (17) investigated 49 different known cancer markers in an EV array in patients with small cell or non-small cell lung cancer and found several of these overexpressed in exosomes from the cancer patients, reaching an AUC of about 0.70 and a combination of 10 of these increased AUC to 0.74–0.76. Thus, exosomes may add to the diagnosis but we need more specific cancer markers, and we are still waiting for the break-through. This is not solved by the ExoCounter. However, if some excellent markers are found, the ExoCounter will certainly be a strong candidate as the equipment of choice to be used for detection of these exosomes. Finally, miRNA and other types of RNA are also potential exosomal cancer-biomarkers (13,16), but that obviously involves other techniques.

In conclusion, the paper by Kabe *et al.* demonstrates a powerful new technique to detect specific exosomes or smaller EVs. The results are convincing regarding sensitivity and specificity, and this methodology could potentially be superior to several presently used methodologies. However, the patient data indicates that in spite of the technical ability of the method it has clinical limitations but this may be resolved with the discovery of improved cancer markers. We still need studies demonstrating that exosomes or EVs as markers of disease can clearly improve the present clinical diagnostic tool box.

Acknowledgments

Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned and reviewed by Section Editor Min Yang (Department of Laboratory Medicine, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China).

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/jlpm.2018.09.12). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/jlpm.2018.09.12
Cite this article as: Kristensen SR, Botha J, Handberg A. Blu-ray beyond music and movies—novel approach to diagnostics measuring specific extracellular vesicles. J Lab Precis Med 2018;3:84.

