Rapid magnetic isolation of extracellular vesicles via lipid-based nanoprobes

Yuan Wan1,2, Gong Cheng1,2, Xin Liu3,4, Si-Jie Hao1,2, Merisa Nisic1,5, Chuan-Dong Zhu1,6, Yi-Qiu Xia1,2, Wen-Qing Li1,2, Zhi-Gang Wang1,2, Wen-Long Zhang1,2, Shawn J. Rice3,4, Aswathy Sebastian7, Istvan Albert5,7, Chandra P. Belani3,4 and Si-Yang Zheng1,2,5,8*

Extracellular vesicles (EVs) can mediate intercellular communication by transferring cargo proteins and nucleic acids between cells. The pathophysiological roles and clinical value of EVs are under intense investigation, yet most studies are limited by technical challenges in the isolation of nanoscale EVs (nEVs). Here, we report a lipid-nanoprobe system that enables spontaneous labelling of nEVs for subsequent magnetic enrichment in 15 minutes, with isolation efficiency and cargo composition similar to what can be achieved by the much slower and bulkier method of ultracentrifugation. We also show that this approach allows for downstream analyses of nucleic acids and proteins, enabling the identification of EGFR and KRAS mutations following nEV isolation from the blood plasma of non-small-cell lung-cancer patients. The efficiency and versatility of the lipid-nanoprobe approach opens up opportunities in point-of-care cancer diagnostics.

Results

Design, optimization and characterization of the LNP. The LNP consisted of a labelling probe (LP) and a capture probe (CP). The LP is composed of a lipid tail for nEV membrane insertion, a polyethylene glycol (PEG) spacer (about 45 ethylene oxide units, corresponding to ~156 Å of spacer length) for increasing reagent solubility, and a biotin tag for subsequent isolation of labelled nEVs (Fig. 1, middle). We first compared the labelling efficiency among fluorescein isothiocyanate (FITC)-conjugated PEYlgated monoclonal lipid (C18), diacyl lipid (DSPE) and cholesterol. Because both cell membranes and EV membranes are lipid bilayers, to facilitate evaluation we used 10³ breast adenocarcinoma MDA-MB-231 cells in Diluent C or 5% human albumin in phosphate-buffered saline (Supplementary Fig. 1a). Although the presence of human albumin significantly decreased the fluorescence intensity of cells labelled with PEYlgated lipids compared with the fluorescence intensity of the Diluent-C group (P < 0.05; two-tailed t-test), the three lipids...
showed differential labelling efficiencies in the presence of albumin (Supplementary Fig. 1b). The average fluorescence intensity of C18-labelled cells in 5% human albumin was slightly higher than that of cells labelled with DSPE, but there was no significant difference between the two groups. Considering that diacyl lipids have been widely used for the manipulation of cells and that the mechanism is known, we chose DSPE–PEG–biotin as the LP for the following studies.

The NA-coated MMPs served as the CP and enabled the enrichment and isolation of nEVs in suspension. The MMPs were prepared as a monodisperse suspension with a mean size of 465.4 nm (Fig. 2a). The MMPs had a negative zeta potential of −32.0 mV (Supplementary Fig. 2a), arising from their silica shell. After aminosilane modification, absorption peaks at 2,920 cm$^{-1}$ and 2,852 cm$^{-1}$ in the spectra of Fourier transform infrared spectroscopy (Supplementary Fig. 2b), associated with the stretching vibration of methylene groups in silane, indicated the immobilization of amine groups. Accordingly, the value of the zeta potential shifted to 9.6 mV and then decreases to −17.7 mV once isothiocyanate was conjugated. The surface-modification process was finalized with the covalent immobilization of NA (see Methods).

Nanoscale extracellular vesicles from MDA-MB-231 cells were isolated via ultracentrifugation, and identified by electron microscopy. The isolated nEV population mainly consisted of vesicles with diameters of 30–200 nm (Supplementary Fig. 3a), exhibiting the characteristic saucer-shaped morphology under electron microscopy (Fig. 2b and Supplementary Fig. 3b) and the usual spherical shape under cryo-scanning electron microscopy (cryo-SEM; Fig. 2c).

Figure 1 | Schematic of the LNP system for nEV enrichment and downstream analyses. Top and middle: nEVs from either serum-free cell-culture medium or blood plasma are marked with the LP (top), followed by magnetic separation with the CP (middle). Bottom: nEVs and their cargo contents can then be analysed by different methods; such as PCR, Sanger sequencing and NGS sequencing for DNA; RNA staining and NGS for RNA; enzyme-linked immunosorbent assay (ELISA), BCA assay and LC–MS/MS for proteins; and cellular-uptake and wound-healing assays for functionality.
nEVs captured on NA-coated MMPs were imaged with cryo-SEM and transmission electron microscopy (TEM) (Fig. 2d and Supplementary Fig. 3c). We also showed that nEVs labelled with DSPE–PEG–FITC can be effectively taken up and internalized by MDA-MB-231 cells (Supplementary Fig. 4). nEV pellets were homogeneously re-suspended in serum-free medium and divided into six replicates, which served as model nEV samples. Each model sample contained approximately 13.2 ng RNA, as determined by a Qubit fluorometer. An isolation efficiency of 48.3% was achieved using 10 nmol of LP and an excess quantity of RNA extracted from the captured nEVs over total nEVs). Isolation of nEVs from 100 μl of blood plasma from a healthy donor was tested using the LNP to isolate nEVs from blood plasma. Because albumin might interfere with insertion of the LP into the membranes of nEVs, we increased the quantity of LP to 200 nmol for labelling and isolation of nEVs from 100 μl of blood plasma from a healthy donor containing approximately 13.2 ng RNA, as determined by a Qubit fluorometer. An isolation efficiency of 48.3% was achieved using period with CP for maximum isolation efficiency could be shortened to 10 min with continuous gentle rotation (Fig. 2g). Altogether, approximately 80% of nEVs from the model sample could be labelled and isolated using 10 nmol of LP and an excess quantity of the CP, with the whole isolation procedure taking about 15 min.

In addition, when we used DSPE–PEG–desthiobiotin as the LP, captured MDA-MB-231 nEVs on NA-coated MMPs could be released through displacement of DSPE–PEG–desthiobiotin with biotin, which binds much more tightly to NA than desthiobiotin. Approximately 84 ± 3% of the nEVs were released within 30 min (cryo-TEM images of MDA-MB-231 nEVs collected by ultracentrifugation and of the released nEVs are shown in Supplementary Fig. 6a,b). Furthermore, the released nEVs were functional. We educated non-invasive MCF7 cells with ~8 × 10^6 nEVs derived from highly aggressive MDA-MB-231 cells. A wound-healing assay showed the wound-closure rate of MCF-7 cells to be about twofold faster after nEV education (P < 0.05; two-tailed t-test), which indicates that the LP-labelled nEVs can induce higher levels of migration than uneducated MCF-7 cells (Supplementary Fig. 6c,d). We then used the LNP to isolate nEVs from blood plasma. Because albumin might interfere with insertion of the LP into the membranes of nEVs, we increased the quantity of LP to 200 nmol for labelling and isolation of nEVs from 100 μl of blood plasma from a healthy donor containing approximately 13.2 ng RNA, as determined by a Qubit fluorometer. An isolation efficiency of 48.3% was achieved using
100 nmol of LP with the CP in excess. Doubling the LP amount only slightly increased the efficiency to 49.5% (not statistically significant; \(P > 0.05\), two-tailed \(t\)-test; Fig. 2h).

Detection of nucleic acids and proteins in model nEV samples. The LNP enables nEV enrichment directly onto a surface, which facilitates subsequent molecular analyses for the quantitative detection of nEVs and profiling of membrane proteins. After incubation of the LP with model MDA-MB-231 nEV samples for 5 min, the mixture was transferred to NA-coated wells in a multi-well plate for nEV capture. Here, NA was immobilized on the well surface, and the NA–biotin reaction time was extended to 30 min, which allowed for over 95% binding efficiency\(^a\). We used a membrane-permselective dye (SYTO RNASelect) to selectively stain nEV RNA, and found that the green fluorescence intensity increased in direct proportion to the total RNA contained in intact nEVs (coefficient of determination, \(r^2 = 0.98147\); Fig. 3a). This shows that the assay can semi-quantify nEV RNA content in 35 min, which could allow it to serve as a useful alternative when nanoparticle-tracking or dynamic-light-scattering equipment is not available.

Proteins in the nEV membrane can also be detected using LNP-mediated capture and enrichment. Model nEVs from SK-N-BE(2) neuroblastoma cells, MDA-MB-231 breast adenocarcinoma cells and SW620 colon adenocarcinoma cells were captured and stained with fluorescently labelled antibodies against cluster-of-differentiation molecule 9 (CD9) or epithelial cell adhesion molecule (EpCAM) (Fig. 3b). CD9 is one of the most ubiquitous molecular markers for all EVs\(^b\), and anti-EpCAM grafted magnetic beads have been widely used for exosome isolation\(^c\). EpCAM expression in nEVs from SK-N-BE(2) cells was barely detected, whereas the expression levels for nEVs from MDA-MB-231 and SW620 cells were weak and strong, respectively. In contrast, CD9 expression levels were comparable for nEVs of these three cell lines. These results parallel the EpCAM and CD9 expression levels determined by immunocytochemistry (Fig. 3b, top).

Nanoscale extracellular vesicles can also be directly collected by CPs, followed by the extraction and analysis of protein and nucleic-acid cargo. CD63 (a commonly used EV marker\(^d\)) and glycerinaldehyde 3-phosphate dehydrogenase (GAPDH; a well-known housekeeping protein\(^e\)) extracted from MDA-MB-231 cell lysates and nEV protein lysates, respectively, were detected by western blot (Fig. 3c and Supplementary Fig. 7). Additionally, DNA and RNA were extracted from isolated MDA-MB-231 nEVs followed by agarose gel electrophoresis to confirm the presence of RNA and long fragments of DNA (Fig. 3d and Supplementary Fig. 8\(^f,g,h,i\)).

We also compared the contents of nEVs collected by ultracentrifugation versus those collected by the LNP. DNA from MDA-MB-231 nEVs and cellular genomic DNA without amplification were analysed by next-generation sequencing (NGS). The purified nEV DNA samples mainly contained DNA fragments longer than 10 kbp (Supplementary Fig. 5c). This differs from circulating cell-free DNA, which shows a typical apoptotic DNA ladder\(^j\). The percentage of reads mapped to the human genome was 99.6% and 99.5% in the ultracentrifugation and LNP groups, respectively. DNA from nEVs isolated by the two methods uniformly spanned all chromosomes. The nEV DNA contents after ultracentrifugation and LNP isolation were similar, with a Pearson correlation coefficient of 0.96 calculated using a 100-kbp window size (Fig. 3e). The nEV DNA content extracted by either of the two methods resembles nuclear genomic DNA from the same cell line, as indicated by the copy-number-variation (CNV) plots of the purified nEV samples and of the genomic DNA sample (Supplementary Fig. 9). In addition, the Pearson correlation coefficient between the nEV DNA content from ultracentrifugation and the genomic DNA content was 0.87, and that between the nEV DNA content from the LNP and the genomic DNA content was 0.92.

Furthermore, cargo RNA was extracted from nEVs isolated by ultracentrifugation and the LNP, and then compared. Quadruplicated samples of MDA-MB-231 nEV RNA, including messenger RNA and microRNA, were analysed by NGS. The average percentage of reads mapped to human total RNA was 89.3% and 86.2% for the ultracentrifugation and LNP groups, respectively. In a Euclidean-distance plot of mRNA from MDA-MB-231 nEVs (Supplementary Fig. 10a), the biological replicates isolated with the LNP and those isolated by ultracentrifugation appeared in separate clustered regions. Using read counts of mapped sequences, we then quantified the RNA cargo of nEVs isolated from MDA-MB-231 cells with the LNP and with ultracentrifugation. nEVs isolated from MDA-MB-231 cells contained diverse cargo RNA, including significant amounts of long intergenic noncoding RNA, ribosomal RNA, small nucleolar RNA and other RNA types in addition to the most abundant RNA type, protein-coding RNA or mRNA (Fig. 3f). There were no noticeable differences in RNA species between nEVs isolated by the LNP and those isolated by ultracentrifugation; in fact, there was a substantial overlap of mRNA (81%) and miRNA (94%) species in the top 1,000 expressed miRNAs and miRNAs (Supplementary Fig. 10b and Supplementary Datasets 1 and 2). In a Bland–Altman plot (Supplementary Fig. 10c) comparing the expression levels of miRNAs isolated by ultracentrifugation or the LNP, we found that the majority of the detected miRNAs had similar expression levels, which was also indicated by the linear correlation coefficients of \(>0.998\) for total RNA content (Supplementary Fig. 10d). A recent report indicated that foetal bovine serum (FBS)-derived miRNAs interfere with subsequent transcription analysis\(^k\). However, we found minimal interference by the reported top 14 FBS miRNAs when comparing the numbers of miRNAs in the ultracentrifugation nEV samples with those in the LNP nEV samples (Supplementary Table 1).

Furthermore, we found that after nEV isolation via the LNP, the weight of protein to RNA in extracts decreased from 12.1 to 4.9 (Supplementary Fig. 11a), indicating that without an additional washing step our approach can eliminate on average 68.5% of total protein. Because nEV isolation via the LNP leads to a 22% loss of nEVs, we speculate that the removed protein consists mainly of protein contaminants. In contrast, ultracentrifugation allowed the collection of 61.4% of the nEVs from a model sample, and additional washing purification by re-suspension in PBS buffer along with further ultracentrifugation reduced the overall efficiency to only 13.9% (Supplementary Fig. 11b). Results from liquid chromatography–tandem mass spectrometry (LC–MS/MS) further revealed the relationship between the cargo proteins of ultracentrifuge-isolated nEVs and LNP-isolated nEVs (Supplementary Dataset 3). We compared our LC–MS/MS data with a recently published report\(^l\) on 30 key proteins in EVs (Supplementary Table 2). We found a similar cargo–protein composition for nEVs isolated by ultracentrifugation and by LNP. Moreover, our results are also consistent with the work in ref. \(^{26}\), which used a combination of ultracentrifugation and density-gradient ultracentrifugation for small EV preparation. In addition, for 8,452 EV cargo proteins archived in the public database Vesiclepedia (www-vesiclepedia.org), we found that ~91% and ~94% of them corresponded to the cargo proteins from nEVs isolated by ultracentrifugation and by LNP, respectively. Similarly, ~94% of the EV cargo proteins reported in ref. \(^{26}\) can be identified in the database (Supplementary Fig. 12a). Also, 76 and 89 out of the top 100 proteins from Vesiclepedia were identified in the nEVs isolated by ultracentrifugation and LNP, respectively, and 96 of the 100 were identified in the nEVs of ref. \(^{26}\) (Supplementary Fig. 12b). Analysis of the cellular distribution of the identified proteins showed that, for the ultracentrifugation, LNP and ref. \(^{26}\) groups, respectively, 51.8%, 64.7% and 57.2% of the proteins localize with exosomes and 34.2%, 39.7% and 47.6% localize with lysosomes (\(P < 0.01\); two-tailed \(t\)-test; Supplementary Fig. 12c). Finally, our MS analysis also confirmed that vimentin, cytokeratin, EGFR, and the mammary cancer stem cell genes are significantly enriched in nEVs isolated by the LNP method.
Figure 3 | Isolated nEVs provide flexibility in downstream molecular analyses.  

**a**, LP-labelled nEVs were enriched on NA-coated well plates followed by RNA-dye staining. Fluorescence intensity (arbitrary units, a.u.) increased in direct proportion to the total RNA contained in intact nEVs; mean ± s.e.m. (n = 4).

**b**, Fluorescently labelled CD9 and EpCAM antibodies were used to detect relevant protein expression in model nEVs released from SK-N-BE(2), MDA-MB-231 and SW620 cells. Error bars, mean ± s.e.m. (n = 20). Top insets show CD9 (green), EpCAM (red) and DAPI (blue) staining of the cells.

**c**, CD63 and GAPDH proteins were extracted and identified from isolated nEVs by western blot.

**d**, DNA and RNA were extracted from isolated nEVs and identified with 2% agarose gel electrophoresis. DNA ladders (1 kbp; labelled M) indicate the length of the fragments.

**e**, Circos plots of nEV DNA from MDA-MB-231 cells isolated by ultracentrifugation (top) and by the LNP (bottom). DNA was sequenced by NGS with 3.3x depth of coverage, mapped to the human genome, and plotted with a size window of 100 kbp. Read coverage is expressed in natural logarithmic scale for 0, 3, 6, 9, 12 and 15 reads, from the inside to the outside.

**f**, Pie charts depicting different RNA species and their mapped read-count distributions from MDA-MB-231 nEVs isolated by ultracentrifugation (top) and by the LNP (bottom). Left: full-scale plots. Right: plots zoomed in on low-abundance RNA species (labelled as ‘others’ in the full-scale plots).
cell marker CD44 appear in the nEVs from MDA-MB-231 cells, which agrees with the phenotype of this triple-negative and aggressively metastatic cancer cell line.

**Detection of mutated DNA in nEVs isolated from blood-plasma samples from NSCLC patients.** By using the LNP, we isolated nEVs from 100 μl of blood-plasma samples of 19 NSCLC patients. To achieve high sensitivity, we implemented mutant-enriched PCR assays for the analysis of mutations in EGFR exons 19 and 21, and a real-time PCR assay for the identification of mutations in KRAS codons 12 and 13 (Supplementary Fig. 13). All PCR products were subjected to Sanger sequence analysis. After conventional PCR amplification, the desired PCR products of EGFR exon 19 and 21 and of KRAS were obtained from all samples (Fig. 4a and Supplementary Fig. 14). Sequencing analysis only identified the KRAS G13D mutation in the plasma sample of patient 42 (Fig. 4b). Mutations were not detectable in the rest of the samples by Sanger sequencing (Supplementary Fig. 15). As the detection limit for the mutant allele fraction is about 10% for Sanger sequencing, we employed a mutant-enriched PCR assay that can reduce this limit to ~0.05%.

After mutation-specific restriction-enzyme digestion and nested PCR, we found an L858R mutation in EGFR exon 21 in the plasma sample of patient 28 (Fig. 4c), which we were unable to confirm by NGS using the patient’s tissue sample because of the low quantity of sample available. Moreover, a deletion mutation in EGFR exon 19 was readily detected in patient 29, which matched the results of the NGS sequencing of this patient’s tissue sample (Fig. 4d). We also used real-time PCR to enrich mutations in KRAS codons 12 and 13 for downstream sequencing (Fig. 4e; according to the manufacturer, the limit of detection can reach 0.01% by targeting the mutant gene and suppressing the wild-type copy). We failed to detect KRAS mutations in patients 25, 27 and 50 (Table 1), presumably because of their extremely low abundance in the nEVs of patient blood samples or because of a change in the mutation status over the period between the primary tissue biopsy and blood draw. All wild-type EGFR and KRAS alleles in patient tissue samples were detected as wild-type after nEV isolation via the LNP.
Discussion

To increase the clinical utility of EVs, efficient isolation and detection methods are needed. As phospholipid derivatives, PEGylated lipids have been used for the labelling and manipulation of cells and liposomes. Similarly, PEGylated lipids can also be used for nEV isolation. The main advantage of the LNP approach described here is rapid nEV isolation. The two-step isolation procedure takes only 15 min; existing methods require longer processing times, from 30 min to over 22 h (ref. 17). Also, the LNP system does not require bulky and expensive instruments or delicate microfluidic devices. Moreover, the nEV isolation efficiency of the LNP system is similar to that of ultracentrifugation. However, the EV isolation efficiency of ultracentrifugation depends on repeated cycles, and such additional purification steps can damage the nEVs and reduce yields from ~70% to less than 10%. In contrast, in the LNP system, repetitive purification is eliminated as ~68% of proteins can be removed by the one-step isolation process, which exerts minimal impact on downstream molecular analyses of the nEV content. Furthermore, the LNP system aids qualitative and quantitative molecular analyses of nucleic acids and proteins. Overall, by significantly shortening the time of sample preparation and by providing relatively pure nEVs via isolation, the LNP system should facilitate nEV-based diagnostics.

With regards to the lipids for membrane labelling, DSPE bearing two hydrophobic fatty acid tails showed stronger non-covalent interactions with the lipid membranes of nEVs than did amphiphatic cholesterol and C18 with its single hydrophobic fatty acid tail, and thus DSPE was found to display more stable retention. It is worth noting that the optimal quantity of LP and the isolation efficiency of nEVs differed between the model samples and plasma.

Table 1 | KRAS and EGFR mutations in samples from 19 NSCLC patients.

| Patient number | Age | Sex | Tissue source | EGFR mutation | KRAS mutation |
|---------------|-----|-----|---------------|---------------|---------------|
| 24            | 61  | M   | Lung          | WT            | WT            |
| 25            | 58  | M   | Small bowel   | WT            | WT            |
| 26            | 58  | F   | Lymph node    | WT            | WT            |
| 27            | 60  | F   | Lung          | WT            | WT            |
| 28            | 90  | M   | NA            | exon 21 L858R | WT            |
| 29            | 65  | F   | Lymph node    | exon 19 Del   | exon 19 Del   |
| 30            | 66  | M   | Bone          | WT            | WT            |
| 31            | 50  | M   | Lymph node    | WT            | WT            |
| 32            | 79  | F   | NA            | WT            | WT            |
| 36            | 65  | F   | Lung          | WT            | WT            |
| 37            | 82  | F   | Lung          | WT            | WT            |
| 42            | 70  | F   | Lymph node    | WT            | WT            |
| 50            | 70  | M   | Lymph node    | WT            | WT            |
| 51            | 70  | M   | Pleura effusion | WT           | WT            |
| 52            | 74  | M   | Liver         | WT            | WT            |
| 54            | 53  | M   | Lung          | WT            | WT            |
| 55            | 86  | F   | Lung          | WT            | WT            |
| 56            | 72  | F   | NA            | WT            | WT            |
| 58            | 65  | M   | Pleura effusion | WT           | WT            |

Bold text indicates detected mutations. WT, wild type; Del, deletion; NA, not available.

either the shrinkage of nEVs during fixation, or from shortcomings in NanoSight that lead to a bias towards the detection of larger EVs.

Cells secrete heterogeneous populations of nEVs with different sizes and compositions, and universal EV markers such as CD63 do not consistently appear in each individual nEV. Similarly, we found that the expression of EpCAM in nEVs collected from three different cancer cell lines varied. This might however reflect low nEV-isolation efficiency with anti-EpCAM-based immunoselction. Conversely, the LNP system is unique in that it selects all lipid vesicles in the sample, thus providing antigen- and size-independent isolation. The method is therefore applicable to all nEVs regardless of size and protein composition. Overall, our genomic, transcriptomic and small-RNA studies indicate that the cargo contents of the LNP-isolated nEVs are similar to those of nEVs isolated by ultracentrifugation. With low-coverage genomic sequencing, CNV profiles can be generated from purified nEV samples that are identical to those of the original cells. Not only could the DNA sequences obtained from LNP- and ultracentrifugation-isolated nEVs be mapped to the human genome, they also contained CNV profiles that were highly similar to those of the original MDA-MB-231 cells. This may provide a way to confirm the tumour from which the nEVs originated. Read-counts-based quantitative analysis of the sequencing data revealed rich RNA content in the nEVs isolated by both LNP and ultracentrifugation. Most of the reported cellular coding and noncoding RNAs were present in the isolated nEVs. In all cases, the RNA from LNP-isolated nEVs was not significantly different to that from nEVs isolated by ultracentrifugation. Furthermore, protein LC–MS/MS analysis showed that the nEV protein compositions were similar for the two isolation methods, and our results are consistent with the Vesiclepedia database and a recently reported proteomic analysis of EV subtypes. A cellular-distribution analysis further confirmed that the LNP-isolated nEVs carried a larger percentage of exosomal and lysosomal proteins.

nEVs contain whole-genomic DNA, and mutated KRAS and p53 have been detected in exosomes pelleted from patient serum.

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In our cohort study of patients with NSCLC cancer, mutation analysis of the tumour tissue revealed that at least one in four carried mutations in EGFR exons 19 and 21 or in KRAS codons 12 and 13. This is in fairly good agreement with the frequency of EGFR (~5%) and KRAS (~15%) mutations in NSCLC. We collected nEVs from NSCLC patients using the LNP system and extracted the genomic DNA for the detection of KRAS and EGFR mutations. Using Sanger sequencing right after traditional PCR, we only identified the KRAS G13D mutation in one patient. Improving the detection sensitivity via mutant-enriched PCR and real-time PCR, we were able to find mutations in the DNA of nEVs from three more patients (we should note, however, that EGFR and KRAS mutations in the tissue samples might not be identical to those in the plasma samples). A L858R mutation in EGFR exon 21 and a G13D mutation in KRAS were identified in nEV DNA from two patients (28 and 42, respectively; however, there was not enough sample available for tissue-based mutation analysis). This demonstrates the feasibility of mutation analysis in nEV DNA, and underscores the advantage of tissue-based mutation analysis (tissue samples might not be identical to those in the plasma samples). This demonstrates the feasibility of mutation analysis in nEV DNA, and underscores the advantage of tissue-based mutation analysis (tissue samples might not be identical to those in the plasma samples).

Optimization of the LNP with cells. FITC-tagged C18–PEG, DSPE–PEG and cholesterol–PEG powder were purchased from Nanocs and used to further purification. The FITC-tagged PEG–cholesterol–PEG hydrogel was dissolved in pure anhydrous ethanol at a final concentration of 1 mM and then stored at −80 °C. Approximately 105 MDA-MB-231 cells were collected and re-suspended in either 250 μl of Diluent C or 5% human albumin in PBS. Ten nanomoles of each LP was added to 250 μl of Diluent C before being added to the cell suspension. The samples were mixed gently at 4 °C for 5 min followed by sonication at 500 g for 5 min to remove redundant LPs, then tenfold concentrated with 4% paraformaldehyde at 4 °C for 10 min. The cells were re-suspended in 1.5 ml of PBS, stained with DAPI solution (1 μg ml−1) at room temperature for 10 min, thoroughly rinsed thrice with PBS and finally re-suspended in 500 μl of PBS. Cell suspension (20 μl) was added onto glass cover slips for fluorescent imaging using a 40× objective lens using an Olympus IX71 microscope. The fluorescence intensities were analysed using the ImageJ software package (National Institutes of Health).

Preparation and characterization of the CP. The magnetic Fe3O4–SiO2 core–shell submicrometre particles were synthesized via a modified Stöber sol–gel process. Briefly, 30 mg of MCA and 250 μl of STM aqueous solutions were ultrasonically dispersed in a solution containing 160 μl of ethanol, 40 μl of water and 10 μl of concentrated ammonia (28% w/w). Tetraethyl orthosilicate (0.3 ml) was then added dropwise under sonication, followed by stirring for 3 h at room temperature. The resulting particles were separated using a magnet, washed thoroughly with deionized water and ethanol, and dried at 60 °C for 12 h. To functionalize the MMs with 5′-end biotin groups, 250 μg of MMPO and 250 μl of 3-aminopropyltriethoxysilane were ultrasonically dispersed in 30 μl of toluene. The mixture was refluxed for 12 h under a nitrogen atmosphere. Finally, the products were collected, thrice rinsed with toluene and ethanol, and dried at 80 °C overnight. The morphology of the particles was examined using a scanning electron microscope ( Nova Nanosem, FEI). Fourier transform infrared spectra were obtained using a Bruker Vertex V70 microscope. The fluorescence intensities were analysed using the ImageJ software package (National Institutes of Health).

Isolation of nEVs using the LNP. BP (biotin-tagged DSPE–PEG) powder was dissolved in pure anhydrous ethanol at a final concentration of 1 mM and stored at −20 °C. The nEVs were labelled with the LP according to the PKH26 labelling protocol, with minor modifications. A 100 μl volume of each nEV model sample was added to 1 ml of Diluent C. LP (0.001, 0.01, 0.1, 1, 5 or 10 μl) was added to the other 1 ml of Diluent C before being incubated with the nEVs and the control. The samples were mixed gently at 4 °C for 5 min and then incubated with ~1013 CP (NA-coated MMPs) at room temperature for 30 min. After isolation, CPs were thoroughly rinsed thrice with PBS to remove non-specific molecules absorbed on the CP surface. The influence of LP mixing times from 2 to 8 min and CP incubation times from 5 to 30 min were assessed and optimized. The morphology of nEV-bound CPs was characterized using SEM.

Aliquots, comprising 1, 10, 50, 100 or 200 nmol of LP in 500 μl of Diluent C, were added to 100 μl of plasma (collected from a healthy volunteer), and then mixed for 5 min at 4 °C and incubated with CPs at room temperature for 10 min. Additionally, 100 μl of plasma was added to 300 μl PBS and ultracentrifuged once at 100,000 × g and 4 °C for 2 h. RNA was extracted as before to evaluate the isolation efficiency. All experiments were performed in triplicate.

Release of captured nEVs using biotin. DSPE–PEG–desethylbiotin (Nanocs) in pure anhydrous ethanol was prepared as above. Following the above-mentioned protocol, nEVs were labelled with DSPE–PEG–desethylbiotin and captured onto CPs. Surplus uncaptured nEVs were removed by rinsing the CPs thrice with PBS. Twenty nanomoles of biotin in PBS was introduced to displace the DSPE–PEG–desethylbiotin. After incubation for 30 min at room temperature, CPs were thoroughly washed with PBS using a pipette. The supernatant was collected for RNA extraction. Release efficiency was calculated as the amount of RNA extracted from the supernatant (released nEVs) divided by the total amount of RNA from captured nEVs.

Characterization of nEVs. For TEM, 5 μl of model nEV sample was placed on a 400-mesh Formvar-coated copper grid and incubated for 3 min at room temperature. Excess samples were blotted with filter paper and then negatively stained with filtered aqueous 1% uranyl acetate for 1 min. Stain was blotted dry from the grids with filter paper, and samples were allowed to dry. Samples were...
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then examined in a FEI Tecnai transmission electron microscope at an accelerating voltage of 100 kV.

For SEM, model nEV samples (5 μl) were seeded onto a poly-L-lysine-coated silicon wafer and fixed in 4% paraformaldehyde for 30 min. The samples were then sequentially immersed in 20, 50, 70, 85, 95 and 100% ethanol solutions for 15 min per solution (ref. 17). Samples were lyophilized overnight followed by sputter-coating with gold at room temperature. The morphology of the nEVs was examined under a Zeiss field-emission scanning electron microscope. The number of nEVs was counted using ImageJ software (NIH).

For cryo-EM, 5 μl of model nEV sample was added to a 200-grid mesh (Quintoil, Ted Pella), blotted for 1 s with FEI Vitrobot before plunging into liquid ethane, and transferred to a cryo-sample holder. Samples were visualized by TEM (FEI Tecnai F20) and SEM (FEI Helios NanoLab 660).

**Wound-healing assay.** Approximately 3 × 10^4 MCF-7 cells were seeded into each well of a 24-well plate and were allowed to attach onto the substrate overnight. When confluence reached 100%, a pipette tip was used to scratch the cell monolayer. Detached cells and medium were removed. The wounds were then incubated at 37 °C in 5% CO₂. To educate cells with nEVs, ~8 × 10^9 nEVs from each of three cell types, SK-N-BE(2), MDA-MB-231 and SW620, were diluted 1:100, placed in the chamber, and analysed using Nanoparticle Tracking Analysis software (Malvern) to count the number of nEVs.

**Sample preparation.** After 24 h, floating cells were removed with phosphate-buffered saline. The wound area.

**Ncific acid and protein extraction.** RNA preparation was conducted using Trizol (Life Technologies) according to the manufacturer's instructions. Trizol (750 μl) and chloroform (200 μl) were added to and vigorously mixed with 1 μl of RNA sample. The whole reaction was centrifuged in a spin column. After two washing steps, the RNA was eluted in 5 μl of DEPC water.

**Whole genome NGS.** The isolated nEV DNA was mechanically fragmented to 400 bp using a focused ultrasonicator (Covaris). DNA sequencing was performed at the Biopolymers Facility at Harvard Medical School. The Wagen Genomics Core Facility of Penn State University.

**NGS of RNA.** The ribosomal RNA-depleted total nEV RNA was extracted using a mirNeasy Mini Kit (Qiagen). DNA sequencing was performed at the Genomics Technology Center at New York University medical center. An Illumina TruSeq Standard mRNA kit was used to prepare the mRNA and small RNA sequencing libraries. Sequencing libraries were pooled together and sequenced on the Illumina HiSeq platform (single-end 50 bp). We obtained more than 20 million 51-bp reads for each of the 16 samples (small RNA-seq, n = 8; total RNA-seq, n = 8). The adapters for small RNA-seq were removed using cutadapt (v.1.3.1). All the reads were mapped to the human reference genome (GRCh37/ hg19) using STAR aligner (v.2.5.0e). The alignment was guided by a Gene Transfer File (GTF version GRCh37.70). The reads per million normalized Bigwig files were generated with BedTools (v.2.17.0) and the signal was visualized using the Genome Browser (v.31). Read count tables were generated using HTSeq (v.0.6.0) based on the Ensembl gene annotation file (Ensembl GTF version GRCh37.70). All read-count tables were then corrected for their library-size differences based on their geometric library-size factors calculated using the DESeq2 R package (v.2.3.0)29, and differential expression analysis was performed. To compare the level of similarity among the samples, we used the Fluorescence-activated cell sorter (FACS) analysis.

**PCR and sequencing.** KRAS analysis (466 bp) was performed using the following primers: forward 5’-AAG GCC TGC TGA AAA TGA CTG-3’ and reverse 5’-TCA CAA TAC GAA ACC CAT-3’. Analysis of EGFR Exons 19 and 21 was performed using the following primers: Exon 19 (372 bp), forward 5’-TCA CAA TAC GAA ACC CAT-3’ and reverse 5’-TCA CAA TAC GAA ACC CAT-3’; Exon 20 (300 bp), forward 5’-TGA GCC TCT CAT CAC GA-3’ and reverse 5’-GCA TGT GTT AAA CAA TAC AGC-3’. PCR was performed in a 25 μl reaction tube containing 12.5 μl of GoTaq Green Master Mix (Promega), 10.5 μl of template DNA, and 1 μl of each primer. Amplification was carried out under the following conditions: 94 °C for 1 min, two cycles of 94 °C for 10 s, 70 °C for 10 s, and 72 °C for 10 s; two cycles of 94 °C for 10 s, 64 °C for 10 s, 70 °C for 10 s; five cycles of 94 °C for 10 s, 60 °C for 10 s, 70 °C for 10 s. PCR products were cleaned using a QIA quick PCR Purification Kit (Qiagen) following the manufacturer’s instructions, and then sequenced by Sanger DNA sequencing (Applied Biosystems 3730XL) at the Genomics Core Facility of Penn State University.

Alternatively, a PointMan KRAS (codon 12 or 13) DNA enrichment kit (EKF molecular diagnostics), a real-time PCR kit, was used to enrich mutations. Relevant samples were purified for Sanger sequencing once variant traces were observed in the real-time PCR. For the EGFR-mutant–enriched PCR assay, 0.3 μl of the first traditional PCR products of EGFR exon 19 and exon 21 were further digested with Mse I and Msc I, respectively, at 37 °C for 4 h. An aliquot was used as a template for the second round of nest PCR amplification under the same first round PCR but for 42 cycles. The exon 19 nest PCR (175 bp) primers were: forward 5’-TAA TAC CCT CAA CCT TTT GGC-3’ and reverse 5’-TGG TGG AGA TGA GCA GGG-3’. The exon 21 nest PCR (213 bp) primers were: forward 5’-CAG CAG GCT GTG TTC-3’ and reverse 5’-GAA AAT GGC TGC TAA GCA AAA G-3’. Products were purified and analysed by Sanger DNA sequencing.
LC–MS/MS. Protein concentrations were measured by BCA protein assay. Approximately 30 μg of proteins were separated by SDS–PAGE using 10% Bis-Tris Napage gels (Life Technologies). Serial gel slices were excised and diced into smaller fragments. Samples were reduced with 10 mM dithiothreitol in 25 mM NH₄HCO₃ at 56°C for 1 h and alkylated with 55 mM iodoacetamide for 45 min at room temperature. In-gel trypsin digestion was performed using 10 ng/μl of sequencing grade modified porcine trypsin (Promega) diluted in 500 mM NH₄HCO₃, 37°C overnight. Peptides were extracted with 0.5% formic acid and 50% acetonitrile. Following evaporation of acetonitrile, peptides were purified using a ZipTipC18 column (Millipore). The volume of each eluted sample was reduced in a Speedvac (Savant, Thermo Fisher) to 5 μl in order to evaporate acetonitrile, and then adjusted to 20 μl with 0.1% formic acid prior to LC–MS/MS analysis. LC–MS/MS analysis was performed on an AB SCIEX TripleTOF 5600 System (Foster City) equipped with an Eksigent nanoLC Ultra and ChipPLC-nanoflex (Eksigent) in the trap elute configuration was employed for LC–MS/MS. The acquired mass spectrometric raw data was processed using ProteinPilot 5.0 software (AB SCIEX) via the Paragon search mode. The ProteinPilot Descriptive Statistics Template (AB SCIEX) was used for alignment of multiple results and evaluation of the false discovery rate.

Data availability. The authors declare that all data supporting the findings of this study are available within the paper and its Supplementary Information. Source data for the figures in this study are available in figshare with the identifier doi:10.6084/m9.figshare.4728856 (ref. 4).

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Author contributions

Y.W. and S.-Y.Z. designed the research. Y.W. conducted experiments and analysed data. G.C. prepared the MMPs, assisted with peptide-sample preparation and performed proteomic analyses. S.-J.H. assisted with the preparation of NGS samples, the analysis of RNA NGS data, and the fluorescence imaging. M.N. prepared blood plasma. C.-D.Z. and W.-Q.L. assisted with the cell culture, nEV collection and gel electrophoresis. Y.-Q.X. performed the wound-healing assay. Z.-G.W. performed the electron microscopy. W.-L.Z. assisted with the image processing. A.S and I.A analysed NGS DNA data and clinical support. Y.W. and S.-Y.Z. wrote the manuscript.

Additional information

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Correspondence and requests for materials should be addressed to S.-Y.Z.

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Competing interests

The authors declare no competing financial interests.