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Targeting Tumor-Associated Exosomes with Integrin-Binding Peptides

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All cells expel a variety of nanosized extracellular vesicles (EVs), including exosomes, with composition reflecting the cells' biological state. Cancer pathology is dramatically mediated by EV trafficking via key proteins, lipids, metabolites, and microRNAs. Recent proteomics evidence suggests that tumor-associated exosomes exhibit distinct expression of certain membrane proteins, rendering those proteins as attractive targets for diagnostic or therapeutic application, yet it is not currently feasible to distinguish circulating EVs in complex biofluids according to their tissue of origin or state of disease. Here, peptide binding to tumor-associated EVs via overexpressed membrane protein is demonstrated. It is found that SKOV-3 ovarian tumor cells and their released EVs express α3β1 integrin, which can be targeted by the in-house cyclic nonapeptide, LXY30. After measuring bulk SKOV-3 EV association with LXY30 by flow cytometry, Raman spectral analysis of laser-trapped single exosomes with LXY30-dialkyne conjugate enables the differentiation of cancer-associated exosomes from noncancer exosomes. Furthermore, the foundation for a highly specific detection platform for tumor-EVs in solution with biosensor surface-immobilized LXY30 is introduced. LXY30 not only exhibits high specificity and affinity to α3β1 integrin-expressing EVs, but also reduces EV uptake into SKOV-3 parent cells, demonstrating the possibility for therapeutic application.

1. Introduction

For the past two decades, the mortality rate has steadily declined for certain cancers (lung, prostate, colon, and breast), yet has changed very little for others (ovarian, pancreatic, liver, stomach, uterine, leukemia, and bladder). The latter group suffers a notable lack of noninvasive early detection methodologies, leaving few treatment options for patients diagnosed at an advanced stage. Fortuitously, all cells expel extracellular vesicles (EVs), whose biomolecular contents reflect the composition and state of their parent cells. A nanosized subset of these vesicles, termed exosomes, holds great promise for cancer diagnostics, and can be detected in numerous biofluids, including blood, urine, saliva, ascites, and cerebrospinal fluid.

Tumor cells can hijack the exosomal pathway for means of immunosuppression, antigen presentation, tumor growth, and metastasis. It has been demonstrated that proteins, genes, lipids, and metabolites exhibit distinct profiles within exosomes derived from tumor cells compared to healthy cells. Combined with the cell's ability to quickly alter EV composition in response to stimulus (e.g., stress, pathology, and drug treatment), exosomes have been implicated as vast mediators of intercellular communication, and provide a framework for diagnostic platform development. Much work is currently underway to develop methods for exploiting the chemical content trafficked in tumor-associated exosomes for use as potent biomarkers.

An ideal point-of-care isolation strategy for cancer diagnostics would address two issues: first, tumor-associated EV subpopulations represent only a small fraction of the circulating secretome, thus the nontumor EV background impedes sensitive detection. Second, tumor EVs should be distinguished...
from nontumor EVs. One potential approach for simple, rapid discrimination of tumor EVs is to exploit differences in their surface composition. Many membrane proteins have been identified to be generally associated with exosome-type EVs (CD9, CD63, CD81, and EpCAM). Others have been reported to be enriched in certain tumor-associated exosomes, such as EGFR and HER2. Given that several recent studies have demonstrated enrichment of particular integrins in exosomes derived from a variety of tumor cells, exosomal integrins are interesting potential targets.

Integrins are transmembrane heterodimer glycoproteins composed of an α and β subunit, and contribute to a variety of normal and pathogenic biological processes including cell adhesion, inflammation, thrombosis, metastasis, and adhesion-mediated drug resistance. Characteristic combinations of integrins are often overexpressed in certain tumor cells. In 2015, Hoshino et al. described that organotropic tumor metastasis is largely mediated via exosomal integrin trafficking. They demonstrated that exosomes expressing integrins αβ₄ and αβ₅, or αβ₃, contributed to metastatic spreading toward the lung or liver, respectively, and that prior “education” with lung-tropic exosomes (by retro-orbital injection in mice) redirected metastasis of bone-tropic tumor cells to the lung. Furthermore, targeting of the αβ₄ or αβ₅ integrins decreased metastasis. Thus, EV-expressed integrins represent an attractive target for both (i) diagnostic discrimination of circulating tumor EVs and (ii) therapeutic application by binding tumor EV integrins in order to block their tumorigenic function.

Recent work in our lab has led to the discovery of several unique ligands capable of binding tumor cells through their overexpressed integrins, including ligands against αβ₁[28,29], αβ₃[30] and αβ₅[31]. These ligands were found by screening tumor cells against focused combinatorial libraries of peptide or peptidomimetic molecules, via the one-bead screening method. This generated the highly potent cyclic disulfide-containing peptide: LXY30 [cdG-Phe(3,5-diF)-G-Hyp-NcR]. LXY30 showed increased in vitro and in vivo tumor targeting across a panel of ovarian, breast, brain, and nonsmall-cell lung carcinoma (NSCLC) cells. Furthermore, the binding specificity of LXY30 to the αβ₃ integrin subunit was confirmed, since binding to αβ₃-expressing U-87MG cells could be inhibited by preincubation of the cells with anti-αβ₃ antibody. Finally, a scrambled version of LXY30 (scr-LXY30: [cGd-Hyp-Phe(3,5-diF)-G-NcR]), featuring switched positions of some amino acids in the peptide sequence, did not significantly bind to αβ₃ integrin-expressing cells, demonstrating the sequence specificity of LXY30.

Here, we describe the characterization of the αβ₁ integrin-binding peptide LXY30 with respect to binding exosomes derived from αβ₁-expressing ovarian tumor cells. Through a combination of bulk and single EV characterization techniques, we demonstrate that LXY30 exhibits high binding affinity to specific tumor cell-derived exosomes, likely via the overexpressed αβ₁ integrin.

2. Results
2.1. Exosome Isolation and Characterization

Exosomes isolated from SKOV-3 ovarian tumor cells overexpressing αβ₁ integrin, Jurkat leukemia cells overexpressing αβ₃ integrin, and healthy human plasma were compared in this study. Classic ultracentrifugation (UC) methodology was used to isolate EVs from both cancer cell culture supernatant (see the Experimental Section for full detail) and human plasma samples. The collected EVs were confirmed as exosomes by employing the International Society for Extracellular Vesicles (ISEV) suggested standards for minimal characterization (Figure 1). Thus, western blot (WB) supported both the presence of multivesicular body-associated proteins CD63, CD9, and tsg101, and also the absence of endoplasmic reticulum-associated protein calnexin (Figure 1a). Imaging by

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**Figure 1.** Confirmation of exosome type for vesicles isolated from SKOV-3 ovarian tumor cells. a) Western blot analysis for CD63, CD9, tsg101, calnexin, and α₁ and β₁ integrin subunits in SKOV-3 cell lysate (CL) and exosome (Exo) preparations (20 μg protein per lane). b) Electron microscopy image of exosomes by negative-stained conventional EM showing the typical cup-shaped morphology. c) AFM amplitude image of isolated exosomes deposited on mica. d) Exosome number concentration and size distribution by NTA, with red bars representing one standard deviation.
negative-stained electron microscopy (EM) demonstrated typical exosome size and “cup-shape” morphology (Figure 1b).\(^{[36]}\)

Atomic force microscopy (AFM) provided complementary size measurement (Figure 1c). Nanoparticle tracking analysis (NTA) was used to determine EV concentration (\(1.70 \pm 0.11 \times 10^{13}\) particles mL\(^{-1}\)) and size distribution (\(177 \pm 87\) nm) (Figure 1d). According to a recently reported metric to assess exosome purity, we measured the ratio of protein concentration to particles mL\(^{-1}\), confirming that our isolation procedure reliably purified exosomes from excessive protein contamination.\(^{[37]}\)

2.2. On-Bead Flow Cytometry

To confirm cell–peptide binding, LXY30 (Figure 2a) and scr-LXY30 were labeled with fluorescein isothiocyanate (LXY30-Fl) dye via PEG\(_2\) spacer at the peptide N-terminus and incubated with SKOV-3 cells. As previously reported, SKOV-3 cells exhibited high affinity for LXY30-Fl but only minimal affinity for the scrambled analogue (Figure 2c, left panel).\(^{[34]}\) Next, SKOV-3 exosomes were premixed with either LXY30-Fl or scr-LXY30-Fl overnight. After purification from unbound ligand by centrifugal filtration, exosomes were mixed with the latex beads (Figure 2b). Similar to the cells, flow cytometry revealed a significant increase in fluorescence intensity for exosomes bound to LXY30-Fl compared to the scrambled-LXY30-Fl (Figure 2c, right panel) (statistical significance of \(p < 0.01\)). On the other hand, \(\alpha\beta\)-expressing Jurkat tumor cells and their released exosomes bound LXY30-Fl and scr-LXY30-Fl to a similar, minimal extent (data not shown). When incubated with anti-\(\alpha\beta\)-Fl, SKOV-3 cells demonstrated a high degree of binding, yet bead-bound SKOV-3 exosomes showed little increase in fluorescence compared to the control. In addition to the Jurkat cell-derived exosome control that tested the specificity of our peptide LXY30 toward its expected \(\alpha\beta\) integrin target, we also tested exosomes isolated from healthy human plasma samples. Following ultracentrifugation isolation (see the Experimental Section for full detail), the plasma-derived exosomes were subjected to LXY30-Fl and scr-LXY30-Fl binding and examination by on-bead flow cytometry (Figure 2c, dotted lines). The healthy human-plasma-derived exosomes did not exhibit significant binding to either LXY30-Fl or scr-LXY30-Fl peptide.

On-bead flow cytometry is limited to measuring bulk exosomes, and therefore may not be sensitive enough to distinguish circulating tumor-associated EVs. While single exosome flow cytometry is becoming feasible, it is still presently too unreliable for timely reporting of our findings. We therefore developed a new Raman spectroscopic method to measure LXY30-exosome binding for single vesicles.

2.3. Laser Tweezers Raman Spectroscopy (LTRS)

We employed LTRS to measure the binding of LXY30 to single vesicles by analyzing variations in Raman-active peaks for optically trapped vesicles, via adaptation of our previously reported methodology for single vesicle LTRS.\(^{[38]}\) As the ISEV-suggested characterization framework\(^{[36]}\) becomes more difficult to apply on a single-vesicle basis, here we refer to the optically trapped vesicles more generally as EVs.

For determining the extent of ligand binding to single EVs, LXY30 and scr-LXY30 peptides were each covalently functionalyzed with a highly Raman-active compound: 4-(phenylbuta-1,3-diyne-1-yl)benzoic acid (RT, for Raman Tag). In a region typically empty of Raman chemical shifts for unlabeled EVs, the di-alkyne and aromatic bonds present in the RT give rise to strong Raman shifts at 1600 and 2230 cm\(^{-1}\), respectively (Figure 3, blue trace). EV samples were incubated with LXY30-RT or scr-LXY30-RT (\(1 \times 10^6\) m) for optical tweezers trapping and Raman spectra measurement by LTRS. For each condition, at least ten individual EVs with sizes in the range of 80–200 nm were trapped and their Raman spectra averaged over 5 min. Typical peaks were indicative of EV trapping, for example, the peak centered at 1066 cm\(^{-1}\) can be assigned to C=C stretching in lipids, the peak at 1450 cm\(^{-1}\) assigned to CH\(_2\) and CH\(_3\) deformations in proteins and lipids, and the peaks at 1651 and 1668 cm\(^{-1}\) as C=C stretching in lipids and protein amide vibrations.

Figure 2. On-bead flow cytometry (FC). a) Chemical structure of the LXY30 peptide. For FC measurement, the N-terminus of the peptide is coupled to a FITC dye molecule via PEGylated spacer. b) Exosomes tagged with fluorescently labeled antibody or peptide are bound to sulfate/aldehyde-coated latex beads for FC measurement. c) FC profiles of fluorescence-labeled LXY30 (green), scr-LXY30 (red), and anti-\(\alpha\beta\) (blue) binding to SKOV-3 cells (left) or exosomes (right). Exosomes from healthy human plasma showed limited binding to scr-LXY30 (dotted red) and LXY30 (dotted green).
I vibrations, respectively.\textsuperscript{[38]} Effectively at the same dilution, the LXY30-RT peptide alone was undetectable (the spectra presented in Figure 3a are far more concentrated), yet when condensed on the vesicle could be observed quite strongly, as evident by the emergence of the two characteristic RT peaks (Figure 3b). Incubation with scr-LXY30-RT resulted in little discernible binding, and furthermore control Jurkat exosomes, which lack αβ, were not detected to measurably bind LXY30-RT. For trapped exosomes isolated from healthy human plasma, too, we could not detect any binding to LXY30-RT or scr-LXY30-RT. To quantify the relative binding stoichiometry of LXY30-RT to a single SKOV-3 vesicle, we further examined the region between 1550 and 1700 cm\(^{-1}\) (Figure 4). This region contains two major peaks, Peak A, centered at 1600 cm\(^{-1}\) arising from the Raman tag’s aromatic/di-alkyne functional group, and Peak B, the group of peaks arising from the aforementioned exosome protein/lipid content. The ratio of these peaks was taken for absolute comparison of chemical contribution from either the RT or the exosome. For this comparison, the Raman intensities were not normalized. We observed that the peaks did not scale together (Figure 4b), indicating that total protein content and LXY30 binding were not linearly correlated.

2.4. Effect of LXY30-Binding on Cell Uptake

Following demonstration of specific binding of LXY30 peptide to SKOV-3 cells and their derived exosomes, we tested whether LXY30-binding could inhibit or otherwise affect cell uptake of SKOV-3 exosomes. For visualization by confocal laser scanning microscopy (CLSM), isolated SKOV-3 exosomes were nonspecifically labeled with the red fluorescent lipophilic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). Dil-labeled SKOV-3 exosomes were incubated with cells for 1 h before washing and cell imaging by CLSM (Figure 5). Prior to peptide binding, exosomes exhibited significant uptake in SKOV-3 cells, with punctate fluorescence patterning indicative of endocytic uptake (Figure 5c). Energy-dependent uptake of SKOV-3 exosomes by SKOV-3 cells has been previously reported.\textsuperscript{[39]} When scr-LXY30 (1 × 10\(^{-6}\) m) was preincubated with Dil-labeled SKOV-3 exosomes (and unbound peptide removed by centrifugal spin columns), we observed a significant (\(p < 0.005\)) decrease in uptake (Figure 5d). Preincubation with LXY30 at the same concentration significantly reduced uptake even compared to the scr-LXY30 case (\(p < 0.005\)) (Figure 5e). Increasing LXY30 concentration continued reducing uptake until 10 × 10\(^{-6}\) m, at which point further increase of concentration did not continue to increase prohibition of exosome uptake (Figure 5f). Furthermore, at incubation concentrations above 10 × 10\(^{-6}\) m, significant cell toxicity occurred. CLSM images were quantified by pixel intensity and normalized by cell area for statistical comparison (Figure 5a).

2.5. Capture of SKOV-3 EVs by Surface Immobilized LXY30

To assess the potential of immobilized LXY30 as a tumor-associated exosome capturing or detection agent, we applied multiparametric surface plasmon resonance (MP-SPR). While SPR has been recently applied to EV characterization, these reports were performed using surface-immobilized antibodies targeted against surface proteins of vesicles, for instance, anti-CD9 or anti-CD63.\textsuperscript{[40]} Since most types of EVs express such generic membrane proteins in their membrane surfaces, they are unable to distinguish between tumor and healthy EVs. Instead, in this proof-of-concept model system, we demonstrate that immobilized LXY30 peptide on an SPR chip surface could...
capture the tumor EVs, and furthermore, that the surface-associated bilayer of captured EVs can be characterized for average thickness ($d$) and refractive index ($n$). Moreover, by comparing the recorded shifts in the position of SPR peak minimum reflectance ($\Theta_{SPR}$), interaction kinetics for peptide–EV binding can be calculated, including association/dissociation rate constants ($k_a$/$k_d$) and total binding affinity ($K_D$).

Figure 6 illustrates the MP-SPR scheme and relevant sensorgrams following EV injection. Several solutions are sequentially introduced by flowing over chips precoated with a self-assembled monolayer (SAM) of biotinylated-alkanethiol (11-mercapto-1-undecanol, MuOH). The MP-SPR instrument used here features two independent channels for parallel chip functionalization. To both channels, streptavidin was flown over and bound to the biotinylated surface, as confirmed by SPR response (Figure 6b). To flow channel 1 (Figure 6b, black), LXY30-biotin was added (in increasing concentration to ensure complete surface coverage), while control flow channel 2 (Figure 6b, red) was not exposed to LXY30-biotin. The subsequent injection of SKOV-3 exosomes in both channels at 100 min resulted in characteristic adsorption in the case of LXY30-decorated surface (flow channel 1) but little to no exosome binding for the control without LXY30-biotin (flow channel 2).

To assist modeling the kinetic parameters of binding, exosomes were serially injected at three different concentrations, representing $50 \times$, $30 \times$, and $10 \times$ dilutions ($3.4 \times 10^9$, $5.7 \times 10^9$, and $1.7 \times 10^{10}$ particles mL$^{-1}$, respectively). The sequential injections produced a stepwise signal reflecting the steady-state binding of exosomes at the sensor surface, corresponding to the three peaks evident in the flow channel 1 (Figure 6b outlined boxes, and examined more in detail in Figure 7a). For modeling the interaction kinetics, we employed a two-site binding model (the relevance of this model is discussed in the following section) allowing for two instances of LXY30 ligand binding to a single vesicle. For three hypothetical cases where the integrin content at the exosome surface represents either (i) 1%, (ii) 5%, or (iii) 10% of the total protein per particle, the $\alpha_3\beta_1$ integrin concentration can be estimated. Hence, using the total protein ($0.65 \mu$g $\mu$L$^{-1}$) concentration, the initial particle concentration determined with the NTA ($1.7 \times 10^{14}$ particles L$^{-1}$), and the average molecular mass for a single $\alpha_3\beta_1$ complex (220 kDa), we estimated the $\alpha_3\beta_1$ integrin concentrations as listed in Table 1. These values were used to fit the model to the data for the three injected concentrations. The resulting interaction kinetics evaluation yielded two association rate constants, $k_{a1}$ and $k_{a2}$, two dissociation rate constants $k_{d1}$ and $k_{d2}$, and two binding affinities, $K_{D1}$ and $K_{D2}$, respectively, for the possible cases (i), (ii), and (iii), as represented in Table 2. The results for binding affinities, $K_{D1}$ varied in the range of 12–26 $\times 10^{-9}$ M, and $K_{D2}$ between 55 and 480 $\times 10^{-12}$ M, suggest strong binding of SKOV-3 exosomes to the surface-bound LXY30 ligands. The low chi-squared values indicate that the data fitting was accurate. This can also be observed in Figure 7a displaying the measured (solid black remaining images represent the conditions where Dil-labeled exosomes were added to cells after premixing with c) no peptide, d) $1 \times 10^{-6}$ M scr-LXY30, e) $1 \times 10^{-6}$ M LXY30, and f) $10 \times 10^{-6}$ M LXY30. The scale bar is 20 $\mu$m. * represents $p < 0.005.$
Figure 6. On-chip capture of tumor EVs. a) MP-SPR detection platform for tumor SKOV-3 exosomes using the surface-immobilized LXY30 capturing peptide. Each step of the peptide immobilization via streptavidin-biotin conjugation can be followed by SPR response. b) Sensorgram of the SKOV-3 exosome detection following biotin-PEG-SH self-assembled monolayer (SAM) formation. First, streptavidin was injected to both flow channels, followed by injections of LXY30-biotin in flow cell 1 only. Finally, SKOV-3 exosomes were injected to both flow channels. The characteristic adsorption outlined by the dotted box was modeled for extraction of kinetic parameters (Figure 7).

Figure 7. Kinetic modeling of MP-SPR sensorgrams. a) The solid black line shows the measured data during the three injections while the red, blue, and pink dashed lines depict the fitted data for the hypothesized cases of (i) 1%, (ii) 5%, and (iii) 10% coverage of integrin on the surface of a SKOV-3 exosome. b) Full SPR angular spectra recorded at various time points during the experiments; the black line represents the initial situation whereby the gold sensor surface and SAM layer were measured; the red line illustrates the events after the streptavidin injection and the following baseline stabilization; and the blue line is a snapshot after all three SKOV-3 concentrations were injected and a stable baseline was observed. The pink dashed line depicts the modeled fitting at the last phase of the interactions, that is, following the three SKOV-3 injections.

3. Discussion

Of the αβ integrin-expressing tumor cell lines found to bind LXY30 in preliminary studies, SKOV-3 ovarian tumor cells reliably produced the largest number of EVs with minimal effort in cell maintenance, and thus were chosen as the model cell line for this study. Jurkat human T lymphoid tumor cells were cultured as a control to measure integrin specificity, since they express αβ and not αβ3. Using western blot on SKOV-3 cell and SKOV-3 exosome lysates, we measured
the presence of both α<i>3</i> and β<i>3</i> integrins (Figure 1a), paving the way for targeting tumor-associated exosomes by (i) reproducing previous findings that tumor cells pass certain overexpressed integrins to their released exosomes,[39] and (ii) identifying a target for LXY30 binding. Interestingly, α<i>3</i> was not readily detectable in the SKOV-3 cell lysate compared to the SKOV-3 exosome lysate, likely because it represents only a minor fractional component of the total loaded protein, yet we do demonstrate the presence of the α<i>3</i> subunit on SKOV-3 cells via significant anti-α<i>3</i> antibody binding to SKOV-3 cells when examined by flow cytometry (Figure 2c).

Flow cytometry is an increasingly important tool for EV characterization.[42] Presently, exosomes themselves are too small to be easily and reliably examined by most flow cytometers, but they can be bound to 4 μm aldehyde/sulfate-coated latex beads for analysis (Figure 2b). The aldehyde functional groups can be covalently linked to lysine side chains on exosomal protein while the anionic sulfate groups confer colloidal stability to the beads during exosome binding. With this technique, we found that both α<i>3</i>β<i>3</i>-expressing SKOV-3 tumor cells and their released exosomes bind strongly to LXY30-Fl. When incubated with scr-LXY30-Fl, SKOV-3 cells exhibited a drastic reduction in binding, while their exosomes retained some nonspecific binding. Yet, SKOV-3 exosomes did not measurably bind anti-α<i>3</i> antibody, nor did prior incubation with anti-α<i>3</i> inhibit LXY30 binding. This finding could be interpreted by considering the state of the integrin membrane protein under extreme membrane curvature, as is the case for nanoscale vesicles. At this scale, a biomembrane’s extreme geometrical curvature can critically influence biochemical properties, with some reports suggesting occurrence of significant protein conformational changes.[45] We hypothesize that this may result in poor antibody affinity for exosomal integrins, despite high affinity toward cell membrane integrins. This point will be investigated further in future studies, but is a good example of an advantage of peptide binding in place of immunoaffinity methods.

Following bulk flow cytometry measurement of LXY30-EV binding, we applied our recently reported technique of LTRS. A complete discussion of the application of LTRS to optically trap and subsequently measure the chemical composition of EVs can be found in our recent publication.[38] While a major advantage of Raman spectroscopy is label-free characterization, we strengthened the technique with the addition of Raman tags, extending the capability of LTRS to measuring ligand binding on a single exosome. As illustrated in Figure 4b, ligand binding can be quantified in a particular region where spontaneous Raman scattering peaks arise from either the LXY30-RT (Peak A, alkyne, 1600 cm<sup>−1</sup>) or exosomes (Peak B, a grouping of 1651 cm<sup>−1</sup> from C=C stretching, and 1668 cm<sup>−1</sup> from amide I vibrations). The table in Figure 4b lists the spectra by descending vesicle peak height (Peak B), and it is evident that the ratio of Peak A to Peak B fluctuates randomly. In other words, for a given single vesicle (i.e., a single trace in Figure 4a), the peaks did not scale together, as might be expected if one assumes that Peak B represents relative exosome amount (protein/lipid content) and that integrin number scales with vesicle size. This may represent a varied expression in integrin distribution across subpopulations of vesicles that may not scale with vesicle surface area, or instead, variation in LXY30-integrin binding. Although we could not extract binding stoichiometry in this case, we regard Raman tagging of small molecules as a major innovation for examining binding to single extracellular vesicles.

Interestingly, LXY30 binding was able to inhibit cell uptake of the SKOV-3 exosomes by their parent cells. By premixing Dil-labeled SKOV-3 EVs with LXY30, we observed a reduction in cell uptake up to ~80%. Preincubation with scr-LXY30 reduced uptake by ~50%, corroborating our flow cytometry results that some weak binding of scr-LXY30 still occurs, though significantly reduced (<i>p</i> < 0.005) compared to LXY30. Some fluorescence was still observed even at saturation of LXY30 (i.e., higher amount of LXY30 did not further decrease uptake), indicating that even by blocking the α<i>3</i>β<i>3</i>-mediated uptake, some other independent mechanisms remain intact. The mechanisms by which integrin blocking reduces cell uptake are unclear, but given that circulating tumor exosomes are heavily implicated in tumorigenesis, this observation renders LXY30 useful for potential therapeutic application, and will be a topic of future investigation in our lab.

Several recent studies and reviews have reported on the feasibility of applying extracellular vesicles to therapeutic or diagnostic application, emphasizing the importance of developing EV marker assays that outperform and/or complement conventional diagnostics.[46–48] In that context, the flow cytometry and LTRS methodologies presented above would be challenging to adopt in clinical settings. In fact, most traditional exosome characterization assays typically rely on various techniques requiring labels that alter the biochemical structures of interest, or are limited to sampling rate, thus not offering information

| Table 1. Estimated αβ<sub>3</sub> integrin concentrations (×10<sup>−9</sup> µM). |
|------------------|------------------|-----------------|-----------------|
| Exosome dilution | 50× | 30× | 10× |
| 1% coverage | 0.62 | 1.03 | 3.09 |
| 5% coverage | 3.09 | 5.15 | 15.5 |
| 10% coverage | 6.20 | 10.3 | 30.9 |

| Table 2. The results of interaction kinetics analysis, biolayer characterization, and surface mass density calculations. |
|------------------|------------------|------------------|------------------|------------------|
| Estimated αβ<sub>3</sub> | k<sub>b</sub> [s<sup>−1</sup> | k<sub>e</sub> [s<sup>−1</sup> | K<sub>D1</sub> [µM] | k<sub>d1</sub> [s<sup>−1</sup> | k<sub>d2</sub> [s<sup>−1</sup> | K<sub>D2</sub> [µM] | χ² |
| (i) 1% | 5.14 × 10<sup>−6</sup> ± 6.97 × 10<sup>−6</sup> | 9.35 × 10<sup>−6</sup> ± 9.11 × 10<sup>−6</sup> | 1.82 × 10<sup>−4</sup> ± 4.23 × 10<sup>−5</sup> | 1.28 × 10<sup>−2</sup> ± 7.28 × 10<sup>−2</sup> | 7.00 × 10<sup>−2</sup> ± 1.88 × 10<sup>−2</sup> | 5.46 × 10<sup>−11</sup> ± 1.47 × 10<sup>−11</sup> | 7.52 |
| (ii) 5% | 6.32 × 10<sup>−2</sup> ± 1.85 × 10<sup>−3</sup> | 7.30 × 10<sup>−3</sup> ± 1.54 × 10<sup>−3</sup> | 1.16 × 10<sup>−4</sup> ± 5.83 × 10<sup>−5</sup> | 2.52 × 10<sup>−2</sup> ± 9.53 × 10<sup>−3</sup> | 6.05 × 10<sup>−2</sup> ± 2.53 × 10<sup>−2</sup> | 2.40 × 10<sup>−10</sup> ± 1.01 × 10<sup>−10</sup> | 6.46 |
| (iii) 10% | 2.90 × 10<sup>−2</sup> ± 1.86 × 10<sup>−3</sup> | 7.40 × 10<sup>−2</sup> ± 1.26 × 10<sup>−3</sup> | 2.55 × 10<sup>−2</sup> ± 2.08 × 10<sup>−3</sup> | 1.26 × 10<sup>−2</sup> ± 6.30 × 10<sup>−3</sup> | 6.07 × 10<sup>−3</sup> ± 2.13 × 10<sup>−3</sup> | 4.82 × 10<sup>−10</sup> ± 1.71 × 10<sup>−10</sup> | 6.47 |
| d [nm] | n | Surface mass density increase [ng cm<sup>−2</sup>] | Δ (F) = 90 |
in real time. One recent review by Lener et al. extensively describes the requirements for EV-based therapeutics, outlining the challenges in costs, reproducibility, effectiveness, throughput, and limit of detection.\[48\] For those reasons, we applied the technique of MP-SPR to measure LXY30 binding. MP-SPR is extremely sensitive label-free optical measurement method based on detecting plasmonic phenomena enhanced by a nearby metal surface. MP-SPR measurements can be made in real time, allowing for constant monitoring of interaction events. It also employs a microfluidic flow system enabling measurements under steady state or dynamic flow conditions, and thus is considered more relevant for characterizing physico-chemical phenomena in biological environments. Furthermore, at their best, plasmonic-based measurements offer at least an order of magnitude in increased detection sensitivity compared to ELISA or fluorescence-based detection methods,\[49\] therefore it will be an excellent direction to explore in future studies examining binding to clinical samples.

Using MP-SPR, we confirmed the ability for SKOV-3 exosomes to bind to LXY30 modified surfaces. We adopted the commonly used one-to-two (1:2) binding model for adi- sorption, taken from previous observations that the CD63 tetraspanin proteins on exosomal membranes exhibit such binding behavior.\[14\] From a physical standpoint there may be more than two binding sites per exosome, but it has been observed that the two strongest sites are domin- ant, and other binding events are typically undetected.\[50\] It is noteworthy that the 1:2 binding model is also applicable to binding schemes where heterogeneous immobilization of the target (i.e., LXY30) at the surface may have occurred. We expect that this is likely the case in our measurements, since the modeling resulted in good data fitting as seen by visual inspection in Figure 7. The calculated interaction kinetics parameters (Table 2) did not show strong depend- ency on the estimated amount of \(\alpha_3\beta_1\) integrin proteins on the membranes of SKOV-3 exosomes. This observation may suggest that the binding of SKOV-3 exosomes occurs as long as there are at least some \(\alpha_3\beta_1\) found on the surfaces of exosomes available for binding. The results of the layer model- ing also suggest that either (i) significant vesicular shape deformation events may occur during the exosome binding or at the surface after binding, (ii) surface-bound LXY30 cap- tures exosomes of certain diameter or up to some maximum diameter, or more likely that (iii) the coverage of exosomes at the surface is uneven. The latter is a technical limitation of optical modeling, as mathematical fitting of the measured angular SPR spectra to the models by Fresnel’s equations is made by averaging over the laser spot area on the gold sensor surface, \(\approx 1 \text{ mm}^2\) in diameter. Therefore, empty spaces in the surface-bound layer under the laser spot would result in underestimating the calculated layer thickness and refractive index. In recent SPR-vesicle literature, physical models have been developed to correlate change of refractive index with exosome concentration.\[51,52\] In those models, both particle shape deformation and surface unevenness (due to wide particle size distribution) were considered. The biolayer thickness modeling in this study strengthens those previous observations, namely, that shape deformation may play a role in exo- some interactions and should be taken into account.

While the layer modeling yielded rational results with satisfactory accuracy within the context of this study, and had resemblance to other recent investigations,\[32\] intriguing ques- tions merit further studies. For example, we plan to examine the differences between interaction kinetics and biolayer com- positions for exosomes from different cell types. A prerequisite for such studies is that additional exosome-specific capturing ligands are found and synthesized, currently underway. Finally, calculation of the limit of detection (LOD) also warrants subse- quent assays in order to find out the feasibility of this platform for early-stage cancer detection by sensing tumor-associated exosomes in human biofluids.

4. Conclusions

EVs isolated from various biological fluids are essentially a mixture of distinct types of vesicles that have originated from various parent cells. Hence, the capability to detect certain types of EVs, such as tumor-associated ones, is essential for their exploitation as diagnostic agents. Since integrins are known to be highly overexpressed on cancer cells, and are trafficked to those cells’ released EVs, we developed a methodology for characterizing the specific targeting of integrin-binding pep- tides to cancer cell released EVs. Our in-house peptide, LXY30, selectively binds the \(\alpha_3\beta_1\) integrin overexpressed on cells comprising many types of epithelial cancers, including ovarian adenocarcinoma cells. In SKOV-3 ovarian tumor cell released EVs (that were determined to be primarily exosome-type), pro-tein quantification revealed the presence of both \(\alpha_3\) and \(\beta_1\) inte- grin subunits. On-bead flow cytometry was used to characterize ensemble LXY30 peptide binding to SKOV-3 exosomes, but not other types of Jurkat-tumor-cell-derived exosomes. Laser tweezer Raman spectroscopy confirmed that LXY30 peptide binding occurs at the single vesicle level. Furthermore, by confocal microscopy, we determined that LXY30 could dramatically reduce SKOV-3 exosome uptake by their parent cells. Finally, we present a convenient and straightforward method to cap- ture and detect cancerous SKOV-3 exosomes in real time by MP-SPR, without a priori labeling. This platform allowed for monitoring the interaction kinetics of and biolayer properties of SKOV-3 vesicles with the surface-immobilized LXY30 capturing ligand. The characterization schemes outlined here, along with the first report of a ligand capable of discriminating tumor and nontumor EVs, represent major steps in improving the poten- tial of EVs for nanomedical-based therapeutic and diagnostic systems.

5. Experimental Section

Unless otherwise noted, all reagents and materials were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and used without further purification. Cell lines were purchased from ATCC (Manassas, VA).

Cell Culture: Human ovarian carcinoma SKOV-3 cells were grown in McCoy’s 5A media and human T-cell leukemia Jurkat cells were grown in RPMI 1640 medium. Each medium was supplemented with FBS (10%), penicillin (100 units mL\(^{-1}\)), and streptomycin (100 µg mL\(^{-1}\)). Cells were subcultured (37°C, 5% CO\(_2\)) and regularly maintained by splitting upon reaching an estimated 80% confluence. Contaminating EVs are found
in high concentration in FBS and must be removed before exosome collection. In vitro serum-free conditions have been demonstrated to increase oxidative stress, leading to generation of unintended EV release,[31] therefore it is preferred to deplete FBS of exosomes by ultracentrifugation. Typically, 30% FBS (150 mL) in appropriate culture medium was loaded in the rotor-type 50.2 Ti (Beckman Coulter, polycarbonate bottles, prod. no. 355618) and spun at 100 000 × g, 4 °C, >18 h. After centrifugation, supernatants were filtered (0.2 µm) and diluted with appropriate medium to 10% FBS. Clearing of bovine EVs is regularly confirmed by NTA. It was observed that exosome yield began to drop significantly if a cell line was grown in prolonged absence of EV-depleted FBS, therefore cells past five generations after thawing from liquid nitrogen were not cultured.

Preparation of exosomes: After 48–72 h of incubation with EV-depleted medium, cell culture supernatant was decanted and subjected to low-speed centrifugation to clear any cells (3000g, 5 min, 4 °C), dead cells (20000g, 10 min, 4 °C), and cell debris (10 000g, 30 min, 4 °C). The resulting supernatant was centrifuged twice at 100 000 × g, 4 °C, 2 h (rotor type: SW 28 Ti, tubes: thickwall polycarbonate), dissolving and diluting the pellet in PBS between spins to remove contaminating free protein. For every 20 million cells at the time of EV harvest, the final exosome-containing pellet was dissolved in sterile DPBS. Samples were stored at −80 °C for up to 1 month. Human plasma was obtained from healthy volunteers and deidentified via the UC Davis biorepository for laboratory use. Plasma obtained from three different patients (>0.5–1.0 mL each) was used as controls in this study. Whole plasma was subjected to identical ultracentrifugation isolation as described above for cell culture supernatant.

Western Blot: All reagents listed by part number in this section were purchased from ThermoFisher Scientific. Following exosome lysis with RIPA buffer (prod. no. 89900), protein concentration was measured by BCA assay (Piecer kit, prod. no. 23225) according to manufacturer’s instructions. For WB analysis, exosome aliquots containing 20 µg protein (as determined by BCA assay) were lysed in SDS sample buffer: 2% (w/v) sodium dodecyl sulfate, 125 µg phenylmethylsulfonyl fluoride, 10 mM EDTA, 100 mM Tris- HCl at pH 8.6, 12% (v/v) glycerol, 0.05% (w/v) bromophenol blue, and DTT (50 µM to 1 mM). For proteins that should not be reduced prior to immunostaining (tetracaspans CD9 and CD63), DTT was omitted and its volume replaced by PBS. To obtain cell lysates, cell suspensions were washed by repeated (3×v) pelleting (300g, 5 min) and dissolution in sterile DPBS. After three washes, the cell pellet was dissolved in RIPA lysis buffer with protease inhibitor (prod. no. 78420), and briefly vortexed before and after 30 min incubation on ice. Following incubation, the solution was centrifuged to precipitate insoluble membrane components and other impurities (18 000g, 30 min, 4 °C) and resulting supernatant frozen for up to six months at −80 °C. Protein aliquots (20 µg) were dissolved in Laemmli sample buffer (prod. no. NP0008), supplemented appropriately with reducing agent, just prior to gel loading. After dissolution in appropriate loading or lysis buffer, EV and cell lysates were incubated at (95 °C, 5 min) while shaken (900 rpm) and then centrifuged (1 min, 13 000g).

Gel electrophoresis of lysed EVs or cell was performed by first loading samples (20 µg protein/well) onto 4%–12% Bis-Tris ten-well precast gels (NuPage Novex, prod. no. NP0335) and filling the electrophoresis rig (Xcell Surelock gel tank) with MOPS buffer (NuPage, prod. no. NP00102). Proteins were separated at 90 V for 30 min, then 120 V until complete. The gel was carefully removed, washed with buffer, and transferred to nitrocellulose membrane (prod. no. 88018) for 1 h at constant current of 400 mA, with the entire WB module submerged in ice. The resulting blots were removed and blocked overnight by submerging in blocking buffer: 5% (w/v) nonfat dairy milk in Tris-buffered saline with Tween (TBST) buffer. For immunostaining, primary antibody was appropriately diluted in blocking buffer according to its datasheet and incubated with blot for 1 h at room temperature while gently shaking. The following primary antibodies were used: mouse monoclonal anti-CD9 (ThermoFisher Scientific), rabbit polyclonal anti-tsg101 (Sigma Aldrich, St. Louis, MO), rabbit polyclonal anti-calnexin (Cell Signaling Technology, Danvers, MA), and mouse monoclonal anti-β5 and anti-fib integrins (Santa Cruz Biotechnology, Dallas, TX). The blots were washed (3× TBST, 5 min) and incubated with HRP-conjugated secondary antibody diluted in blocking buffer (1:2000) for 1 h. Secondary antibodies were goat antirabbit and goat antimouse immunoglobulin (Santa Cruz Biotechnology, Dallas, TX). ECL substrate (prod. no. 35000) was used to develop blots according to manufacturer’s instructions and immunonoave bands detected by chemical imaging (Bio-Rad ChemiDoc Hercules, CA). Representative WB for SKOV-3 cell lysate and exosomes is shown in Figure 1a.

**Electron Microscopy:** EVs were contrasted and embedded for whole-mount EM analysis according to previously reported methods.[48] Briefly, fresh EV-containing UC pellets were directly resuspended in 2% (w/v) formaldehyde in PBS and 10% (v/v) coated EM grids. The grids were sequentially imaged by EM at 80 kV (Philips CM120). A representative EM image of SKOV-3 derived EVs is displayed in Figure 1b.

**Atomic Force Microscopy:** An aliquot (50 µL) of diluted (1:200) purified SKOV-3 EVs was pipetted onto a freshly cleaned mica sheet and air dried. Images were acquired with an Oxford-Asylum Research MFP-3D AFM and T-KTEK Nanotechnologies Tetra14 silicon lever with a spring constant of about 5. Pixel resolution was 512 × 512 and scan rate was 0.5 Hz. Topography and Z-sensor images were flattened using mean surface data to establish a reference plane. Amplitude images were also taken in order to show edge enhancements, approximating the EV surface gradient. A representative AFM amplitude image for SKOV-3 EVs is shown in Figure 1c.

**Nanoparticle Tracking Analysis:** NTA was recorded on a NanoSight LM10 instrument with illumination at 488 nm. Following dilution of purified EVs (1:1000) in triple-filtered PBS, samples were introduced by perfusion pump. For a typical experiment, three 60 s videos were recorded, with 30 s of sample flow between replicates. Concentration was confirmed to be in the acceptable range for NTA analysis (3–20 × 10^10 particles mL^{-1}), or else diluted/concentrated appropriately and reanalyzed.

**Peptide Synthesis:** Solid-phase peptide synthesis was used to synthesize LXY30 and scr-LXY30 peptides. Rink amide resin (0.52 mmol g^{-1} loading) was chosen as the solid support in order to facilitate peptide synthesis and eventual cleavage from the resin. HPLC (high-performance liquid chromatography) grade dimethylformamide (DMF) was used for coupling and analytical reagent grade DMF, dichloromethane (DCM), and methanol (MeOH) were used for washing of the resin throughout synthesis. Resin was swollen overnight in DMF in a polystyrene column with frit and tight-sealing cap, enabling easy washing via bead retention during coupling steps. The protecting Fmoc group was removed from the resin by 20% (v/v) 4-methyl-piperidine/DMF solution (2 × 15 min), and the resin was washed by DMF (15 mL × 3), MeOH (15 mL × 3), and DCM (15 mL × 3). Standard Fmoc/t-But peptide chemistry was used to couple the amino acids to the resin. For each coupling step, 5 molar eq. of COMU ([1-cyano-2-ethoxy-2-oxoethyldiminoxy] dimethylamino-morpholino-carbenium hexafluorophosphat) and 10 molar eq. DIEA (N,N-diisopropylethylamine) were added with 5 molar eq. of Fmoc-protected amino acid. After 1 h of light agitation, Kaiser test was used to confirm complete reaction. Following coupling, the Fmoc group was again removed by 4-methyl-piperidine/DMF solution, confirmed by Kaiser Test. Prior to cleavage from the bead, the disulfide in the peptide was formed. Iodine (8 molar eq. to resin, dissolved in DCM) was used to simultaneously deprotect tryt chemical groups on the cysteine residues and oxidize the thiols to form a ring. The resin was subsequently washed with copious DMF and aqueous acetic acid to quench and remove excess iodine. Finally, the resin was mixed for 3 h with slight agitation at room temperature with a cleanup cocktail (82.5% trifluoracetic acid, 15% triisopropylsilane, all % are v/v), resulting in both removal of side chain protecting groups and cleavage from the resin. The peptide-containing
filtrate was collected and concentrated under a gentle stream of air for 30 min. A large volume of cold diethyl ether was added to precipitate the crude peptide, which was collected after brief centrifugation (3000g, 5 min), washed again with diethyl ether, and dissolved in water for HPLC purification. 5 × 10^{-5} M peptide stock solutions were stored at 4 °C until needed. For flow cytometry detection, peptide analogues were additionally conjugated at their N-terminus with a short PEG spacer (N-[8-(9-fluorenylmethyloxycarbonyl)amino-3,6-dioxaoctyl]succinimide acid, Ebes) and FITC dye. For both reactions, 4 molar eq. 8 eq. DIEA were mixed with beads in DMF for 2 h before washing. Reaction steps were confirmed by Kaiser test. For LTRS measurement, 4-(phenylbuta-

1,3-diyn-1-yl)benzoic acid (RT) was covalently conjugated to the peptide N-terminus via PEG spacer (Figure S1, Supporting Information) under similar coupling conditions. Peptide molecular weight was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry (Bruker UltraFlextreme). Figure S1 (Supporting Information) displays a representative mass spectrum for purified LX30-RT peptide.

**Flow Cytometry:** For whole cell flow cytometry, cells were trypsinized (if necessary) and aliquoted in 3% (v/v) FBS in PBS with LX30-Fl or scr-LX30-Fl peptide (to 1 × 10^6 u). After 1 h, the cells were washed thrice with PBS and finally resuspended with 3% FBS and 1% sodium azide. On-bead flow cytometry of antibody–exosome binding was performed according to previous reported methods, by passive adsorption to 3.9 µm latex beads with aldehyde/sulfate groups grafted to the polymer surface (ThermoFisher Scientific, prod. no. A37304). Only one change to the protocol was made for peptide incubation: exosomes were labeled with peptide prior to bead adsorption. Briefly, exosomes (10 µg in 15 µL, as measured by Bradford) were mixed overnight with LX30-Fl or scr-LX30-Fl (1 µL, 5 × 10^{-5} m). Labeled exosomes were purified from free peptide by centrifugal spin column (ThermoFisher Scientific, MWCO: 3000 Da, prod. no. 4484449) according to manufacturer’s instructions. Prepared whole cells or exosome-coated latex beads were promptly analyzed by flow cytometry (Becton Dickinson Fortessa). Three side-by-side replicates were performed for each condition and at least 10 000 gated cells or beads were analyzed for each replicate. Typical gating strategies for SKOV-3 cells and latex beads are displayed in Figure S2 (Supporting Information).

**Laser Tweezers Raman Spectroscopy:** Raman measurements were made on a purpose-built LTRS system that was described previously. Briefly, 785 nm laser light (CrystalLaser, Reno, NV) was coupled into a multimode optical fiber and launched into a spectrograph (BioNavis Ltd., Tampere, Finland) instrument was used for the surface plasmon resonance measurements. The system was equipped with two wavelength lasers, 670 and 785 nm, for plasmon excitation at the metal surface. In addition, it consisted of two independent microfluidic channels having inflow and outflow PEEK tubing, a controllable peristaltic pump for buffer flow, and a 12-port chromatography injector for the sample injections. Both of the channel surfaces were scanned with both incident wavelengths (i.e., 670 nm laser spot in flow channel 1 and flow channel 2; 785 nm laser spot, correspondingly) over a wide angular area of ~40°−78°. Such a scanning method allows for recording full SPR angular spectra instead of the narrow angular window near the SPR peak minimum that is typically measured with traditional SPR instruments.

The sensor surface was designed as a biofunctional multilayered composition whereby the bottom molecular layer consisted of a SAM structure. The protocol for SAM establishment on a plain gold sensor surface was adopted from previous works describing the robustness and versatility of such molecular construction. In brief, the gold sensor was incubated overnight ex situ in a glass vial sealed with wax membrane in MuOH: biotin–PEG–thiol mixture in molar ratio 90%/10% in 88% EtOH. Immediately before using, the sensor was washed three times with excess amount of 99% ETOH and gently dried under nitrogen gas flow and mounted into the instrument. Consequently, in order to associate streptavidin to the biotin moieties of SAM, streptavidin solution representing autofluorescence in the system. For the AsLS modeling, an asymmetry parameter of p = 0.001 to heavily penalize negative residuals was used, allowing to obtain spectra free of any background contamination. Following background correction, all spectra were normalized to the area under the curve (vector normalization). Thus, the y-axis of the Ramana spectrum then represents the proportion of individual chemical groups relative to the total chemical content within the sampling volume. Instead, for comparison of absolute quantity of chemical content (Figure 4a), no normalization was performed.

For peptide labeling experiments, LX30-RT or scr-LX30 were added to the vesicles during imaging on the coverslip to a final concentration of 100 × 10^{-6} m. At this concentration in the absence of EVs, the notable peaks arising from the RT could not be discerned. To measure the LAS signal for liposome monolayer aggregates of LX30-RT were trapped before full dissolution in water.

**Exosome Uptake by Confocal Laser Scanning Microscopy:** To label the membrane of EVs for cell uptake visualization, the lipophilic dye DiL (Life Technologies, Catalog No. D9311) was used. A stock solution (10 × 10^{-6} µL) of DiL in MeOH was diluted 100-fold with SKOV-3 EVs (30 µg) to a final volume of 200 µL in PBS containing 0.5% BSA. Dilabeled EVs were diluted 50-fold with McCoy’s media supplemented with 10% EV-cleared FBS and repelleted at 10 000 g, 4 °C, 2 h. The pellet was resuspended in McCoy’s media. Aliquots (5 µg) Dil-labeled SKOV-3 EVs were mixed with either LX30 or scr-LX30 to final concentrations of 1 or 10 × 10^{-6} µL. For blank controls, the peptide solution was replaced by PBS. After overnight incubation at 4 °C with gentle agitation, EVs were purified from unbound ligand by centrifugal spin column (ThermoFisher Scientific, MWCO: 3000 Da, prod. no. 4484449) according to manufacturer’s instructions.

SKOV-3 cells were plated in an eight-well ibiTreat µ-slide chambers (ibidi USA, Inc.) at a starting concentration of 10 000 cells per well in serum-containing McCoy’s media (300 µL). After 24 h, the cells were washed thrice with PBS, finally replaced in fresh PBS (200 µL), and promptly imaged under confocal microscopy with the appropriate filter set. Images were exported to ImageJ for analysis. The average fluorescence intensity and standard deviation (normalized by cell area) were measured and corrected by representative background areas. The statistical significance of differences in mean values was assessed using a two-sample independent Student’s t-test at the 99.5% confidence level. Differences were reported using P values.

**Multiparametric Surface Plasmon Resonance:** The MP-SPR Navi 200 (BioNavis Ltd., Tampere, Finland) instrument was used for the surface plasmon resonance measurements. The system was equipped with two wavelength lasers, 670 and 785 nm, for plasmon excitation at the metal surface. In addition, it consisted of two independent microfluidic channels having inflow and outflow PEEK tubing, a controllable peristaltic pump for buffer flow, and a 12-port chromatography injector for the sample injections. Both of the channel surfaces were scanned with both incident wavelengths (i.e., 670 nm laser spot in flow channel 1 and flow channel 2; 785 nm laser spot, correspondingly) over a wide angular area of ~40°−78°. Such a scanning method allows for recording full SPR angular spectra instead of the narrow angular window near the SPR peak minimum that is typically measured with traditional SPR instruments.
(4 × 10^{-6} \text{ m}) was flown over the SAM surface in situ at constant flow speed (20 \text{ mL min}^{-1}) in TRIS-EDTA (25 \times 10^{-3} \text{ m} \text{ TRIS}, 1 \times 10^{-3} \text{ m} \text{ EDTA}, pH 8.0) running buffer. Finally, after the streptavidin immobilization, using two independent microfluidic channels in the MP-SPR instrument, one channel surface was functionalized with biotinylated LXY30 peptide (LXY30-biotin) in constant TRIS-EDTA buffer flow (15 \text{ mL min}^{-1}) onto the gold sensor slide, while the other channel remained without LXY30 and was exposed to only buffer flow. The interactions of three different concentrations of SKOV-3 exosomes with the surface-associated LXY30-biotin were carried out using a flow speed of 10 \text{ mL min}^{-1}. Evaluation of interaction kinetics was performed using TraceDrawer 1.6 software (Ridgeview Instruments AB, Vänge, Sweden). LayerSolver 1.2.1 software (BioNavis Ltd., Tampere, Finland) was used for the biolayer thickness and refractive index modeling.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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