Assessing the effect of protein corona formation in the process of EV surface engineering

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ABSTRACT

To be profitably exploited in medicine, nanosized systems must be endowed with biocompatibility, targeting capability, the ability to evade the immune system, and resistance to clearance. Currently, biogenic nanoparticles, such as Extracellular Vesicles (EVs), are intensively investigated as the platform that naturally recapitulates these highly needed characteristics.
EV native targeting properties and pharmacokinetics can be further augmented by decorating the EV surface with specific target ligands as antibodies. However, up to date, works dealing with the functionalization of EV surface with proteins have never considered the biomolecular corona (BC) “variable”, namely the fact that extrinsic biomolecules, mainly proteins, may spontaneously adsorb on the EV surface in biofluids, contributing to determine the biological identity of the EV.
In this work, we explore and compare EVs modified with the antibody Cetuximab (CTX) by chemisorption (covalent binding of CTX via bioorthogonal click-chemistry) and by physisorption (formation of a CTX corona). Results (surprisingly) indicate that (i) no differences exist between the two formulations in terms of binding affinity imparted by molecular recognition of CTX versus its natural binding partner (epidermal growth factor receptor, EGFR), but (ii) significative differences emerge at the cellular level, where CTX-EVs prepared by click chemistry display superior binding and uptake toward target cells in Fetal Bovine Serum (FBS)-supplied culture medium, very likely due to the higher robustness of the CTX anchorage that resists to the formation of a BC due to interaction with the FBS proteins.

KEYWORDS: extracellular vesicles, red blood cells, surface functionalization, biomolecular corona, cetuximab, bioorthogonal chemistry
INTRODUCTION

Extracellular nanoparticles (ENPs) are nanoparticles originating from cells and biological systems, including Extracellular Vesicles (EVs), lipoproteins, and protein nanoaggregates. They constitute the nanostructured secretome and differentiate each other in biological roles and biophysical properties. Due to their biological origin, ENPs present lower cytotoxicity and higher colloidal stability in biological fluids than their synthetic counterparts (e.g., liposomes, and synthetic nanoparticles - SNPs). For these reasons, ENPs are emerging in different medical fields as promising therapeutics and imaging agents. Among the ENPs, EVs have gained much interest during the last decade mainly due to their outstanding therapeutic performances in cancer therapy and regenerative medicine. EVs are composed of a phospholipid bilayer membrane enriched with lipids, proteins, and carbohydrates enclosing an aqueous core containing soluble proteins, nucleic acids, and metabolites. The complexity of EV structure allows their application as up-and-comer biological delivery systems. However, despite promising preclinical data, EV-based therapeutic approaches have been hampered by different issues, including EV-based therapeutics stability, scalability, safety, and accumulation in specific organs (e.g., liver and spleen). A promising approach to tackle EV-based therapeutics pharmacokinetics and tropism limits is represented by the functionalization of EV surface that can be achieved by endogenous or exogenous strategies. Endogenous EV surface functionalization exploits cellular machinery and involves genetic modification of EV-secreting cells. This challenging approach could lead to unintended cell changes and may not be suitable for introducing unnatural or short-term stable molecules. Contrarily, exogenous EV surface functionalization can be achieved by manipulating the EVs after their isolation, thus overcoming some of the limitations mentioned above related to the endogenous strategy. Indeed, exogenous modification of EVs is not affected by cellular activity and exploits the richness of chemistry conferred to the biological surface by the presence of tunable molecules such as proteins and carbohydrates. Finally, it can be applied to introduce both natural and artificial moieties. One of the most investigated exogenous surface functionalization methods is based on covalently binding target ligands to suitable moieties of membrane proteins or phospholipids present on EV membranes. While small molecules such as fluorophores or drugs can be added with a one-step reaction to EV surface components, larger moieties as antibodies require the preliminary introduction of spacers and clickable groups onto the EV surface, followed by a reaction with suitable units on antibodies or proteins to achieve EV functionalization. During this procedure, EVs are generally reacted with a target protein-enriched solution to achieve a satisfactory increase in the kinetics of the reaction and the conjugation yield. It has been widely acknowledged that synthetic nanoparticles, when immersed in biofluids, are almost immediately covered by multiple layers of macromolecules (i.e., biomolecular corona - BC). The BC is a dynamic entity made by some tightly adsorbed macromolecules (the so-called hard corona) and surrounded by weakly adsorbed layer(s) of macromolecules (soft corona). The overall composition is in equilibrium with the medium with which it constantly exchanges macromolecules. The BC plays a pivotal role in defining the biological properties of the SNPs (e.g., nanoparticle uptake, biodistribution, and toxicity). The most common type of BC is the protein corona (PC), where proteins are the most abundant macromolecules adsorbed on the SN surface. Very recent studies have demonstrated PC also forms around EV surface during in vivo circulation (and since then, exponentially increasing efforts have been launched to tackle understanding of its role on EV colloidal, biophysical, and biological properties). Therefore, it is reasonable to hypothesize that a PC can also form in vitro during the incubation of EVs with macromolecule-enriched fluids, i.e., during the exogenous surface functionalization of EVs. In this work, we explore this aspect for the first time, using EVs derived from Red Blood Cells (REVs) functionalized with an anti-epidermal growth factor receptor (EGFR) monoclonal antibody (Cetuximab, CTX). Functionalized and pristine EVs were compared in terms of morphology, functionalization efficiency, binding activity, and in vitro uptake.

RESULTS AND DISCUSSION

Cetuximab modification and characterization
Cetuximab (CTX) chemical modification was optimized for REV surface functionalization. We exploited the coupling between primary amines (mainly the amino group on the side chain of lysines of the antibody) and activated esters (see experimental section). We adopted this strategy to covalently bind the fluorophore (Sulfo Cyanine 7.5, Cy7.5) and the alkyne unit (dibenzocyclooctyne, DBCO) in order to permit tracking of CTX and coupling with the azido groups added on the REV surface (see REV surface functionalization section), respectively (see Fig. 1). The complete procedure used to functionalize and characterize CTX is resumed in Fig. S1A. CTX modification was optimized to introduce functional units while keeping antibody binding ability. Two reaction ratios were tested to find the better condition to bind approximately 1 DBCO and 2 Cy7.5 to the CTX (see Table S1 for the detailed summary of CTX modification). In the optimized protocol, CTX was reacted with 12 equivalents of Cy7.5 and 6 equivalents of DBCO. After purification, modified CTX (mCTX), was characterized by UV-Vis spectroscopy (Fig. S1B) to determine the number of dyes attached to the antibody by the following equations:

\[ M = \frac{A_{280} - (A_{\text{max}} \times C)}{\varepsilon} \times D \]  

Equation (1) was used to estimate the CTX molar concentration (M), while equation (2) was used to calculate the antibody/dye ratio. In the equations, D is the dilution factor, \( A_{280} \) is the absorbance at 280 nm, \( A_{\text{max}} \) is the absorbance of the peak of the fluorophore, C is the correction factor, while \( \varepsilon \) and \( \varepsilon' \) are the molar extinction coefficients of the antibody (210000 M\(^{-1}\) cm\(^{-1}\)) and the dye (Cy7.5: 220000 M\(^{-1}\) cm\(^{-1}\), Cy3: 150000 M\(^{-1}\) cm\(^{-1}\)) respectively. DBCO quantification was performed by reacting an aliquot of mCTX with 6 equivalents of azido-sulfo Cyanine 3 (Cy3). This step is required due to the low molar extinction coefficient of the DBCO and to the proximity of its peak with the more intense protein signal at 280 nm, which hampers direct DBCO quantification by UV-Vis spectroscopy. UV-Vis spectra of mCTX-Cy3 (Fig. S1C) were used to calculate the degree of CTX labeling by Cy3 using equations (1) and (2), and assuming that DBCO is completely consumed to bind Cy3, due to the high kinetics and excess used for the reaction. Our calculations showed that each CTX binds on average 1.3 molecules of DBCO and 2 molecules of Cy7.5. The approximately 1:1 ratio between CTX and DBCO is pivotal to avoid that CTX with multiple DBCO units could bind different REVs at the same time. To create a control sample unable to bind azido groups (de-activated modified CTX, DmCTX), an aliquot of mCTX was reacted with NaN\(_3\) to consume any DBCO (see experimental section). DmCTX showed not to bind Cy3 ligation, indicating the successful deactivation of DBCO (Fig. S1D).

**Figure 1.** Strategy for Cetuximab (CTX) modification (mCTX). CTX was reacted with 12 equivalents of Cy7.5-NHS-Ester and 6 equivalents of DBCO-STF-Ester in buffer NaHCO\(_3\) at pH 8.4, for 16 hours at 4°C. The isolated reaction product is named mCTX.
Biophysical and biochemical characterization of Red Blood Cell-EVs

Red Blood Cell-EVs (REVs) were obtained following the protocol described by Usman et al.\textsuperscript{2} and characterized according to the most updated guidelines for EV characterization.\textsuperscript{30} Specifically, Nanoparticle Tracking Analysis (NTA), BCA, Atomic Force Microscopy (AFM), Western Blot, and CONAN assay\textsuperscript{31} were used to determine EV particle number and size distribution, protein concentration, morphology, protein markers, and the presence of soluble protein contaminants, respectively (Fig. S2). All these data highlighted that REV preparations were suitable for functionalization purposes: indeed, the monomodal size distribution and the negligible amount of soluble proteins\textsuperscript{32,33} avoid possible interferences during functionalization processes. Furthermore, Western Blot analyses confirmed the absence of EGFR on REVs, avoiding unspecific, undesired immunological binding of mCTX during functionalization processes (Fig. S2C).

**REV surface functionalization**

For REV surface functionalization, we followed the experimental workflow depicted in Fig. 2A. We adopted a two-step chemical labeling strategy\textsuperscript{16} (see experimental section), exploiting the SPAAC (Strain-Promoted Alkyne-Azide Cycloadditions) reaction between an azido group and an alkyne to covalently bind mCTX to the EV surface. REVs were first reacted with a N\textsubscript{3}-(PEG)\textsubscript{4}-NHS ester (reaction ratio 1:200 REVs:N\textsubscript{3}-(PEG)\textsubscript{4}-NHS ester) to introduce a clickable group onto the REV surface. A non-clickable (without azido-PEG) control sample was prepared to explore the eventual physisorption of mCTX onto the EV surface (REVs underwent the same workup procedure as pegylated REVs). Clickable and non-clickable samples were reacted with mCTX (reaction ratio 1:200 REVs:mCTX) and washed by dilution with PBS followed by ultracentrifugation to remove unreacted antibodies, obtaining REVs-click-mCTX and REVs-physi-mCTX, respectively. As shown in Fig. 2B, the size distribution of REVs was not affected by the functionalization process, indicating no significant morphological changes due to surface functionalization. The CONAN assay was performed to verify the presence of non-EV-associated antibodies\textsuperscript{31}. The samples tested showed the same AuNP aggregation properties of native EVs, thus indicating that the eventual amount of free antibody present in the solution is negligible (see Fig. 2C). CTX binding on REV was evaluated by SDS-PAGE followed by fluorescence imaging exploiting the fluorescent dye (Cy7.5) linked to mCTX. The densitometric fluorescence profile was acquired from the gel image, and differences between REVs-click-mCTX, REVs-physi-mCTX, and mCTX were observed (Fig. 2D), strongly suggesting the successful covalent ligation of mCTX to EV proteins by click-chemistry reaction. Indeed, lane 1 of SDS-PAGE displays the electrophoretic profile of REVs-click-mCTX as composed of higher molecular weight bands in respect to free mCTX (lane 3). This profile is likely ascribable to an increase in mCTX heavy and light chains’ molecular weight due to the covalent bonding with EV membrane proteins. Interestingly, the intensity of light chain and heavy chain band signals was lower in REVs-click-mCTX than mCTX, further confirming the covalent bonding. REVs-physi-mCTX (lane 2) showed the presence of mCTX heavy chain and light chain with a densitometric profile similar to mCTX (lane 3), suggesting that mCTX might be physiosorbed on EV surface without the azido-PEG unit. In order to quantify CTX on functionalized REVs, a calibration curve based on total lane fluorescence intensity was obtained by loading different amounts of mCTX (0.02 – 2 μg range) in an SDS-PAGE gel (Fig. S3C). Quantification of CTX molar concentration, normalized for REV molar concentration, is shown in Fig. 2E. Data indicated that a similar number of antibodies was present on REVs-click-mCTX and REVs-physi-mCTX (approximately 90 molecules of mCTX per EV), despite the differences in the densitometric profile described above. Furthermore, we performed a control reaction of the functionalization process using the DmCTX, and the reaction yield was quantified as above. As shown in Fig. S3D, about 90 DmCTX molecules per EV were co-isolated with REVs-physi-DmCTX, while REVs-click-DmCTX showed a negligible co-isolation yield, suggesting that active DBCO is required to react with the azido group on pegylated REVs. This result fosters the hypothesis of the anti-fouling properties exerted by PEG on REV surface. PEG anti-fouling effect was already largely explored and exploited for synthetic NPs,\textsuperscript{34,35} and our data highlight the role of pegylation in inhibiting the physisorption of DmCTX. All these experimental evidence strongly indicate that our strategies led to REV surface functionalization through the two different mechanisms we aimed at: the first mechanism featuring
chemisorption, through the formation of a covalent bond by click-chemistry reaction, while the second featuring the formation of a physisorbed PC.
Next, we performed a dose-dependent functionalization assay to optimize the functionalization process by modifying REVs by CTX chemisorption or physisorption as described above, with different mCTX-to-REV ratios. mCTX/REV amount relative to samples produced with three different REV:mCTX reaction ratios of 1:200, 1:500, and 1:1000 (azido-PEG were reacted in the same excess of mCTX) was quantified immediately after the first wash following functionalization (Fig. 3A and B, red bar). Moreover, to evaluate the stability of the binding, a second and third wash was performed (red and green bars, respectively, Fig. 3A and B), as described in the experimental section. Concerning REV-click-mCTX, results indicated that 200 equivalents of antibodies per REVs allowed the saturation of the binding sites present on the REV surface, and theoretical calculations (normalizing the total area generated by the antibodies for the surface of the REVs and approximating mCTX to a sphere with a radius of 5.5 nm) indicated that under these conditions mCTX covered approximately 10-15% of the REV surface. In addition, REV-click-mCTX showed a constant mCTX amount per REV for all the reaction ratios after every washing step (see Fig. 3A), again supporting that mCTX was covalently bound to the REV surface. Considering that REV surface is for the 23% occupied by membrane proteins, such a result about surface coverage is probably due to a combination of the saturation of the binding sites on the REV surface, the antifouling properties of PEG chains, and the steric hindrance generated by mCTX attached to the REV surface. On the other hand, the REVs-physi-mCTX samples showed a proportional increase in the co-isolation of mCTX per EV with increasing reaction ratios after the first wash (see Fig. 3B, blue bars). In the higher reaction ratio (1:1000, blue bar), mCTX covers up to 30% of the total REV surface, but after further washes, the mCTX amount per REV dramatically decreases, indicating that antibodies are likely to physisorbed on the REV surface rather than being stably bound. For 1:200 and 1:500 ratios, after the second wash, the amount of mCTX per REV reached a stable value of ~50 mCTX/REV, representing 5% of the surface coverage. This data could indicate the formation of a first layer of mCTX more strongly physisorbed onto the REV surface than the others. These considerations are in accordance with the general description of the PC as a multilayered entity with the stability of the protein layers decreasing with the distance from the EV surface: the more stable layer constitutes the so-called hard PC, while the soft PC is made by the layers more loosely attached.

**Figure 2.** REV surface functionalization by chemical or physical strategy. (A) Schematic representation of the functionalization processes followed. (B) Size distribution of functionalized samples determined by NTA shows no significant differences compared to the native REVs. Data are expressed in frequency %, and the diameter bin has been selected as 20 nm. N = 3. (C) In the CONAN assay, the Int-REF AI ratio defines the threshold below which the spectral redshift is only due to the interaction between the AuNPs and the EVs. Functionalized REVs were tested undiluted and after dilutions of 10 and 100-fold. The dotted line represents the CONAN assay threshold for soluble protein detection (<20% AI ratio means that the soluble protein content is ≤ 0.05 μg/μL) (D). SDS-PAGE and relative fluorescence densitometric profile of REVs-click-mCTX (lane 1, 20 μL), REVs-physi-mCTX (lane 2, 20 μL) and mCTX (lane 3, 1 μg). (E) mCTX per REV yields of functionalized samples. Data are elaborated as the molar concentration of mCTX normalized by the molar concentration of REVs of the sample. N = 5.

**Figure 3.** mCTX dose-dependent functionalization assay and mCTX stability assay on REVs. (A) mCTX per REV ratio and % of REV surface coverage by CTX of REVs-click-mCTX, obtained by different REV/mCTX reaction ratios
Evaluation of the binding capacity of REVs-click-mCTX and REVs-physi-mCTX to EGFR

We next evaluated the binding of REVs-click-mCTX and REVs-physi-mCTX towards EGFR by Surface Plasmon Resonance (SPR) technology, already exploited by our group for binding studies of growth factors and EVs. To this aim, EGFR was immobilized onto a SPR sensorchip by amine-coupling procedure (see experimental section). Firstly, the functionality of the EGFR-containing biosensor was validated using the epidermal growth factor (EGF), the natural ligand of EGFR. Saturation curves plotted with the binding values at equilibrium (Fig. 4C) were obtained that allowed the calculation of the dissociation constant ($K_d$) value, which is inversely proportional to the binding affinity. As shown in Fig. 4A, EGF bonded to EGFR in a dose-dependent and saturable way with a $K_d$ equal to 67.3 nM (supporting table S2), thus in line with the values already reported in literature. The EGFR-containing biosensor was then used to evaluate the binding of CTX before or after its chemical modification. As shown in Fig 4B, CTX bonded to EGFR in a saturable and dose-dependent way with a $K_d$ equal to 1.8 nM (supporting table S2), as already reported in literature.

The chemical modification introduced in mCTX leads to an apparent, limited decrease of affinity ($K_d$ equal to 3.6 nM, supporting table S2). On the bases of the positive results obtained with control proteins, the EGFR-containing biosensor was then used to evaluate the EGFR-binding affinity of the differently functionalized REVs (Fig. 2A). As shown in Fig. S4, injection of increasing concentrations of REVs-click-mCTX onto the EGFR-containing biosensor provided saturable and dose-dependent sensorgrams. The binding of the vesicle to EGFR is very stable, as indicated by their slow detachment from the surface after the end of the injection phase. Also, the binding is CTX-dependent, as demonstrated by the lack of binding of native REVs, devoid of surface-associated antibodies (Fig. S4). The $K_d$ for the REVs-click-mCTX/EGFR interaction is equal to 0.17 nM (Tab. S2). Also, REVs-physi-mCTX binds to EGFR in a specific, stable, dose-dependent, and saturable way (Fig. 4 and S4), with an affinity that is similar to that of REVs-click-mCTX (Tab. S2). This result suggests that the association between mCTX and REVs is quantitatively similar despite the different functionalization strategies. It is also worth of mention also the high affinity of the EGFR/REVs-click-mCTX interaction, which is 10 times higher than that of free mCTX (supplementary table S2). It is tentative to hypothesize that a cooperative interaction process, occurred at the interface between the sensorchip surface and the vesicle that generate a very high affinity binding. Interestingly, a high affinity cooperative binding has been already demonstrated for the interaction of immunoliposomes carrying anti-ephrin A2 single chain fragment with ephrin A2. The saturation response ($R_{max}$) is known to be dependent on the amount of surface-immobilized ligand available to the binding. REVs-physi-mCTX showed a $R_{max}$ value that is four times lower than that of REVs-click-mCTX (33 ± 2.5 and 150.0 ± 35.5, respectively, Fig. 4C). It is tentative to hypothesize that, when weakly adsorbed on REVs-physi-mCTX, the antibody “moves” from the vesicle to the EGFR-coated surface, where it remains firmly bound, decreasing the amount of EGFR available to interact with other REVs. Conversely, this does not occur in REVs-click-mCTX, where mCTX is covalently anchored to the REVs surface. The stability of the association of CTX to REVs obtained by physisorption or by chemical strategy considerations may have important implications on the vesicle uptake in vitro (see below) and their practical therapeutic use. In this work, DmCTX has been generated to demonstrate the essential role of DBCO in mCTX association with REVs and the anti-fouling properties of pegylation (see above). As expected, based on the similar amount of antibody associated (Fig. S3D), REVs-physi-DmCTX retains an EGFR-binding affinity very close to that of REVs-physi-mCTX, confirming that DmCTX behaves as mCTX in interacting with EGFR and that it can be absorbed on REV surface as mCTX. Although endowed with a negligible amount of associated antibody REVs-click-DmCTX binds EGFR with high capacity (measured as maximal RU bound) but occurs in a non-saturable manner (Fig. 4C) and with a
very low affinity ($K_d > 33 \mu M$, supporting table S2), indicating a non-specific interaction$^{47}$. This anomalous behaviour is likely due to the presence on REVs of unreacted azido-PEG that, as already reported$^{44,45}$, could interact in a non-specific way with biosensor surfaces. Whatever the cause of this non-specific binding, these results confirm that the interaction of CTX with the azido-pegylated EV surface occurs only when it possesses a reactive DBCO group. To summarize, SPR experiments confirmed that REVs can be functionalized with mCTX following biorthogonal and physisorption strategies, where both methods produce samples with a similar affinity for EGFR but with different stability of the antibody association. Furthermore, SPR analysis of pegylated and un-pegylated REVs functionalized with DmCTX confirmed the co-isolation reported above (Fig. S3D).

**Figure 4.** SPR binding analysis on the EGFR-containing biosensor. Exemplificative saturation curves drawn by fitting the data plot of the amount of formed complex at equilibrium (in resonance units, RU) from the single cycle analysis versus concentrations in solution of EGF (A), CTX or mCTX (B). For the analysis of functionalized and pristine REVs (C), their actual concentration and not that of associated mCTX was used.

**In vitro** cell uptake of REVs-click-mCTX and REVs-physi-mCTX

Modifying the EV surface by introducing suitable molecules for targeted drug delivery increases the chances for EVs to be taken up by cells *via* specific receptor-mediated endocytosis$^{42,43}$. SPR data showed that REVs-click-mCTX and REVs-physi-mCTX have the same affinity towards EGFR. Therefore, in principle, at the cellular level, both functionalized EVs are in the position to trigger the same uptake pathway by activating EGFR-mediated endocytosis. We assessed the targeting and uptake of the modified REV samples *in vitro* using MDA-MB-231 triple-negative breast cancer cells expressing high levels of EGFR$^{44,45}$. EGFR expression in MDA-MB-231 cells was confirmed by Western blot analysis, showing a 1.55-fold expression level when compared with control cells, namely HEK293T epithelial-like immortalized kidney cells$^{52}$ known to express basal levels of EGFR (Fig. S5)$^{50}$. Cells were treated with equal particle number of REVs-click-mCTX and REVs-physi-mCTX for 4 and 24 h in the presence or absence of Fetal Bovine Serum (FBS), used here to best mimic the physiological environment. The cellular internalization of the REVs was evaluated by following both mCTX and REV uptake quantifying the fluorescence of Sulfo Cyanine 7.5 (mCTX, red) and MemGlow™ 488 (REVs, green), respectively (see Fig. S6 for characterization). At first glance, it was evident that the addition of FBS decreases the overall uptake of both mCTX and REVs at 4 and 24 h (Fig. 5), thus suggesting a competition mechanism between the heterogeneous FBS components with REVs, as observed by Salvati et al. for transferrin-functionalized NPs.$^{51}$ After 4 h treatments, REVs-click-mCTX showed a significantly higher mCTX uptake than REVs-physi-mCTX whether the treatments were carried out with or without FBS (Fig. 5A). However, we did not observe relevant differences in REV uptake between functionalized samples and the control pristine REVs, suggesting other interactions, independent from mCTX-EGFR recognition, can still happen at early time points$^{52}$ (Fig. 5B). After 24 h, REV-click-mCTX showed significantly higher mCTX uptake compared to REV-physi-mCTX only in presence of FBS (Fig. 5C). This difference is also reflected in REV uptake related to REV-click-mCTX, that is significantly higher than pristine REVs and REV-physi-mCTX (Fig. 5D). Interestingly, REVs and REV-physi-mCTX uptake was experimentally equal at each time point in FBS-containing conditions. These data can be, at first instance, explained by the fact that REV-physi-mCTX should be more sensitive to protein exchange when
immersed in biological media, leading to decreased EGFR-mediated cellular uptake. As mentioned earlier, pristine NPs and EVs are immediately coated by a BC once immersed in a biofluid, which (re)sets their targeting, uptake, and biodistribution profiles\textsuperscript{22,29}. Mostly due to its dynamic properties\textsuperscript{53,54}, in biofluids, BC features and morphology evolve due to the continuous adsorption/desorption of loosely bound proteins, primarily enriched in the soft corona\textsuperscript{24,55}. Altogether, these results suggested that in biological fluid containing a physiological concentration of proteins, such as the one containing FBS, REVs-click-mCTX and REVs-physi-mCTX did not maintain the same EGFR-targeting ability and that mCTX functionalization by physisorption did not improve EV targeting ability in respect to pristine REVs (Fig. 5D).
**Figure 5.** (A). *In vitro* mCTX uptake assay. Fluorescence image and quantification of MDA-MB-231 cells after the treatment with REVs-click-mCTX and REVs-physi-mCTX at 4 h in the absence (No FBS) and in presence of FBS. The green fluorescence is related to mCTX. * = p value < 0.05. (B). *In vitro* REV uptake assay. Confocal microscopy image and quantification of MDA-MB-231 cells after the treatment with REVs-click-mCTX and REVs-physi-mCTX at 4 h in the absence (No FBS) and in presence of FBS. The green fluorescence is related to MemGlow™ 488, while the blue is to DAPI. * = p value < 0.05. (C) *In vitro* mCTX uptake assay. Fluorescence image and quantification of MDA-MB-231 cells after the treatment with REVs-click-mCTX and REVs-physi-mCTX at 24 h in the absence and in presence of FBS.
Ultracentrifugation for large volumes

Instrumentation

The green fluorescence is related to mCTX. * = p value < 0.05. (D) In vitro REV uptake assay. Confocal microscopy image and quantification of MDA-MB-231 cells after the treatment with REV-click-mCTX and REVs-physi-mCTX at 24 h in the absence (No FBS) and in presence of FBS. The green fluorescence is related to MemGlow™ 488, while the blue is to DAPI. * = p value < 0.05; ** = p value < 0.01.

CONCLUSIONS

The present work opens to the exploration of the influence and role BC has in the exogenous surface functionalization of EVs with targeting antibodies. In parallel, it evidences the key importance of the rigorous understanding and control of all the functionalization processes – from the choice and preparation of the antibodies to the final in vitro functional evaluation, a characteristic too often underrated in the literature. We developed and optimized the modified CTX by introducing a red-emitting fluorophore (Cy7.5) and clickable unit (DBCO). mCTX was then used to functionalize REV surfaces by chemisorption (covalent binding of mCTX via biorthogonal click-chemistry) and physisorption (formation of a CTX corona). Both approaches did not affect REV morphology. Covalent bonds established between REV surface proteins and mCTX by click-chemistry allowed the functionalization of REV surface, resulting in changes in the mCTX electrophoretic profile. Besides, mCTX physisorbed on REV surface was dynamic and in equilibrium with the media. mCTX-EGFR molecular recognition was carefully verified by comparing mCTX and CTX’s binding curves on an EGFR-functionalized biosensor, demonstrating that the modification of the antibody did not affect its binding. The same biosensor was then used to study the binding of functionalized REVs. SPR functional assay demonstrated that both REVs functionalized by chemisorbed and physisorbed mCTX bind EGFR with fairly consistent affinity. In vitro tests showed that in the presence of FBS, a condition closer to the physiological milieu, only the covalent anchorage of mCTX led to increased uptake of the REVs correlated with the increased uptake of mCTX. On the other hand, the presence of FBS-related proteins forces an exchange of biomolecules between the PC made by mCTX on the REV surface and the medium, leading to a loss of functionality. Although EV-BC derived from biofluids has been shown to bestow functional features to EVs, the PC derived from EV surface functionalization seems not functional for drug delivery applications. Thus, the covalent anchorage of antibodies onto the EV surface is required to improve specific EV recognition by the cells and guarantee the delivery of therapeutic cargo. These findings give a first evaluation of the effect of the BC during EV surface functionalization, and they should be carefully considered (and further elaborated) when designing and preparing surface-functionalized EVs. Further work is needed to characterize the role of BC in the formulation of surface-functionalized EVs with other molecules, such as proteins and peptides, to support the exploitation of different functionalization strategies for developing delivery vehicles, imaging agents, or bio nano-therapeutics.

EXPERIMENTAL SECTION

Reagents

Solen-tes were purchased from Merck and Carlo Erba and used as received without further purification. PBS, DMEM, FBS, and Penicillin/Streptomycin were purchased from Corning (Mediatech Inc., Manassas, VA, USA). NaNO3 was purchased from Sigma Aldrich (St. Louis, MO, USA). Sulfo-Cyanine7.5 NHS ester was purchased from Lumiprobe GmbH (Germany). DBCO-STP Ester, Azido-PEG4-NHS Ester, and Cy3 Azide were purchased from ClickChemistryTools, (Scottsdale, Az, US). MemGlow™ 488 was purchased from Cytoskeleton, Inc. (Denver, USA). Calcium ionophore (A23187) was purchased from Sigma Aldrich (St. Louis, USA).

Instrumentation

Centrifugation was performed with a 5804R Eppendorf Centrifuge, A-4-44 rotor, 15 mL tubes. Ultracentrifugation for large volumes (up to 50 mL) was performed with Optima XPN-100, TY45 Ti rotor
(Beckman Coulter, USA) while for small volumes (up to 1.5 mL) we used an Optima MAX-XP, TLA-55 rotor, and MLS-50 (Beckman Coulter, USA). Images from western blot and gel fluorescence were acquired with Syngene G:BOX Chemi XX9 (SYNGENE, UK) and were analyzed and quantified with the software Genesys and GeneTools (SYNGENE, UK). NanoDrop™ OneC (ThermoFisher, Rockford, USA) was used to characterize mCTX. Zeiss LSM510 with a Plan-Apochromat 63x/1.4 Oil DIC objective (Germany) was used to perform fluorescence confocal microscopy. All the VivaSpin columns used in this work were purchased from Sartorius Stedim Lab Lid (Sperry Way, Stonehouse, UK). NanoSight NS300 system was used to determine the size distribution and the particle concentration of REV samples (Malvern Technologies, Malvern, UK). BIACore X-100 instrument was used for SPR (Cytiva Life Science, Marlborough, MA, USA).

**Red Blood Cells EV (REVs) collection (isolation/separation)**

REVs were isolated following the guidelines from Usman et al.\(^5\) Briefly, after blood collection (100 mL), RBCs were pelleted by centrifugation at 1000 × g for 8 minutes at 4 °C and washed three times in PBS without calcium and magnesium. RBCs were further washed two times with CPBS (PBS + 0.1 g/L calcium chloride) and transferred into a 75 mm\(^2\) tissue culture flask. Calcium ionophore was added to the flask (final concentration 10 mM) and incubated overnight at 37 °C. RBCs (75 mL) were gently collected from the flask, and cellular debris was removed by differential centrifugation (600 × g for 20 min, 1600 × g for 15 min, 3260 × g for 15 min, and 10,000 × g for 30 min at 4 °C). The pellet was discarded at every step, transferring the supernatant into a fresh tube. The supernatants were filtered through 0.45 μm nylon syringe filters. EVs were collected by ultracentrifugation at 100,000 × g for 70 min at 4 °C. EV pellets were then resuspended in cold PBS, layered above 2 mL frozen 60% sucrose cushion, and centrifuged at 100,000 × g for 16 h at 4 °C, with deceleration speed set to 0. The red layer of EVs was collected and washed twice with cold PBS and spun at 100,000 × g for 70 min at 4 °C. Finally, EVs were resuspended in 1 mL of cold PBS.

**Cell culture**

For this project we used two cell lines, the epithelial breast cancer cells MDA-MB-231 (ATCC #HTB-26) and the kidney embryo cells HEK293 (ATCC #CRL-1573). Both the cell lines were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA. Cells were cultivated in DMEM supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % Penicillin/Streptomycin and maintained at 37 °C under 5% CO\(_2\). Cells were routinely tested for mycoplasma.

**Cetuximab functionalization**

Cetuximab (CTX) was provided by Prof. Fabio Corsi (University of Milan) in storage buffer (sterile PBS + 0.1 mM NaN\(_3\)). The storage buffer was replaced with 0.1 mM NaHCO\(_3\), pH 8.4 using VivaSpin2000 column with 50 kDa cut-off. CTX solutions were centrifuged at 2000 × g for 15 minutes. The buffer exchange step was carried out thrice by adding 2 mL of NaHCO\(_3\) 0.1 mM, pH 8.4 at each step. CTX concentration after the buffer exchange process was quantified by NanoDrop™ OneC. For antibody functionalization, 200 μL of CTX 0.016 mM was incubated overnight on continuous mixing at 4 °C with 12 equivalents of Sulfo Cyanine 7.5 NHS ester (0.2 mM in DMSO) and 6 equivalents of DBCO STF ester (0.1 mM in DMSO), thus obtaining modified CTX (mCTX). For deactivation, mCTX was incubated overnight at 4 °C with 1 μM of NaN\(_3\) in PBS pH 7.4. DBCO conjugation yield was estimated by incubating a small aliquot of functionalized mCTX with azido-Sulfo Cyanine 3 overnight on continuous mixing at 4 °C. Excess of reagents (Sulfo Cyanine 7.5 NHS ester, DBCO STF ester, azido-Sulfo Cyanine 3 or NaN\(_3\)) was removed after every step with Vivaspin500 column with 30 kDa cut off by centrifugation the mCTX solution at 12000 × g for 15 min and washing with 500 μL of PBS until no significant signal from the free fluorescent dyes could be detected by UV-Vis spectroscopy. mCTX characterization is reported in SI.

**Bicinchoninic acid (BCA) assay**

Protein concentrations of REVs and RBC homogenate samples were determined with Pierce™ BCA Protein Assay Kit (ThermoFisher, Rockford, USA), following the manufacturer's instructions.
SDS-PAGE and Western Blotting

Samples were boiled in reducing SDS sample buffer (80 mM Tris, pH 6.8, 2% SDS, 7.5% glycerol, 0.01% bromophenol blue) supplemented with 2% 2-mercaptoethanol for 5 min at 95 °C and separated by SDS-PAGE on acrylamide/bisacrylamide 10% gel. For densitometric analysis, we used the Image Lab software (Biorad, Hercules, CA, US). For CTX quantification, after the electrophoresis run, the gel was imaged by Syngene G:BOX Chemi XX9 with an acquisition time of 2 min and 800 nm wavelength. For the WB analysis, samples were transferred onto a PVDF membrane and blocked overnight with 5% fat-free milk in PBS-0.05% Tween-20 (PBST). The PVDF membranes were incubated with the following antibodies (at dilution 1:1000) for 90 min in PBST + fat-free milk 1%: mouse anti-GM130 (610823, clone 35/GM130, BD Biosciences, Germany), mouse anti-Alix (sc-53539, 2H12, Santa Cruz Biotechnology, USA), rabbit anti-Annexin XI (GTX33010, polyclonal, GeneTex, USA), mouse anti-CD47 (sc-59079, BRIC 126, Santa Cruz Biotechnology, USA), mouse anti-CD45 (sc-1178, 35-Z6, Santa Cruz Biotechnology, USA), rabbit anti-EGFR (4406, clone 15F8, Cell Signalling Technology Inc.), mouse anti-HBA1 (H00003039-M02, clone 4F9, Abnova, Jhouzih St., Taipei, Taiwan), mouse anti-HBB (2H3Abnova, Jhouzih St., Taipei, Taiwan). The membranes were washed thrice for 10 min with PBST and incubated for 1 h with the following secondary antibodies (at dilution 1:3000): rabbit anti-mouse and goat anti-rabbit (Zymed). Blots were detected using Luminata Classic HRP western substrate (Millipore). Images were acquired using a G: Box Chemi XT Imaging system, as described in Alvisi et al.

Colorimetric NANoplasmonic (CONAN) assay

The purity of REV preparations from soluble contaminants was tested with the CONAN assay. The assay is a colorimetric test that exploits the aggregation of citrate-capped gold nanoparticles (AuNPs) onto the EV membrane and the formation of the protein corona onto the AuNP surface to detect soluble proteins in EV preparations. CONAN assay was performed according to Zendrini et al.

Atomic Force Microscopy (AFM) imaging

Atomic Force Microscopy (AFM) imaging was performed on a Nanosurf NaioAFM equipped with a Multi75-Al-G tip (Budget Sensors). For sample preparation, EVs were resuspended in 100 µL sterile H2O (Milli-Q, Merck Millipore) and diluted 1:10 in H2O. 5 µL of sample were then spotted onto freshly cleaved mica sheets (Grade V-1, thickness 0.15 mm, size 15 × 15 mm²) and dried at 37 °C for 10 minutes. Images were acquired in tapping mode, with a scan size ranging from 1.5 to 25 µm and a scan speed of 1 s per scanning line. Image processing was performed on Gwyddion ver. 2.61.

Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis (NTA) was performed according to the manufacturer's instructions using a NanoSight NS300 system configured with a 532 nm laser. Samples were diluted 1:1000 in filtered PBS (0.22 µm) to a final volume of 1 mL to obtain the optimal particle per frame value (20–100 particles/frame). A syringe pump with constant flow injection was used (20 µL/min), and the temperature was set constant at 25 °C. Particles were detected at a camera level of 10 and three videos of 60 s were captured and analyzed with NTA software ver 3.2. The mean, mode, and median EV sizes from each video were used to calculate sample concentration, expressed in particles/mL.

REV functionalization and labeling

REVs were functionalized following a two-step labeling strategy. 200 µL of 8x10^11 particles/mL REV was reacted with 200, 500, or 1000 equivalent of -ester (diluted in PBS) overnight on continuous mixing at 4 °C. PEG excess was removed using Vivaspin500 column with 10 kDa cut-off, washing the samples five times with 500 µL of PBS. Pegylated REVs were recovered from the column with 200 µL of PBS. 200 µL of pegylated REVs (8*10^11 prt/ml) were then reacted overnight on continuous mixing at 4 °C with 200, 500, or 1000 equivalent of mCTX (in PBS, pH 7.4). The unreacted antibody was removed by ultracentrifugation (100,000 × g, 2 h), and the pellet was resuspended in 100 µL of PBS and stored at 4 °C until use. REV
labeling with MemGlow™ 488 was performed following standard customer protocols. REVs were incubated with MemGlow™ 488 100 nM for 15 min, then MemGlow™ 488 excess was removed by ultracentrifugation (100,000 × g, 2 h).

**Surface Plasmon Resonance (SPR)**

The BIAcore X-100 instrument was used. Human EGFR was immobilized by standard amine-coupling chemistry on a research grade CM5 sensorchip, whose surface consists of a carboxymethylated dextran matrix. In detail, EGFR was resuspended at 15 µg/mL in 10 mM sodium acetate pH 4.8 and injected for 180 sec. at a flow rate of 10 mL/min on one cell of the sensorchip previously activated with a mixture of 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (0.2 M) and N-hydroxy-succinimide (0.5 M). Following EGFR binding the surface was deactivated with ethanolamine. With this procedure, three different biosensors have been generated and used for the analysis presented in the work with an amount of immobilized EGFR receptor varying between 1,400 and 2,500 resonance units (RU) (equal to 8-13 fmol/mm²). On the second cell, used as a negative control for blank subtraction, was performed a blank immobilization. For binding analysis, increasing concentrations of the various proteins and EVs were resuspended in 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3.0 mM EDTA, 0.005% v/v Surfactant P20 (HBS), injected over the sensorchips for 180 sec. at a flow rate of 30 µL/min to allow their association with the immobilized EGFR and then washed until dissociation. To preserve the immobilized receptor, the single cycle model was adopted, where the compounds are injected at increasing concentrations within a single scan without regeneration of the surface after each injection.

**In vitro** uptake assay

MDA-MB-231 cells were seeded on 12 mm-sized coverslips, precoated with 50 µg/mL. Type I Bovine Collagen (TB03, Alphabioregen) as 200000 cells/mL in 150 µL of complete media. After 24 h, cells were treated for 4 h and 24 h with MemGlow™ 488 labelled REVs, REVs-physi-mCTX, REVs-click-mCTX and mCTX in media with or without FBS, as described above. Each treatment was carried out using comparable amounts of REVs (10¹⁰ EVs/mL) and CTX (1.61 x 10⁻¹ nM). Cells were washed once with PBS 1X without CaCl₂ and MgCl₂ (21-040-CV, Corning) and then fixed with 3% paraformaldehyde (PFA) solution for 15 minutes at room temperature (RT). PFA was quenched with 50 mM NH₄Cl for 10 minutes at RT. Cells were washed twice with PBS 1x and permeabilized with 0.3%. Saponin in PBS 1x for 10 minutes at RT. Cellular nuclei were stained with DAPI (Invitrogen, 1:600). Cells were washed twice with PBS 1x, once with bidistilled H₂O, and coverslips were mounted using ProLong™ Gold Antifade Mountant (P36934, Invitrogen). For EV uptake analysis, 512 x 512 pixel images were acquired with a Zeiss LSM510 with a Plan-Apochromat 63x/1.4 Oil DIC objective, with a 0.2% 405 nm laser for DAPI and 0.2% 488 nm for MemGlow™ 488. Images were quantified with the software ZEN 3.6. For mCTX uptake analysis, coverslips were also acquired with Syngene G:BOX Chemi XX9 following standard protocols present in the software for detecting Cy7.5 and DAPI. For each treatment, MemGlow™ 488 and CTX fluorescence signals were normalized to DAPI fluorescence. Data from three independent experiments were expressed as mean ± Standard Error of the Mean (SEM).

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Please refer to the CREdiT taxonomy for full details.

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