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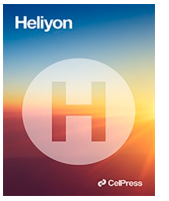

Research article

Sonication is a suitable method for loading nanobody into glioblastoma small extracellular vesicles

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ABSTRACT

Glioblastoma is one of the deadliest cancers, therefore novel efficient therapeutic approaches are urgently required. One of such are nanobodies, prospective nano-sized bio-drugs with advantageous characteristics. Nanobodies can target intracellular proteins, but to increase their efficiency, the delivery system should be applied. Here, we examined small extracellular vesicles as a delivery system for anti-vimentin nanobody Nb79. Nb79 was loaded in small extracellular vesicles either by incubation with glioblastoma cells, by passive loading into isolated small extracellular vesicles or by sonication of isolated small extracellular vesicles. Small extracellular vesicles secreted by glioblastoma cells were isolated by ultracentrifugation on sucrose cushion. The size distribution and average size of sonicated and non-sonicated small extracellular vesicles were determined by nanoparticle tracking analysis method. The loading of Nb79 into small extracellular vesicles by incubation with cells, passive loading or sonication was confirmed by Western blot and electron microscopy. The effect of small extracellular vesicles on cell survival was determined by WST-1 reagent. Loading of small extracellular vesicles by incubation of cells with Nb79 was unsuccessful and resulted in substantial cell death. On the other hand, as confirmed by Western blot and electron microscopy, sonication is a successful method for obtaining Nb79-loaded small extracellular vesicles. Small extracellular vesicles also had an effect on cell viability. Small extracellular vesicles without Nb79 increased survival of U251 and NCH644 cells for 20–25%, while the Nb79-loaded small extracellular vesicles decreased survival of NCH421k by 11%. We demonstrated that sonication is a suitable method to load nanobodies into exosome, and these small extracellular vesicles could in turn reduce cell survival. The method could be translated also to other applications, such as targeted delivery system of other protein-based drugs.

1. Introduction

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults [1]. Its histopathological features, that include vascular proliferation, mitotic activity, and necrosis, assign GBM in grade IV classification of brain tumors according to the World Health Organization [2,3]. Five-year survival rate for glioblastoma is less than 5% and it decreases when diagnosed at older age [4]. Currently best-known treatment for GBM includes surgical resection, radiotherapy and chemotherapy with temozolomide [5,6]. High

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degree of intra-tumoral cellular heterogeneity and infiltrative nature of glioblastoma cells lead to high rate of recurrence and poor efficiency of a single therapy [7,8]. Therefore, modern approaches are investigated as alternative treatment methods, including nanobodies, prospective bio-nanodrugs. Nanobodies are the smallest available antigen-binding fragments that are part of naturally occurring heavy chain only antibodies that are fully functional in the absence of a light chain [9]. The size of nanobody is 4 nm × 2.5 nm × 3 nm and its molecular mass is 12–14 kDa [10]. The advantages of nanobodies compared to classical IgG antibodies are their strong affinity towards their targets, ability to penetrate solid tumors, ability to cross blood-brain barrier and treating nervous system diseases [11,12]. A potential target for GBM treatment is type III intermediate filament vimentin that is found in mesenchymal cells and is a marker of mesenchymal phenotype [13]. It is also abundantly expressed in GBM [13]. In our previous research, we identified *anti*-vimentin nanobody Nb79 and have shown elevated expression of vimentin in GBM on both gene and protein level [14,15]. However, vimentin is intracellular target, therefore delivery system would be required to enhance the efficacy of targeting. There is evidence that nanobodies enter the cell [16], but the uptake is usually limited. Applying delivery systems such as small extracellular vesicles can circumvent the problem. Small extracellular vesicles are the smallest extracellular vesicles, have the capacity to communicate with other cells, and to modulate local or distant microenvironments [17]. They carry proteins, lipids, nucleic acids and metabolites and their diameter ranges from 50 nm to 150 nm [17,18]. Compared to other delivery systems like liposomes, small extracellular vesicles are naturally occurring, have higher biocompatibility, lower toxicity and penetrate deep tissues faster [19,20]. They also enter cell faster, presumably due to the presence of membrane ligands, such as tetraspanins, heparin sulphate proteoglycans and receptor SR-B1 [21–23]. Small extracellular vesicles could also be applied for treating brain diseases, including glioblastoma, they can penetrate or evade blood brain barriers by several ways, including binding vesicle to RVG protein or intranasal application [22]. In contrary, artificial vesicles cannot pass blood-brain barriers. There is also evidence, that small extracellular vesicles resist stomach acid and hypoxic environment [24]. Moreover, several clinical trials are undergoing, evaluating small extracellular vesicles as either biomarkers or delivery system. In phase 2 clinical trial, exosomes with dendritic cells loaded with tumor antigen were used as a vaccine in patients with non-small cell lung cancer. In another clinical trial, exosomes were used to deliver curcumin to colon cancer tissue (obtained from ClinicalTrials.com on February 28, 2023) [25].

The purpose of this study was to examine small extracellular vesicles as potential delivery system and monitor the effect of Nb79-loaded glioblastoma small extracellular vesicles on glioblastoma cells.

2. Methods

2.1. Cell culture

Glioblastoma differentiated cell line U251 (obtained from American Type Culture Collection) was cultured in EMEM Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 1% non-essential amino-acids solution (Gibco, Waltham, MA, USA) and 1% antibiotic–antimycotic solution (penicillin/streptomycin/amphotericin B; Gibco). Glioblastoma stem cell lines NCH644 and NCH421k (obtained from CLS Cell Lines Service GmbH) were cultured in Neurobasal medium (Gibco) supplemented with 1% GlutaMax (Gibco), B27 supplement (Gibco), 20 ng/mL epidermal growth factor (EGF; Gibco), 20 ng/mL basic fibroblast growth factor (bFGF, Gibco), 1 U/mL heparin (Sigma-Aldrich), and 1% antibiotic–antimycotic solution (penicillin/streptomycin/amphotericin B; Gibco). Cell lines tested negative for Mycoplasma using Venor®GeM Classic kit (Minerva Biolabs). Prior Mycoplasma testing, cells were cultured in medium without antibiotic-antimycotic solution for 14 days.

2.2. Small extracellular vesicles isolation

For exosome isolation, FBS without small extracellular vesicles was prepared by ultracentrifugation for 24 h at 100,000 g and 4 °C. U251 cells were seeded on T150 cell culture flask in cell medium composed of EMEM Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with small extracellular vesicles-depleted 5% fetal bovine serum (FBS) (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 1% non-essential amino-acids solution (Gibco) and 1% antibiotic–antimycotic solution (penicillin/streptomycin/amphotericin B; Gibco). After 72 h incubation, medium was collected and centrifuged for 10 min at 300 g, 4 °C and then 10 min at 2000 g, 4 °C. Afterwards, medium was passed through 0.22 μm-pore-size filter with cellulose acetate membrane (Corning) and concentrated by centrifugation at 2100*g*, 4 °C to final volume of 3 mL using Amicon® Ultra concentrators with Ultracel-100 regenerated cellulose membrane (Merck). Concentrated media was diluted with PBS to 8 mL, loaded on 2 mL of 20% sucrose cushion and ultracentrifuged for 135 min at 100,000 *g*. The pellet was thoroughly resuspended in PBS and stored at −80 °C.

2.3. Nanoparticle-tracking analysis

Nanoparticle tracking analysis (NTA) was performed by Nanosight NS300TM (Malvern Panalytical, Malvern, UK) equipped with syringe pump and autosampler. For analysis, small extracellular vesicles were diluted 1:200 in filtered (0.22 μm pore size) and sonicated PBS. For each sample five videos for 60 s were filmed at camera level 14, threshold five [26]. Software NTA 3.3.- Sample Assistant Dev Build 3.3.203 was used for the detection and analysis.

2.4. Nb79 production and FITC labelling

Nb79 was produced in *E. coli* following the protocol described by Vincke et al. [27]. Nanobody contained 6 × His tag and was purified using nickel immobilized metal affinity chromatography and size-exclusion chromatography. The purity was confirmed by SDS-PAGE. Afterwards, the nanobody was labeled with FITC using FluoroTag™ FITC Conjugation Kit (Sigma Aldrich) as described by the manufacturer.

2.5. Nanobody loading methods

For Nb79 loading into small extracellular vesicles we used four different methods as described below.

a) Indirect loading: We plated 700,000 U251 cells per T150 cell culture flask and added 50 μg of Nb79 per plate. After 24 h of incubation, medium was collected and processed for exosome isolation. For processing, sample was divided into three equal parts, one remained untreated, second was treated with 0.05 μg/μL of proteinase K (in presence of 2.5 mM $CaCl_2$) for 2.5 h and third was treated with 0.05 μg/μL of proteinase K for 2.5 h in presence of 1% Triton X-100. Afterwards, Halt protease inhibitors were added and incubated 10 min at 90 °C. Then, the sample was lysed in 10 × RIPA lysis buffer and incubated for 10 min on ice.
b) Passive loading: We incubated 20 μg of Nb79 together with 2×10^8 small extracellular vesicles for 24 h at room temperature. Afterwards, sample was stained with DiL (ThermoFisher) following manufacturer's instructions. Then, sample was ultracentrifuged for 70 min at 100,000 g, 4 °C. For processing, sample was divided into three equal parts, one remained untreated, second was treated with 0.05 μg/μL of proteinase K (in presence of 2.5 mM $CaCl_2$) for 2.5 h and third was treated with 0.05 μg/μL of proteinase K for 2.5 h in presence of 1% Triton X-100. Afterwards, Halt protease inhibitors were added and incubated 10 min at 90 °C. Then, the sample was lysed in 10 × RIPA lysis buffer and incubated for 10 min on ice.
c) Incubation at 37 °C: We incubated 20 μg of Nb79 together with 2×10^8 small extracellular vesicles for 2 h at 37 °C. Afterwards, sample was stained with DiL (ThermoFisher) following manufacturer's instructions. The sample was then ultracentrifuged for 70 min at 100,000 g, 4 °C. For processing, sample was divided into three equal parts, one remained untreated, second was treated with 0.05 μg/μL of proteinase K (in presence of 2.5 mM $CaCl_2$) for 2.5 h and third was treated with 0.05 μg/μL of proteinase K for 2.5 h in presence of 1% Triton X-100. Afterwards, Halt protease inhibitors were added and incubated 10 min at 90 °C. Then, the sample was lysed in 10 × RIPA lysis buffer and incubated for 10 min on ice.
d) Sonication: For sonication, protocol of Haney et al. was followed [28]. Briefly, we incubated 10 μg of Nb79 together with 3×10^8 small extracellular vesicles. Sonication was performed by UP100H (Roth) sonicator with the following settings: 100 W, 30 kHz, 20% power; 6 cycles 4 s on/2 s off, 2 min incubation on ice, 6 cycles 4 s on/2 s off, 60 min incubation at 37 °C. Afterwards, sample was ultracentrifuged for 70 min at 100,000 g, 4 °C. For processing, sample was divided into two equal parts, one was treated with 0.5 μg/μL of proteinase K (in presence of 2.5 mM $CaCl_2$) for 2.5 h and second was treated with 0.5 μg/μL of proteinase K for 2 h in presence of 1% Triton X-100. Afterwards, Halt protease inhibitors were added and incubated 10 min at 90 °C. Then, the sample was lysed in 10 × RIPA lysis buffer and incubated for 10 min on ice. For cell viability assay, 50 μg of Nb79 was loaded with sonication. After 60 min incubation at 37 °C, small extracellular vesicles were ultracentrifuged for 1 h 15 min at 100,000 g and 4 °C. The pellet containing small extracellular vesicles was then resuspended in 20 μL of PBS.

2.6. Western blot

For Western blot, proteins were first separated on 4–12% NuPAGE Bis-Tris polyacrylamide gel (Thermo Fisher) for 70 min at 200 V. The proteins were transferred to PVDF membrane for 90 min at 100 V. Residual binding sites were blocked using 5% milk/PBS for 1 h, and then membrane was probed with anti-His (Invitrogen), anti-FITC antibody (1:200; Santa Cruz) or flotillin antibody (1:1000; Cell Signalling) overnight at 4 °C, shaking at 50 rpm. Next day, membrane was incubated with HRP-conjugated secondary antibody for 2 h at room temperature, shaking at 50 rpm. Signal was detected with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher) or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher) with LAS-4000 CCD camera (Fujifilm; Tokio, Japan). Images were processed with ImageJ software.

2.7. Electron microscopy

Small extracellular vesicles were prepared and immunolabelled as described [29]. Briefly, small extracellular vesicles were pelleted by centrifugation at 100,000×*g* for 1.5 h and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at 4 °C. Samples were post-fixed with 2% OsO_4 for 1 h at 4 °C, dehydrated in a graded acetone series and embedded in the Spurr low viscosity embedding medium. Ultrathin sections (60 nm) were collected on gold grids, incubated in 0.02 M glycine to quench free aldehyde groups and unspecific labelling was blocked in 1% BSA/PBS. Then sections were incubated with mouse anti-FITC antibody (1:50; Santa Cruz) at 4 °C overnight, washed and incubated with goat anti-mouse IgG conjugated to 12 nm colloidal gold (1:40). In PBS containing 0.1% BSA). Sections were stained with 2% uranyl acetate and lead citrate. Grids were examined in Philips CM100 transmission electron microscope at 80 kV.

2.8. Cell viability assay

Cell viability was determined using WST-1 (Roche). First, glioblastoma cells NCH421k, NCH644, and U251 (4×10^3) were seeded in total of 100 μL of medium and incubated for 2 h at 37 °C, in a 5% CO_2 atmosphere. Afterwards, 2.5×10^7 Nb79-loaded small extracellular vesicles per well were added. As controls we used untreated cells, equal number of pure small extracellular vesicles, and Nb79 amount at 10% loading efficiency. Growth medium was used as a blank. After 72 h incubation, 10 μL of Cell Proliferation Reagent WST-1 per well was added and incubated for 3 h. After color had developed, the absorbance was measured at 450 nm and 690 nm using Agilent BioTek Synergy H4 Hybrid Microplate Reader (BioTek). Cell survival was determined by subtracting blank from each absorbance value and comparing non-treated with treated cells. At least three independent experiments were performed in three technical replicates.

2.9. Statistical analysis

Statistical analysis of sonicated and non-sonicated small extracellular vesicles was performed by student t-test, because there were two groups to compare. Statistical analysis of survival assay was performed with Kruskal-Wallis un-parametric test with Dunn's multiple comparison. Results with $p < 0.05$ were considered as statistically significant. The analyses were performed by GraphPad Prism 9.3.0.

3. Results

3.1. Indirect loading with incubation of cells

First loading method tested was incubation of cells in presence of nanobody Nb79 and isolation of small extracellular vesicles from the cell medium. Analysis of the efficacy shows that Nb79 was present in the unprocessed sample, while there was no signal observed after proteinase K and proteinase K in presence of Triton X-100 (Fig. 1a, Supplementary Figure 1), indicating that the Nb79 is present outside the small extracellular vesicles, either on the cell surface or free, unbounded to small extracellular vesicles. Also, higher percentage of dead cells were observed when treated with Nb79 compared to untreated (Fig. 1b).

3.2. Passive loading

As a second method, passive loading with incubation of small extracellular vesicles in presence of nanobody Nb79 for 24 h at room temperature or 2 h at 37 °C was tested.

The results show that strong signal for Nb79 was present in untreated small extracellular vesicles that underwent passive loading (Fig. 2, Supplementary Figure 2). For small extracellular vesicles incubated for 24 h with Nb79, the signal was also observed after proteinase K treatment and was absent when treated also with Triton X-100, indicating that the Nb79 is present also inside small extracellular vesicles. On the other hand, sample which was incubated for 2 h at 37 °C, there was no signal observed neither after proteinase K treatment nor when treated also with Triton X-100, indicating that Nb79 is not present in the small extracellular vesicles.

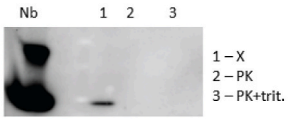
3.3. Sonication

As the third method, sonication was tested for loading nanobody Nb79 into small extracellular vesicles.

Results show that the presence of Nb79 was detected in the sample after treatment with proteinase K as it can be seen in Fig. 3 a. Higher amount of Nb79 was observed in small extracellular vesicles when treating only with proteinase K (small EVs + PK) compared to treatment with proteinase K in the presence of Triton X-100 (small EVs + PK + Triton). In the sample treated only with proteinase K we also detected a positive signal of the exosomal marker flotillin. Flotillin signal was absent from the sample treated also with Triton X-100, indicating the absence of intact exosomes.

With the electron microscopy we additionally confirmed the presence of nanobody Nb79 inside small extracellular vesicles (Fig. 3b). The image on the left shows small extracellular vesicles with labeled Nb79, which is seen as black dots indicated with black

a)



b)

	+ Nb79	- Nb79
% dead cells	55 %	17 %
Number of small extracellular vesicles	8.68×10^8	4.88×10^8

Fig. 1. Indirect loading of Nb79 into small extracellular vesicles. Sample order: pure nanobody (Nb), untreated small extracellular vesicles (X; 1), small extracellular vesicles treated with proteinase K (PK; 2) and sample treated with proteinase K (PK) in presence of Triton X-100 (PK + trit; 3). a) Representative Western blot membrane of the sample. b) Percentage of dead cells and number of small extracellular vesicles in treated (+Nb79) and untreated (- Nb79) cells. Nb79 was detected through its FITC tag using an *anti*-FITC antibody. The experiment was performed in one biological replicate.

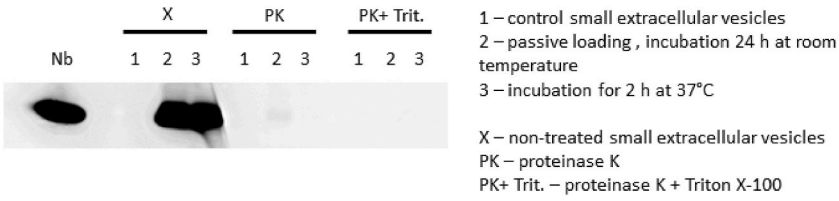


Fig. 2. Direct passive loading of Nb79 into isolated small extracellular vesicles. Sample order: pure nanobody (Nb), untreated small extracellular vesicles (X), small extracellular vesicles treated with proteinase K (PK), and small extracellular vesicles treated with proteinase K + Triton X-100 (PK + Trit.). Each sample was divided into three equal parts as explained above. Nb79 was detected through its FITC tag using an anti-FITC antibody. The experiment was performed in one biological replicate.

arrows. To note, Nb79 is present also outside the small extracellular vesicles as indicated with the orange arrows. The electron microscopy images also show that the small extracellular vesicles are intact after sonication and no rupturing is observed.

The NTA analysis results show that the exosome population after sonication is more heterogenous compared to pure small extracellular vesicles and the average size is reduced from 180 nm to 140 nm (Fig. 3c).

3.4. Viability assay

Application of pure small extracellular vesicles increased survival of differentiated U251 and stem glioblastoma cell line NCH644 for 21–25% (Fig. 4). There was no effect of pure exosome application on the survival of glioblastoma stem cell line NCH421k. The application of Nb79 alone resulted in small increase in survival of all glioblastoma cell lines compared to untreated cells. The application of Nb79-loaded small extracellular vesicles to glioblastoma cells decreased survival of NCH421k by 11%.

4. Discussion

Biological drugs have revolutionized treatment of cancer, and antibody-based drugs are currently the fastest growing branch of drug development [30,31]. At this time, 76 biological drugs are used in the clinics, and many more are in the last stages of testing in clinical trials [30]. Despite the progress in evolvement of monoclonal antibodies as biological drugs, they face several limitations, such as difficult production and limited solid cancer penetration [30,32]. As a consequence, advanced non-traditional approaches such as nanobodies are being examined. Due to several favorable features nanobodies have a huge potential to change the way cancer is treated. Nanobodies are highly stable, can be economically produced in microbial systems, and have low immunogenicity [33]. They can also be used for targeting intracellular targets, which is not possible by monoclonal antibodies [33].

The purpose of our research was to determine the most successful method for loading of nanobodies into small extracellular vesicles. We tested two different loading approaches i.e., indirect by incubation of cells, and direct loading in already isolated small extracellular vesicles. When cells were incubated with Nb79, they underwent a substantial cell death and the number of vesicles released was around 2 × higher compared to untreated cells, probably at the expense of apoptotic bodies or vesicles that are released during cell death [34]. The failure of this type of loading method can be attributed to the low amount of nanobody applied to the cells, intracellular processing of the nanobody and/or inappropriate cell line.

On the other hand, passive loading with 24 h incubation of small extracellular vesicles in the presence of Nb79 was successful. The results were similar to Haney et al. who observed that incubation at room temperature for 24 h yields a positive result [28]. When sonication was tested, a strong signal was observed when treated with proteinase K, indicating the presence of Nb inside the small extracellular vesicles. The electron microscopy additionally confirmed the presence of Nb79 inside the small extracellular vesicles. However, nanobody was also present outside the small extracellular vesicles, on co-isolated contaminants, which was expected as completely pure small extracellular vesicles are hard to achieve with existing methods and this is also one of the biggest current challenges in exosome study [22]. The average size of small extracellular vesicles at sonication was smaller compared to non-sonicated and the peak of the size-distribution graph was shifted to the smaller size. Similar observations were published by Nizamudeen et al. who showed that sonicated vesicles are smaller compared to non-sonicated [35]. Contrary to this, Haney et al. and Kim et al. report increase in exosome diameter after sonication [28,36]. To note, the integrity of small extracellular vesicles was not damaged, and the small extracellular vesicles retained clear, circular shape. Sonication has also been extensively studied for loading small-molecule drugs, e.g. paclitaxel [36,37], but this study shows that it is suitable also for proteins loading.

At last, we analyzed the effect of loaded small extracellular vesicles on glioblastoma differentiated cell line U251 and two glioblastoma stem cell lines, NCH644 and NCH421k. Loaded small extracellular vesicles decreased survival in only one of the tested cell lines, NCH421k, for approximately 10%. In addition, pure small extracellular vesicles increased survival of U251 and NCH644 by 20–25%. Our results indicated that the proposed cell origin might not be suitable for GBM application, or the loading efficiency is too low and could be further optimized. Choosing the right cell source is not straightforward. The advantage of using autologous, cancer small extracellular vesicles is specific membrane structure, that makes the uptake more specific. Cancer small extracellular vesicles also contain tumor antigens, which may induce immune cell response and facilitate tumor eradication. Nevertheless, using tumor small extracellular vesicles might be too risky, as they are known to have a role in tumor progression [38], supported also by our study where pure small extracellular vesicles increased proliferation rate. Brain endothelial cells would be a better alternative as a cell origin, while

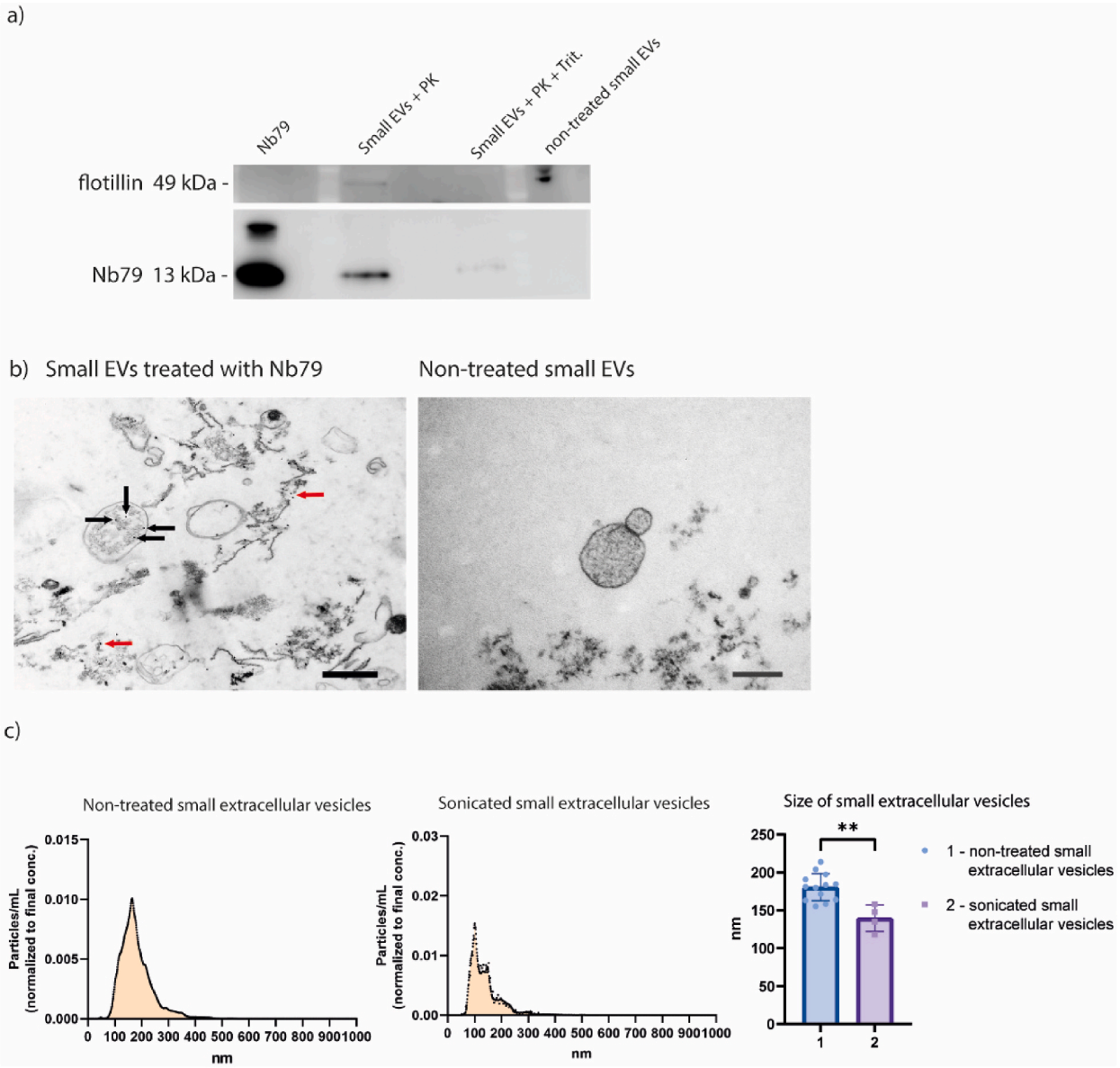


Fig. 3. Characterization of small extracellular vesicles loaded with Nb79 using sonication method. a) Western blot analysis of small extracellular vesicles loaded with Nb79. The following samples were included, pure Nb79 bound with FITC (Nb79-FITC), loaded small extracellular vesicles treated with proteinase K (exo + PK), loaded small extracellular vesicles treated with proteinase K and Triton X-100 (exo + PK + Triton) and pure small extracellular vesicles (Pure exo). The signal of Nb79 is present at 14 kDa and flotillin at 49 kDa. The experiment was performed in one biological replicate. b) Electron microscopy images of small extracellular vesicles. Left – black arrows show labeled Nb79 inside the exosome and red arrows shows labeled Nb79 outside of the small extracellular vesicles, scale bare = 500 nm. Right – control small extracellular vesicles without Nb79, scale bar = 200 nm. The experiment was performed in one biological replicate. c) NTA analysis of exosome size and distribution. Left and middle figures show size distribution of pure (left) and sonicated (middle) small extracellular vesicles. Right figure shows mean size of pure and sonicated small extracellular vesicles. Individual small extracellular vesicles are presented as mean value. Student t-test was applied for statistical analysis. Height of the column present mean ± SD. $^{**}p < 0.01$. N (non-treated small extracellular vesicle) = 13, N (sonicated small extracellular vesicles) = 4.

their small extracellular vesicles can penetrate blood brain barrier, therefore they could be deployed for brain disease treatment, including glioblastoma. Small extracellular vesicles could also be further engineered to contain RVG protein (derived from Rabies virus), which facilitates passage through blood-brain barrier. To achieve tumor-specific targeting, small extracellular vesicles could also contain molecules, that specifically recognize tumor-specific membrane biomarkers, e.g. FREM2 [39].

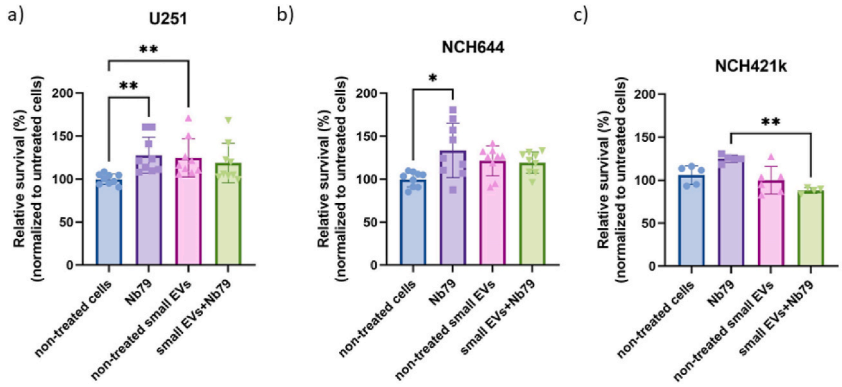


Fig. 4. Relative survival of U251, NCH644 and NCH421k cell lines in presence of loaded small extracellular vesicles. Three different treatments were tested, pure Nb79 (Nb79), non-treated small extracellular vesicles (non-treated small EVs) and Nb-79 loaded small extracellular vesicles (small EVs + Nb79) on three different glioblastoma cells U251 (a), NCH644 (b), and NCH421k (c). WST-1 assay was used for cell viability determination. Three biological replicates with three technical replicates were used for U251 and NCH644. Two biological replicates with three technical replicates were used for NCH421k. Column height represent mean ± SD. Kruskal-Wallis statistical analysis was used for analysis. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

5. Conclusion

Classical biological drugs are limited to target either membrane surface or extracellular proteins. Nanobodies, on the other hand present an advantageous alternative that enable also targeting intracellular proteins, but their uptake should be facilitated by use of a delivery system such as small extracellular vesicles. In this study we proved that sonication is a suitable method for loading small proteins such as Nb79 into glioblastoma small extracellular vesicles which decreased survival of glioblastoma stem cell line NCH421k. This method could be further used to incorporate proteins into small extracellular vesicles and target tumor cells.

Author contribution statement

Sara Colja: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ivana Jovčevska; Neja Šamec; Rok Romih: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Alja Zottel: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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Disclosure statement

The authors report there are no competing interests to declare.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15674.

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