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Nanofilaments on glioblastoma exosomes revealed by peak force microscopy

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Exosomes are sub-100 nm extracellular vesicles secreted by normal and cancer cells. We present a high-resolution structure of previously unidentified nanofilaments on glioblastoma-derived exosomes, using nanoscale peak force imaging. These stiff, adhesive, trypsin- and RNase-resistant surface nanofilaments add a new dimension to the current structural knowledge of exosome-mediated intercellular communication.

1. Introduction

The ability of cells to communicate with each other is essential for multicellular organisms. A number of mechanisms, including small nano-sized, cell-derived extracellular vesicles called exosomes [1], have been investigated as mediators of cellular interactions. In particular, the surfaces of exosomes form fascinating interfaces that may mediate their transport between cells, e.g. both as autocrine and paracrine factors in tumour microenvironments, as well as effectors for distant cellular targets, analogous to endocrine signalling. Exosomes are known to play an important role in intercellular communication by extracellular signalling [2] and horizontal mRNA/microRNA transfer [3]. Their roles in tumour proliferation [4,5], pre-metastatic niches [6] and tumour microenvironment modulation, including glioblastoma as a model disease system, have also been proposed [7].

The quantity, surface molecular composition and dynamics of exosomes released vary depending on the cellular origin and physiological state of the cell [8]. Understanding exosome structure at nanometre resolution can provide us detailed insights into the mechanisms of exosome-mediated cell–surface interactions including transport, binding to and uptake by target cells. Electron microscopy has been the gold-standard method [9] to characterize exosomes, but is limited by potential artefacts [10]. No structural details can be resolved with optical imaging as exosomes are smaller than the diffraction limit (200–300 nm) [11]. Recently, atomic force microscopy (AFM) [12] has emerged as a powerful biophysical nanoscale characterization technique that offers three-dimensional imaging of unlabelled single exosomes, and provides additional information about the structure, mechanical properties and their specific biomolecular composition [13,14]. So far, detailed surface characteristics of glioblastoma exosomes at the nanoscale level have not been reported to date.

Here, we provide first evidence of the presence of surface nanofilaments on glioblastoma-derived exosomes and analyse their biophysical characteristics at the nanoscale using AFM peak force imaging [15]. Compared with normal exosomes, glioblastoma exosomes displayed abundant nanofilaments, and the nanofilaments were trypsin- and RNase-resistant. We quantified the distinct structural, biophysical and biochemical properties of the exosomes and surface nanofilaments. Based on *in vitro* uptake assays, glioblastoma exosomes showed

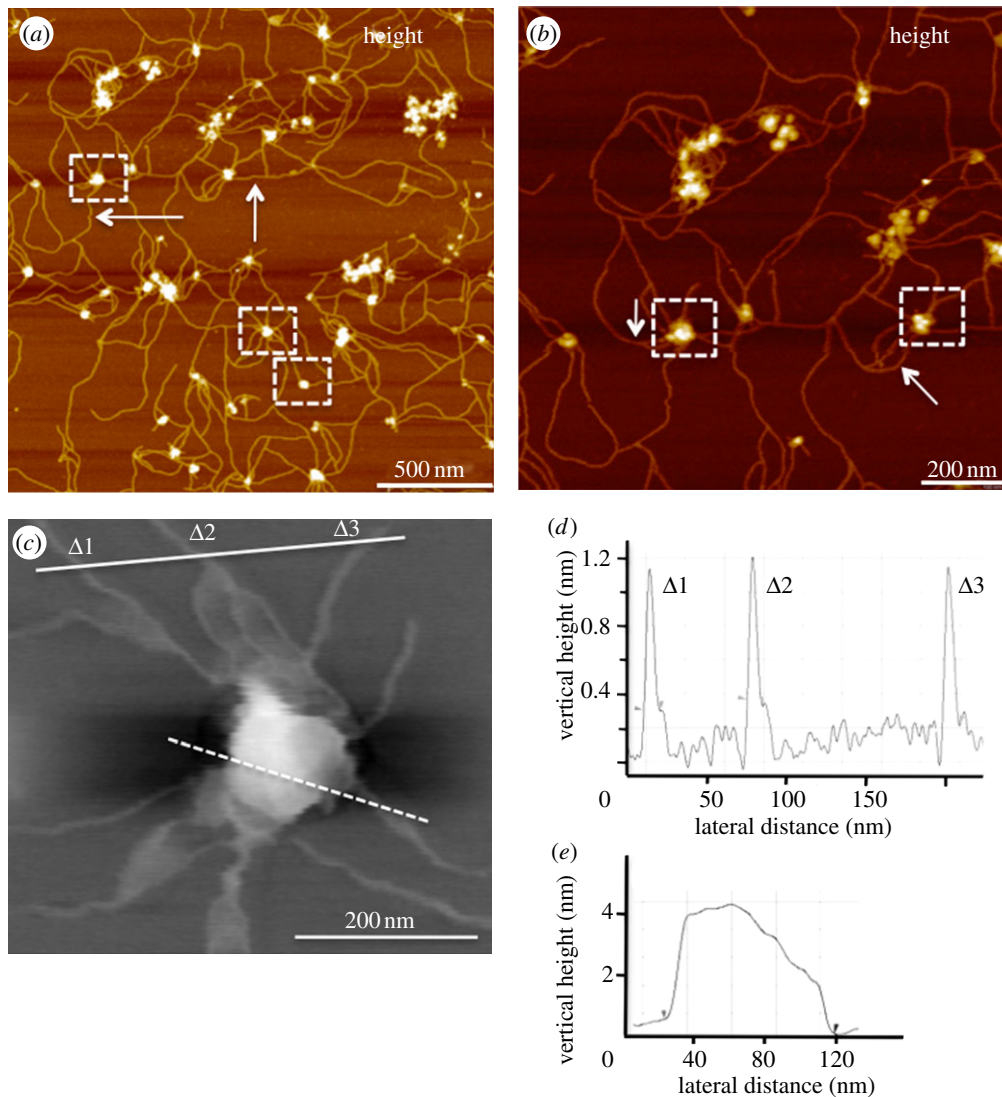


Figure 1. PFM reveals glioblastoma exosomes with abundant nanofilamentous surface extensions. (a) Topographic image ($z = 10$ nm) of glioblastoma U87-derived exosomes showing round bulging vesicles (some shown in dashed boxes) surrounded by a network of nanofilaments. (b) The nanofilaments are shown at a higher resolution ($z = 6$ nm). Size of U87 exosomes measured from typical high-resolution PFM topographic image (c) is 89.3 nm in diameter and 4 nm in height (e; dashed white line). (d) Cross-section profiles show approximately 1.2 nm in height and 10 nm wide nanofilaments (marked $\Delta 1$ –3, solid line). Fewer nanofilaments were seen in U251 exosomes, but not seen in NHA-derived exosomes (see the electronic supplementary material, figure S1). The results were confirmed by imaging samples obtained from two independent and commonly used isolations, with and without sucrose gradient purification. Size distributions show an average vesicle size of 89 ± 3.2 nm, 80.8 ± 2.2 nm and 70.9 ± 2.2 nm for U87, U251 and NHA, respectively ($n \sim 100$ individual exosomes).

a significantly higher uptake in cells compared with normal exosomes. We discuss how these newly recognized nanofilaments may help in modulating, tethering and transport of exosomes for intercellular communication.

2. Results and discussion

2.1. Glioblastoma cell-line-derived exosomes show abundant networks of surface nanofilaments

Exosomes derived from several human cell lines were studied, including glioblastoma (U87 and U251), melanoma (SKMEL) and normal human astrocytes (NHA). U87 and U251 are human primary glioblastoma cell lines from grade IV tumours [16]. SKMEL28 is a malignant melanoma cell line. Normal control exosomes were isolated from a primary human astrocyte cell line (NHA). Key structural features not previously reported for exosomes were observed at

nanoscale resolution, using the recently developed variation of AFM called peak force microscopy (PFM) [15]. This imaging mode is non-destructive to both tip and sample because it directly controls the peak normal force. PFM operates at low imaging forces (less than 100 pN), permitting subnanometre imaging of soft biological structures (see the electronic supplementary material). The most striking feature of the recorded PF images was the ultra-structural details of exosome surfaces (figure 1). Although ‘microscale’ cellular extensions [17] and nanotubes are known to exist between cells [18], their functional mechanisms in intercellular communication remain obscure. However, PFM images reveal new and distinct nanofilaments extending from the surface of glioblastoma exosomes (figure 1). Our current work shows that the surface extensions of glioblastoma exosomes form a branched network of nanoscale inter-exosomal connections (figure 1*a,b*, marked by white arrows). Individual nanofilaments within the network were measured to be 10–20 nm in diameter and up to several micrometres in

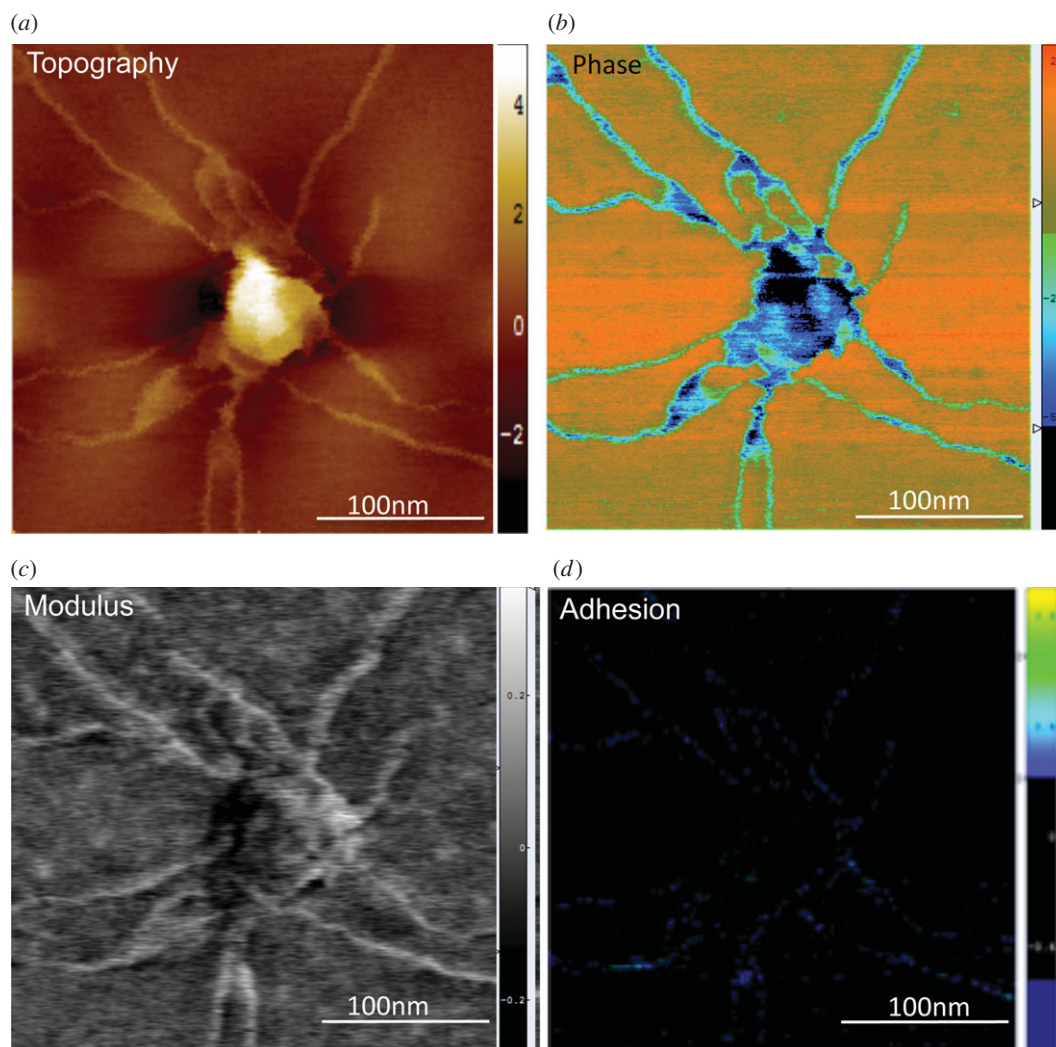


Figure 2. PFM imaging enabled independent extraction and mapping of biophysical parameters at less than 5 nm lateral resolution, revealing relatively stiffer and more adhesive nanofilaments, in comparison with parent vesicle region. Quantitative mapping of biophysical parameters such as (a) topography (range $z = 5$ nm), (b) phase ($z = 10.6$ mV), (c) modulus ($z = 2$ GPa) and (d) adhesion ($z = 1.6$ nN) was simultaneously mapped, as represented by U87 exosomes with extending surface nanofilaments.

length (figure 1*c,d*). Similar, though fewer, nanofilaments were also observed in U251 exosomes. Interestingly, the nanofilaments were rarely observed in the case of normal control NHA exosomes (see electronic supplementary material, figure S1). Sequential ultracentrifugation, both with and without sucrose gradient purification, yielded exosomes with indistinguishable nanofilaments under PFM. Additionally, we found that trypsin–EDTA treatment did not disrupt the network of nanofilaments or exosomes (data not shown), indicating either their non-protein nature or inherent resistance to trypsin digestion.

Exosome morphology was measured, using PF topographic images. The findings revealed three-dimensional spherical vesicles, approximately 80–90 nm in diameter for all three cancer cell lines (U87, U251 and SKMEL) and slightly smaller NHA exosomes (see electronic supplementary material, table S1). PFM images provide support to our previous findings showing exosomes as round spherical structures without cup-shaped indentations [19]. As might be expected for the low force of imaging in PFM, the vesicles were measured to be higher (approx. 4 nm) compared with those measured under standard tapping mode imaging owing to minimal compression of the vesicle lipid bilayers [19].

2.2. Mapping of biophysical characteristics (Young's modulus and adhesion strength) of nanofilaments

Next, we explored the biophysical properties of glioblastoma exosomes, including their surface nanofilaments. AFM tapping mode height and phase imaging has been widely used to study the structure and morphology of biomolecules [20–22]. Additionally, quantitative mechanical information on surface modulus can be obtained by nanoindentation and force volume AFM imaging [23,24], using appropriate mechanical contact models. However, nanoindentation and force volume imaging are limited in lateral resolution and time extensive. Using PFM, less than 5 nm lateral-resolution maps were typically obtained to determine quantitative information about the topography, change in phase, stiffness (elastic modulus) and adhesion strength (stickiness) of the vesicles (see the electronic supplementary material) as represented in figure 2.

Figure 2*a* shows a nanoscale topographic image of a single isolated exosome and extending surface nanofilaments at nanoscale resolution. Complementary to the three-dimensional topography, the phase images can detect inhomogeneous chemical, structural or mechanical properties [25] of the sample. Here, local phase heterogeneities in exosomes (figure 2*b*) indicated a

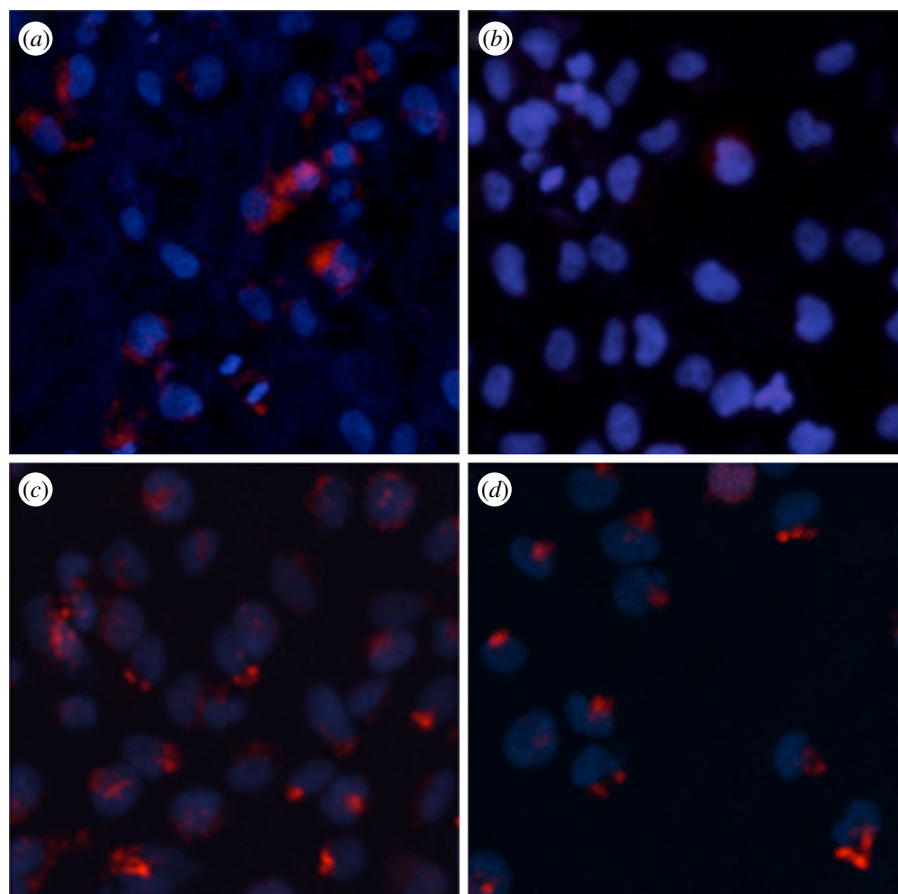


Figure 3. *In vitro* cell assays show greater binding of glioblastoma-derived U251 exosomes compared with normal NHA exosomes (images taken at 200 \times magnification). (a) NHA cells+ U251 exosomes, (b) NHA cells+ NHA exosomes, (c) U251 cells+ U251 exosomes and (d) U251 cells+ NHA exosomes. Cell nuclei are stained with 4',6-diamidino-2-phenylindole (blue) and exosomes are stained with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (red).

dense exosomal core relative to surface filaments. Furthermore, the prominent differences in nanofilaments and vesicle regions were revealed in simultaneously recorded elastic modulus and adhesion maps (figure 2*c,d*). Figure 2*c,d* corresponds to variations in the elastic modulus and adhesion forces, respectively, both showing significant mechanical heterogeneity between the exosomes and nanofilaments. In PFM, Young's modulus is calculated using a Derjaguin–Muller–Toporov model that is applied to the unloading portion of the force–separation curve, which takes into account the adhesive forces between the tip and the surface [15]. The elastic modulus map obtained by PFM imaging (figure 2*c*) revealed stiffer (bright) nanofilaments (up to 2 GPa) compared with the exosomal core regions (dark). While surface effects can be expected to affect the obtained modulus values, the contrast between the modulus was also clearly distinct in regions where the stiffer filaments are observed on top of the vesicle regions. Additionally, we performed single force–indentation curve testing over various regions using sharper tips (approx. 1 nm) and thin-film Hertz model [26], which also indicated stiffer nanofilaments. The measured stiffness of exosome nanofilaments was comparable in range with other filamentous biomolecules (see the electronic supplementary material, table S2) including peak force measurements to identify stiffness profiles of nanoscale fibrils such as Tau and alpha-synuclein. Additionally, the non-specific adhesion of the exosomes to bare AFM probes was quantified across the topography. The nanofilaments were found to be significantly more adhesive ($p < 0.05$) than vesicular regions (figure 2*d*) with adhesion forces up to 1.6 nN (blue).

2.3. Glioblastoma exosomes show greater cellular uptake compared with normal control exosomes

One intriguing question is the functional consequence of exosome nanofilaments in patho-physiological states such as glioblastoma. Our structural data highlight that nanofilaments appear on the surface of glioblastoma exosomes. Consequently, we investigated their potential strength in binding to target cells by assaying the cellular binding and uptake of glioblastoma-derived exosomes compared with normal exosomes. To achieve this, we incubated purified exosomes derived from U251 and NHA cells (labelled with membrane dye CM-DiI) with U251 or NHA cells, respectively (figure 3). We found that *in vitro* cellular uptake of glioblastoma (U251) exosomes was significantly higher than that of normal control NHA exosomes. Although the exact mechanism of increased uptake of glioblastoma exosomes, which express many fold more surface nanofilaments (with respect to NHA exosomes), still needs to be identified, these preliminary results support our hypothesis that increasing numbers of nanofilaments could play a functional role in cellular binding and uptake of exosomes and may be relevant in intercellular interactions in glioblastoma exosomes.

Filamentous connections between cells that allow intercellular communications are typical of multicellular organisms. Recent reports demonstrate the existence of a network of intercellular membrane nanotubes enabling long-distance communication in mammalian cells [17,18]. These tunnelling nanotubes have been shown to facilitate intercellular transfer of cytoplasmic molecules and even organelles and viruses

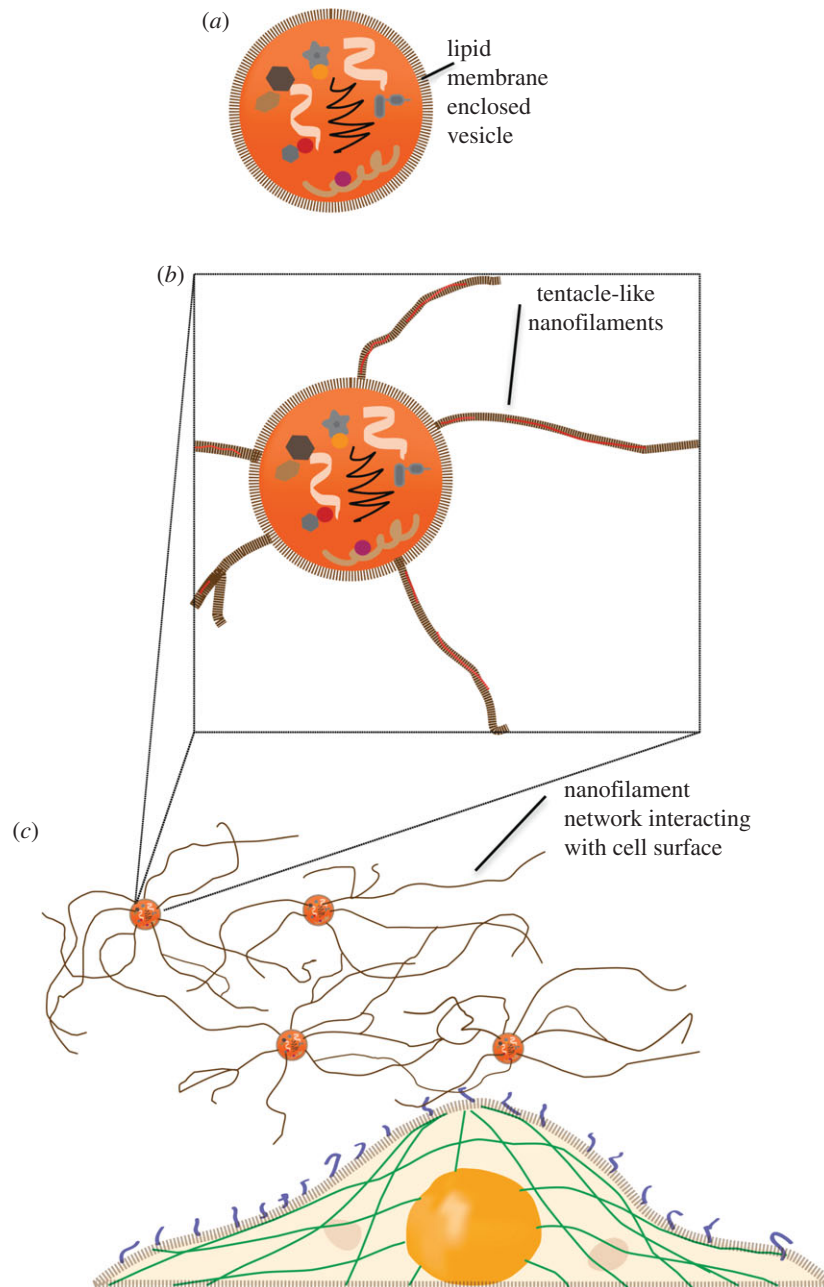


Figure 4. Proposed hypothetical schematic model for exosomes with nanofilaments mediating intercellular transport and communication. (a) Current model of exosomes with membrane-enclosed proteomic and genomic content. (b) Glioblastoma exosomes with surface nanofilaments. (c) Nanofilaments of glioblastoma exosomes interact with target cell.

[27,28]. Using correlative field emission scanning electron microscopy (FESEM) and AFM, we previously demonstrated the structure of saliva-derived exosomes and oral cancer exosomes [19,29]. Evidence of associated inter-vesicular structures was observed under FESEM and AFM, but not resolved in detail. Our current findings on structural, nanomechanical and cellular uptake of exosomes provide the first high-resolution nanoscale characteristics of exosome nanofilaments. The adhesive characteristics and cell binding assays suggest their likely role in a direct physical contact with target cells for intercellular communication (figure 4*b,c*). The nature and function of these observed nanofilaments need to be further investigated, but could well provide a significant advance over existing models of exosome–cell interactions, which are largely based on exosomes being ‘distinct membrane-enclosed vesicles’ (figure 4*a*) capable of intercellular communication and transport of proteomic and genomic content among distant cells [30].

We found a positive correlation between abundance of nanofilament extensions and glioblastoma cell-line-derived exosomes. These nanofilaments were measured to be 10–20 nm wide, compared with the exosome vesicular diameters of around 100 nm. It is known that tip convolution likely results in broadening effect on measured exosome dimensions, yet the images provide good estimates of upper limits of exosome diameters and nanofilament heights and widths. The lengths of nanofilaments extend up to several micrometres, comparable with dimensions of target cells themselves. We tentatively suggest that these structures are composed of lipids and nucleic acids (based on filamentous shape, close to 1.2 nm height in AFM images and similar elastic modulus values ranging from MPa to a few GPa). It is noteworthy that the general physical characteristics and appearance of exosomes, their nucleic acid contents (noted to contain microRNA, mRNA and even potential retro-transposable elements) [3,31], and even their

subcellular biological underpinnings suggest their strong relationship to viruses [32]. Within this context, we suggest that the biophysical and potential functional characteristics of these nanofilaments may be analogous to virulence factors for microbes, such as gp120/gp41 for human immunodeficiency virus [33], or haemagglutinin and neuraminidase for influenza virus infection [34,35]. Further exploration of nanofilaments may not only hold significance for exosome biology and their role in intercellular communication, but also for their role as virulence factors in exosome-associated diseases such as cancer, autoimmune diseases and infectious diseases. Further experimental investigations are needed to establish the occurrence and relevance of nanofilaments in a diverse population of exosomes imaged under physiological buffer conditions.

Nanofilaments may serve as long tethers for anchorage, increasing the probability of exosome binding to target cells. They may provide enhanced rigidity or structural integrity to parent exosomes. These rigid and adhesive nanofilaments may also harbour specific recognition motifs for cognate receptors on target cells, perhaps thereby further modulating exosome targeting, uptake and effector functions. Therefore, we propose a tentative model (figure 4) of nanofilament augmented exosome interactions with target cells.

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