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Lipid nanotube networks: Biomimetic Cell-to-Cell Communication and Soft-Matter Technology

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The exchange of information on the molecular level is a vital task in metazoan organisms. Communication between biological cells occurs through chemical or electrical signals in order to initiate, regulate and coordinate diverse physiological functions of an organism [1]. Typical chemical modes of signaling and communication are cell-to-cell interaction in the form of release of small molecules by one, and receptor-controlled uptake by another cell, also transport of molecules through gap-junctions [2], and transport via exosome carriers [3]. During the last decade a new mode of intercellular cross-talk has been discovered, and over time firmly established [4]. Thin tubular structures composed of lipid membrane material and actin polymer, which facilitate the selective transfer of membrane vesicles and organelles, have been identified as cell-bridging channels between cells [5]. The structures, known as membrane nanotubes, or tunneling nanotubes (TNT), have become the focus of a growing research field which generated significant results, as it became apparent that these interconnecting conduits are involved in fundamental mechanism of cell-to-cell communication [6,7]. TNTs have been identified in a variety of cells, including immune cells and neurons. Nanotubes between cells have been shown to form in different ways, for example through membrane protrusions originating from one cell and connecting to another, adjacent cell. The discovery of the membrane nanotubes *in vitro* in 2004 by Gerdes and coworkers has generated great optimism regarding deeper insights into cellular communication, signaling and disease progression in the light of pathogen transport pathways and other forms of cell stress [8] (Figure 1A). Several new studies have appeared which successively unearthed more and more details of the capabilities of tunneling nanotube structures [1,9]. It has been established that it is indeed mainly stress-related information exchange, for example caused by virus infection, which is propagating *in vitro* via nanotube interconnections [4,10]. In this particular case, the pathogenic virus particles can travel along the nanotubes from an infected to a healthy cell. Other possible sources are oxidative stress, or endotoxins. One topical study has reported that the *in vitro* formation of TNTs is essential for the regulation of osteoclastogenesis; the multi-complex cellular process during which the bones are resorbed [11] (Figure 1B-C). Another newly published work reported the formation of TNTs by exosomes released from tumors, acting as chemotactic stimuli [12]. In a new report related to cancer development, the preferential transfer of mitochondria between endothelial and cancer cells has been shown [13]. According to these findings, the mitochondria acquired by the cancer cells are more resistant to chemicals such as doxorubicin; a cytostatic drug used in cancer chemotherapy. TNTs have been also shown to conduct the transfer of toxic polyglutamine protein aggregates among neurons. Polyglutamine aggregates cause Huntingtons disease, and the findings provide evidence on how huntingtin (Htt) misfolding progresses through the brain [14].

However, as of today the important question whether the same variety of transport and intercellular communication tasks can be performed by these channels *in vivo* remains largely unanswered, since only a few recent studies have conclusively shown that membrane tubes are in fact present inside biological systems. For example, Rupp *et al.* reported that malaria parasites utilize nanotube connections in order to facilitate intercellular contact between gametes during reproduction in the mosquito midgut [15]. The full picture of the contribution

Article note: Dedicated to our friend and colleague Prof. Dr. Hans-Hermann Gerdes, †18.8.2013

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of lipid nanotubes to the dynamic and highly complex cell-to-cell signaling machinery still awaits completion [6]. Earlier speculations that tunneling nanotubes could be acting as an additional layer of information exchange between neuronal cells have so far not been supported by any evidence. However, the idea of adding a new dimension to brain neuro-glial networks by means of a novel type of wiring transmission (WT) based on TNTs, which was initially formulating by Gerdes, has started a broader discussion in neuroscience [16,17].

Besides, membrane nanotubes have long been of considerable interest to the biophysical research community. Investigation of the mechanical and chemical properties of artificially generated lipid nanotubes preceded the discovery of tunnelling nanotubes, and the steady advances of analytical instrumental techniques towards nanoscale resolution have provided deeper insights into composition, formation mechanisms and function of lipid membranes in general, and nanotubes in particular [18]. Furthermore, on-demand fabrication of nanotubes and their application in man-made biomimetic systems has also moved into the focus of biophysical research, with a growing desire to develop technologies and applications for membrane protein science, biosensing and others. Orwar and co-workers have provided a series of methods to generate networks consisting of lipid nanotubes and microscale membrane capsules (vesicles), which opened up opportunities for studies of transport phenomena and communication between cell-sized biomimetic containers produced from phospholipids [19]. An entire set of procedures for manipulation of nanotubes, generation of networks of changing topology and internal functionalization, and new information on nanotube properties and transformations were derived from these experiments [20]. However, the initial promise of entirely new application fields within this branch of soft matter science and technology has not quite been fulfilled, which can possibly be attributed to difficulties in stabilizing the generated structures for prolonged use, and to the lack of simple means of manipulation and exchange of matter between the interior and the exterior of the networks. On the other hand, several unique features of nanotube networks have been clearly demonstrated. In particular, the flexibility, ease of reconfiguration, and biocompatibility can be utilized to mimic and investigate important aspects of cell-to-cell communication in an artificially created environment, where the properties of the membrane largely define the physical, and even chemical, abilities of the system. Propagating enzymatic reactions in reconfigurable geometries is one important example of the capabilities of these networks [21].

Membrane nanotubes form spontaneously in vitro from existing lipid structures, for example, by the interaction of nanoparticles with vesicle membranes [22], by application of electric fields [23,24] or from lipid material adsorbed on a functionalized surface upon application of a shear flow [25]. Their features and properties depend upon the formation conditions, most notably on lipid composition. Tubes can also be generated by exerting a mechanical point load on model cell membranes [26], which was the foundation of the protocols developed for the building of nanotube-vesicle networks [20] (Figure 1D-E).

It has been shown in the early works by Orwar *et al.* that it is possible to connect biological cells via lipid nanotubes with artificial lipid containers [28], where the migration of vesicle contents into the cell was also demonstrated. In related studies, nanotubes could be directly generated from cells after chemically weakening the integrity of the cytoskeleton, which led to the formation of cell-"blebs" [29], *i.e.*, unilamellar vesicles consisting of plasma membrane material (zeiosis). Subsequent application of an earlier developed mechanical tube-pulling technique produced nanotubes, which in turn were used to generate new vesicles, and ultimately, networks of tube-interconnected containers. In contrast to the previously reported vesicle-cell connection, the viability of the cell was lost in these experiments. Very recently, a study was published in which networks of viable cells were generated by means of a similar tube-pulling procedure [27] (Figure 1F-I). Here, nanotubes were created from cultured biological cells, and sequentially connected to adjacent cells, without compromising cell integrity and viability. Examples of materials transport, in this case calcium ions and a prefluorescent enzyme substrate, were supplied to the interconnected cells by diffusion into an open-ended tube, connected to a glass microneedle, and shown to propagate through the interconnection. This represents a promising approach to generating artificial cell networks, even though in this early study the number of nanotubes emanating from a single cell is limited to two. The use of electrical pulses in the 1-10 V range with millisecond duration for penetrating the cellular membrane in the pulling step, and again for the fusion of the tube with the target cell induced considerable damage, and could not be repeated on a single cell more than twice. Although still somewhat limiting, this experimental work demonstrated the validity and effectiveness of artificially-created nanotube interconnections for chemical cell-to-cell communication studies. It has provided an advanced experimental model for cell-cell interactions, and can be viewed as one important step towards man-made network

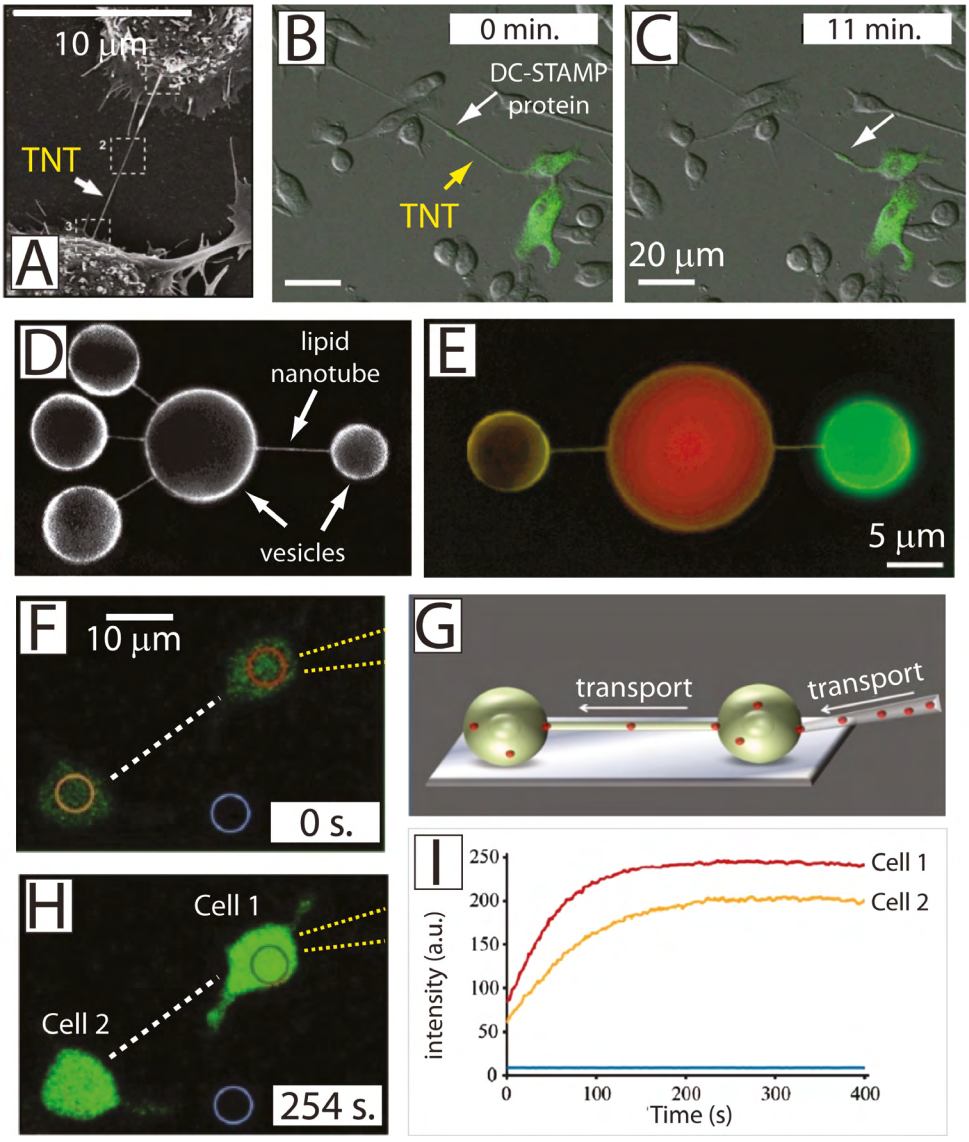

Figure 1. Lipid nanotube networks. (A) Scanning electron micrograph of a tunneling nanotube (TNT) between PC12 cells. Adapted from Ref. [8] - Copyright © 2004, The American Association for the Advancement of Science. (B-C) Confocal micrographs showing DC-STAMP - a key protein which is associated with osteoclastogenesis (bone resorption) - migrating along a TNT among RAW-D cells. The green fluorescence arises from DC-STAMP-GFP expressed by the cells transfected with the DC-STAMP-GFP vector. The protein has been observed to migrate along the tube bi-directionally. Adapted from Ref. [11] - Copyright © 2012, Wiley Periodicals, Inc.. (D-E) Artificially created lipid vesicle-nanotube networks. In (E) a differentiated network where three vesicles are loaded with different fluorescent molecules. Adapted from Ref. [26] - Copyright © 2001, American Chemical Society. (F-I) Artificially created cell-TNT networks. (F)Two HEK-293 cells are connected via a TNT-like conduit (white dashed line), as schematically depicted in (G). Cell 1 is still in contact with the micro injection needle (yellow dashed line) filled with fluorescein diphosphate (FDP). (H-I) Over time (min.) FDP is injected from the micropipette. Due to its conversion by intracellular alkaline phosphatase into fluorescein, the fluorescence signal increases in both interconnected cells. The blue flat line in (I) represents the background signal. Adapted from Ref. [27] - Copyright © 2013, Elsevier Ltd.

architectures involving biological cells. The new model can greatly facilitate fundamental studies of cell-to-cell communication modes, the exchange of cell constituents and components, and the dynamics of biochemical reactions in nearly native (*in vitro*) network environments.

The experimental methods for the on-demand creation of nanotubes and, in particular, their networks are still rather limited. A number of articles have been published on the generation and alignment of lipid nanotubes for the purpose of templating solid materials in different ways, for example by filling the internal space or building desired structures around the tubes' exterior [30]. These studies were directed towards the development of new functional materials on the

nanoscale, and neither communication nor transport of material through the tubes was in their scope. However, technologically interesting ideas, for example the alignment of a multitude of nanotubes by means of electric fields, have resulted [24]. Sugihara *et al.* reported on a simple on-demand fabrication procedure to directly write long-term stable lipid nanotubes onto solid surfaces [31]. A different approach, aiming at controlled generation of lipid nanotubes in the context of artificial cells involved the exploitation of membrane-polymer interactions [32]. A thermoresponsive polymer was introduced into a lipid vesicle, and by means of a heat stimulus, polymer chains linked to the phospholipid bilayer membrane resulted in the extraction of lipid nanotubes towards a contracting polymeric compartment. This clearly demonstrated that contact-less, yet controlled generation of membrane nanotubes is already a possibility. In a similar experimental context, heat-controlled hydrogel compartment formation was employed to modulate the transport rate of small molecules through nanotubes interconnecting two giant vesicles [33]. Czolkos *et al.* reported in 2011 on temperature-controlled self-spreading of a lipid monolayer assembly [34]. Their experimental approach involved elevated temperature to change the phase of a lipid towards a fluid state, thereby lowering the friction of the lipid assembly and hence enabling self-spreading. An even more promising practical approach was reported very recently, where a focused infrared laser was used to change the adhesion properties of surface adhered flat giant unilamellar vesicles so that they were rapidly drifting apart under formation of an interconnecting lipid nanotube [35]. These instances were the first observations of thermoactuated migration of supramolecular (lipid) assemblies. The latter was well enough controllable to be viewed as a contactless fabrication strategy for large area lipid nanotube networks on solid surfaces. The new procedure is a particularly promising approach to on-demand assembly of tube-interconnected container networks. Since the containers are in this case flat giant vesicles, *i.e.*, lipid double bilayers with a very thin separating internal water layer, a truly nanofluidic network can be generated without the need for microneedle manipulation, liquid injection or electrical pulses. In contrast to giant vesicles, the double bilayer structures are not subject to osmotic stress, so their integrity can be maintained for prolonged periods of time. The creation of arbitrary, mechanically stable soft-matter nanotube architectures has now come within reach.

In summary, the current literature on fabrication of, and communication through, lipid nanotubes in the context of cells and artificially generated mimics indicates that the field is progressing both on the biological and the nanotechnology front. Knowledge about the communication functions of lipid nanotubes in biological systems is rapidly increasing, while at the same time new ideas for practically feasible fabrication routes with increased application potential are emerging. Reports on sophisticated, artificially-created lipid nanotube assemblies as well as new approaches to utilizing and modifying nanotube assemblies have been reported in the last few years. The various means to artificially create nanotube networks, connecting either biological cells, or vesicles, or combinations thereof on demand demonstrate the potential to create new experimental possibilities for investigating transport of molecular cargo, and modes of communication through the tubes in a controlled fashion, in particular in the context of intercellular transport between cells of choice. Artificial nanotube interconnections between biological cells have been reported, yet it remains to be firmly established which cell types can be manipulated by the procedure. Moreover, it has not yet been attempted to interconnect cells of different types, or to supply cargo with well-defined function, e.g., for cell growth, migration or development. Further points of interest might also be the stimulated exchange of membrane proteins between cells, or well-defined studies of exchange of pathogens between infected and healthy cells. The described manipulation technique can possibly be extended for creating complex, and far-reaching intercellular networks, including the building of networks among cells in biological tissue, or, as mentioned earlier, the connection of cells to artificial membrane structures for the purpose of membrane protein collection and separation. Such transfer of proteins from cells to biomimetic membrane structures has been reported before, but required the induction of zeiosis, leading in all reported experiments to necrosis [29].

Interesting directions for future studies in the context of cellular communication certainly exist. It has not yet been investigated in detail how artificially created tubes compare to those of natural origin in structure, composition and function, nor have details on the mechanisms involved in transport of chemical compounds and particles through the man-made tubes been obtained. For example, tubes derived from cells by the micropipette technique can be expected to lack the F-actin polymer core that is driving the formation of TNTs [1]. It is certainly also of interest to extend the scope towards electrical cell-to-cell communication, and possibly to evaluate the prospects for using such architectures for computation.

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