Nanomedicine

Localization and mobility of glucose-coated gold nanoparticles within the brain

Aim: To identify the localization of glucose-coated gold nanoparticles within cells of the brain after intravascular infusion which may point to the mechanism by which they cross the blood–brain barrier. **Materials & methods:** Tissue distribution of the nanoparticles was measured by inductively-coupled-mass spectrometry and localization within the brain by histochemistry and electron microscopy. **Results & conclusion:** Nanoparticles were identified within neurons and glial cells more than 10 µm from the nearest microvessel within 10 min of intracarotid infusion. Their distribution indicated movement across the endothelial cytosol, and direct transfer between cells of the brain. The rapid movement of this class of nanoparticle (<5 nm) into the brain demonstrates their potential to carry therapeutic biomolecules or imaging reagents.

Radka Gromnicova[1], Canan Ugur Yilmaz[2], Nurcan Orhan[3], Mehmet Kaya[4], Heather Davies[1], Phil Williams[5], Ignacio A Romero[1], Basil Sharrack[6] & David Male*[,1]

[1]Department of Life, Health & Chemical Sciences, The Open University, Milton Keynes, MK7 6AA, UK
[2]Department of Laboratory Animal Science, Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey
[3]Department of Neuroscience, Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey
[4]Department of Physiology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey
[5]Midatech Pharma Plc, Abingdon, UK
[6]Department of Neurology, University of Sheffield, Sheffield, UK
*Author for correspondence:
Tel.: +44 (0) 1908 659226
D.K.Male@Open.ac.uk

Gold nanoparticles have great potential for the treatment of brain disorders as they may be able to carry therapeutic biomolecules across the blood–brain barrier where there is normally limited entry. Brain endothelial cells possess tight junctions which restrict paracellular movement of small molecules, proteins and nucleic acids. Moreover, many xenobiotics including cytotoxic drugs and antibiotics, are actively removed by multidrug transporters present on the endothelium [1]. Gold nanoparticles (5–80 nm) are able to penetrate the brain parenchyma [2–6], and could therefore be used to carry a therapeutic agent into the brain. However, little is known about the detailed ultrastructural fate of the nanoparticles in the brain. It is vital to gain understanding on which cell types they target in order to develop a functional brain therapy, while reducing possible adverse effects on other cell types.

Glyconanotechnology is a new field of nanotechnology, promising to enhance understanding of glycans and their interactions with cells as well as the use of glycan-coated nanomaterials in nanomedicine [7]. Glycan-coated gold nanoparticles have been used to prevent cell–cell adhesion or interaction between cells and pathogens and in diagnostics [8]. They are also emerging as nanomaterials for drug delivery [9]. In our previous study, we have found that gold nanoparticles with glucose coating (<5 nm) are able to enter and cross human brain endothelium *in vitro* [10], implying that they could be used for delivery of brain therapies such as siRNA, cytokines and small drugs. However, these different therapeutic agents have distinct target zones within the brain. For example, cytokines act within the extracellular space, whereas siRNA must reach the cytosol of the correct target cell type. Therefore, it is essential to determine exactly

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where nanocarriers localize at the ultrastructural level. This study demonstrates that gold nanoparticles can cross the blood–brain barrier *in vivo* and move rapidly through the brain parenchyma. Transmission electron microscopy revealed details of the subcellular distribution of the nanoparticles within different cell types of the brain and elucidated the possible transport mechanisms across the blood–brain barrier and through the brain.

Materials & methods
Full details of all the methods are given in the Supplementary Material.

Synthesis & characterization of glucose-coated gold nanoparticles
Gold nanoparticles with covalently bound glucose on a 2-carbon spacer were prepared in a single step modification of the Brust–Schiffrin method [11]. Coating with ligands was achieved during the reduction of $HAuCl_4$. Thioethyl β-D-glucopyranoside was added into the reaction mixture with $HAuCl_4$ in methanol before reduction with $NaBH_4$. The resulting nanoparticles were <5 nm in diameter with a 2-nm gold core.

Animals & nanoparticle administration
The experimental protocols used in this study were approved by the Local Ethics Committee for Animal Experimentation of Istanbul University (2014/06). Detailed protocols are given in the Supplementary Material. Briefly, the nanoparticles (50 μg Au in 100 μl) were injected into the left carotid artery of anesthetized adult male Wistar rats (250–300 g, n = 4) over the course of 2 min using an infusion pump. The nanoparticles circulated for 10 min before the animals were transcardially perfused with 75 ml 0.9% saline, followed by fixation with 200 ml of fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M PB (phosphate buffer) for 15 min. Brain, liver, lung and kidney were taken for analysis.

Analysis of gold content in brain, liver, kidney & lung
Tissue samples were digested in boiling HNO_3 before analysis by ICP-MS (inductively-coupled mass spectrometry) to quantify gold. The results were first expressed as [μg gold/mg tissue] and the values were then compensated to account for variation in the weights of the animals.
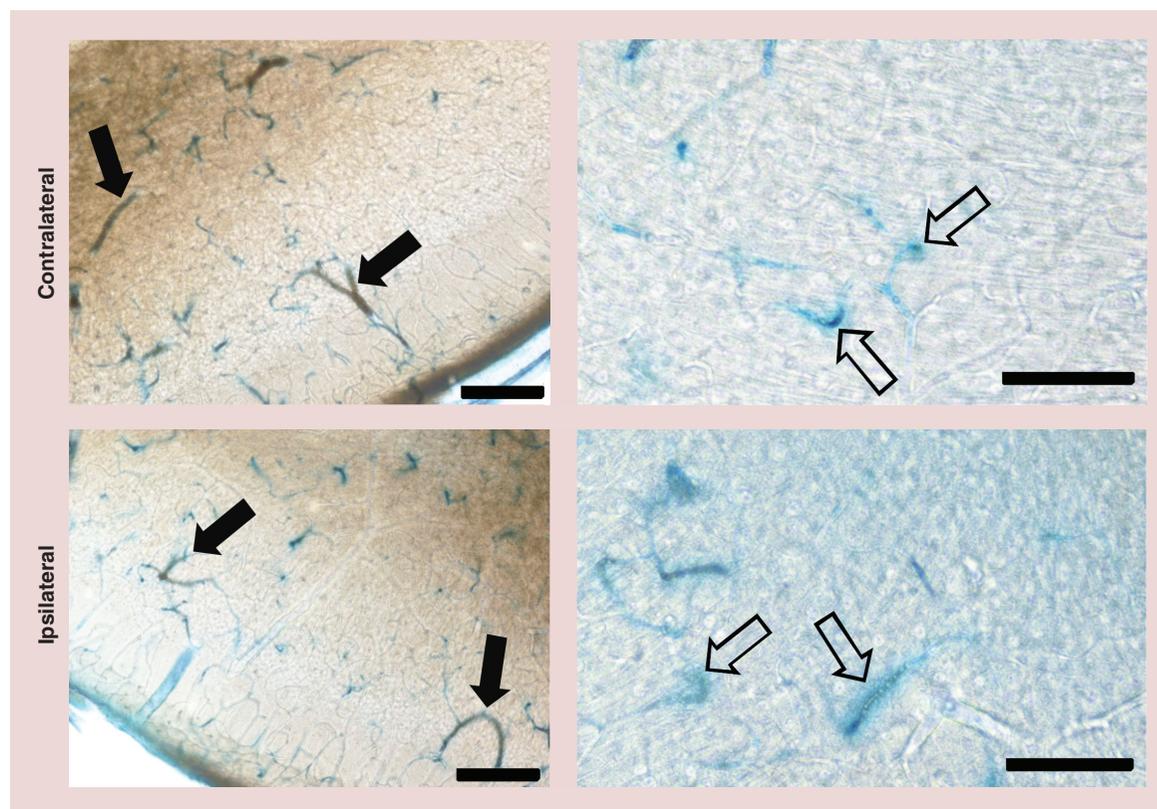

Figure 1. Determination of blood–brain barrier integrity by immunohistochemistry. Immunoglobulins were stained (blue) in coronal sections. Staining appeared in poorly perfused blood vessels (full arrows). Slight leakage of the immunoglobulin (outlined arrows) was observed in both hemispheres of the brain indicating that the leakage itself could not be caused by the injection procedure. Scale bars on the left 500 μm, on the right 100 μm. Representative images are shown.

Immunohistochemistry & electron microscopy

Fixed brains were initially sectioned (100 μm) with a vibratome and a coronal section corresponding to the start of the hippocampus in each animal was analyzed. Then they were permeabilized (0.01% Triton-X100) and enhanced by silver enhancement in order to increase the size of nanoparticles to approximately 20 nm, which also produced brown staining corresponding to the areas containing high levels of nanoparticles. The 100 μm sections were imaged by light microscopy. The sections were then stained with 1% osmium tetroxide to increase contrast and further processed for electron microscopy. Ultrathin sections (80 nm) were counter-stained with uranyl acetate and lead citrate and imaged by a transmission electron microscope, JEOL 1010, at 80 kV.

To check that blood–brain barrier integrity had not been compromised by the nanoparticle infusion, parallel sections to those imaged by electron microscopy were stained for the presence of rat immunoglobulin to determine whether any protein leakage from the microvessels had occurred (Note that the molecular diameter of IgG, ~5.5 nm, is similar to the nanoparticles). The density of the blood vessels in rat cortex was measured on the electron microscope, using imaging software AmtV 600, to determine the distance to the nearest microvessel.

Results

Previous studies have shown that gold nanoparticles can enter the brain following intravenous [2–3,12] or intraperitoneal [5] injection. In this study, we injected 50 μg of glucose-coated gold nanoparticles into the left carotid artery. This protocol allowed for a high local concentration of gold nanoparticles in the left hemisphere of the brain (ipsilateral side) while keeping the blood–brain barrier undisturbed (Figure 1). Nanoparticles that exited the cerebral circulation after passing through the ipsilateral side of the brain then passed through the pulmonary circulation. They then distributed via the systemic circulation into other organs, including the liver, kidney and right side of the brain (contralateral side). Analysis of the distribution of gold in the different tissues by ICP-MS (Figure 2) showed highest concentrations of nanoparticles in the kidney, which is characteristic of nanoparticles of this size [13]. The nanoparticles were also detected in the brain; the levels in the ipsilateral side were similar to the levels in the lung and liver. Moreover, the level of accumulation was approximately three-times higher in the ipsilateral side than in the contralateral side of the brain, although the difference was not statistically significant (one-way paired t-test, p = 0.09).
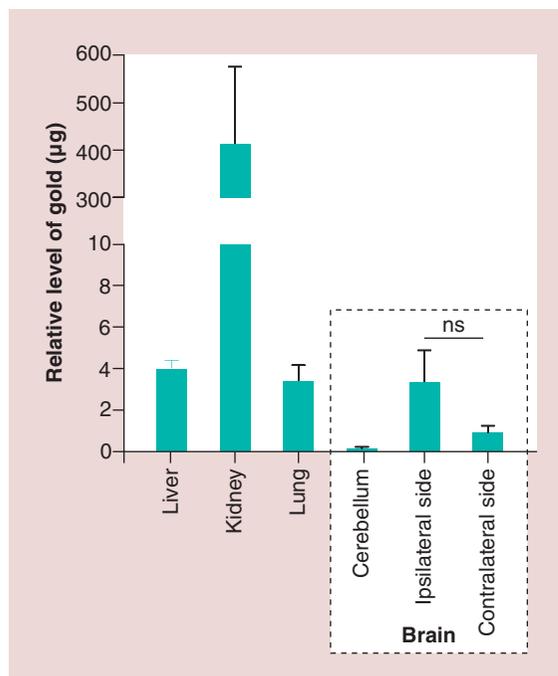

Figure 2. Analysis of gold content in tissues 10 min after intracarotid injection. The values are mean ± standard error of the mean from four animals, initially calculated as μg gold/mg of tissue and then compensated for variation in the initial concentration of the nanoparticles according to the weight of each animal, here shown as relative amount to the animal weight. The difference between ipsilateral and contralateral side was ns (paired t-test).
ns: Not significant.

The cellular localization of the nanoparticles in the brain was determined by transmission electron microscopy. Silver enhancement of gold present in coronal sections identified areas of the cortex with the highest levels of gold (Figure 3A). Note that the silver enhancement is not directly proportional to the amount of gold present – gold was also detected in areas of the contralateral cortex by ICP-MS and electron microscopy, albeit at lower levels than in the stained areas. Silver-enhanced nanoparticles were observed in the cytosol of endothelial cells (Figure 3B), but not in tight junctions between the endothelial cells or in their vesicles. We also confirmed that the silver enhancement does not cause nonspecific staining, by silver-enhancing a brain section of an uninjected animal (Supplementary Figure 1).

Beyond the brain endothelium, the nanoparticles were observed at the basal lamina and in pericytes (Supplementary Figure 2). In brain parenchyma, the nanoparticles were detected in the cytosol and nucleus of glial cells (Supplementary Figure 3) as well as in neurons, both in the cell bodies and in axons of myelinated and unmyelinated neurons (Figure 4 & Supplementary Figure 4). A very small number of
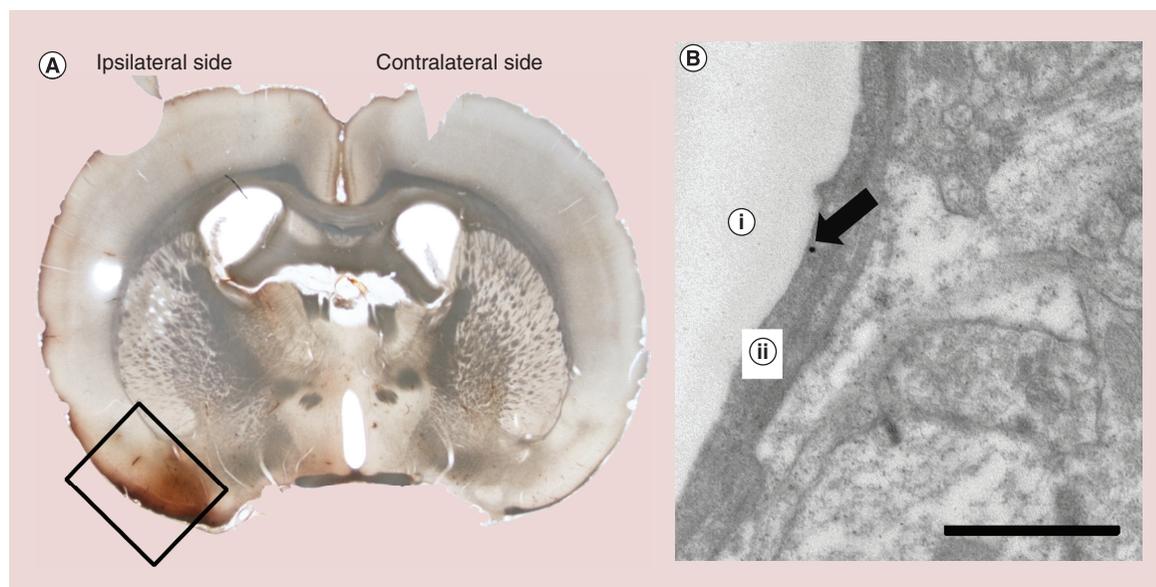
Figure 3. Glucose-coated gold nanoparticles in rat brain. (A) Silver-stained coronal section; the rectangle corresponds to the area of cortex examined by electron microscopy. **(B)** Gold nanoparticle (arrow) in the cytosol of an endothelial cell **(ii)**. **(Bi)** Blood vessel lumen. Scale bar: 1 μm.

nanoparticles were seen in the extracellular spaces (Figure 4).

We also analyzed the distance that these nanoparticles were observed from the blood vessel in the brain parenchyma. Within the 10 min time-frame of the experiment, many nanoparticles have been observed more than 10 μm from the nearest visible capillary (Figure 5 & Supplementary Figure 5).

Discussion

This study demonstrated that glucose-coated gold nanoparticles (<5 nm) can enter the brain of rats 10 min after intracarotid injection. At the cellular level, the nanoparticles localized in the cytosol of brain endothelium and were able to enter glial cells and neurons in the cortex. Nanoparticles had moved more than 10 μm from the nearest visible capillary.

The intracarotid administration used in this study is regularly employed to enhance uptake of substances by the brain. For example, it is common in applications of substances to open the blood–brain barrier with mannitol or TNF-α [14,15] as well as in a clinical setting to help to treat neurological conditions such as ischemic stroke or intracranial malignancies [16]. Intracarotid injection has not been used previously to administer nanoparticles to animals, possibly because of the necessary surgical expertise required. We showed that this route can be useful to study nanoparticle migration into the brain, with the advantage of having a two-in-one picture: one hemisphere was directly affected by the bolus injection whereas the second hemisphere functioned as a control, where the nanoparticle local-

ization would be similar to a more classical route, such as intravenous injection via the tail vein.

The tissue distribution showed that most of the nanoparticles were detected in the kidney, an organ which has fenestrated endothelium. This finding agrees with previously published work, which has shown that smaller nanoparticles (<8 nm) localize in kidney, whereas larger ones (>10 nm) are often taken up by mononuclear phagocytes, particularly in the liver and spleen [13].

At the end of the 10 min infusion, the amount of gold in the blood was approximately 1 μg/ml, while the mean amount in the ipsilateral side of brain was 15 ng/g tissue as measured by ICP-MS. This amount (~1.5% of the blood concentration) may appear low; however, it is high in comparison with the expected level of transport of a molecule of this size. It is possible to assess brain penetration of a molecule by comparing the levels in the cerebrospinal fluid with the levels present in serum (CSF:serum ratio). In this case, hydrophilic molecules of 5 nm, which are not actively transported (e.g., IgG), are present in cerebrospinal fluid at approximately 0.2% of the serum concentration (CSF:serum ratio = 0.002) [17]. Even ligands designed to improve drug delivery across the blood–brain barrier only demonstrate brain penetration between 0.2 and 3% (e.g., monoclonal antibodies to transferrin receptor, or insulin receptor at 3 h after infusion) [18]. The level of entry of these gold nanoparticles into cortex is therefore remarkably high, particularly given the short time-frame of the experiments.

It is essential to distinguish nanoparticles present in the brain parenchyma from any present within the brain vasculature. In our experiments the animals were perfused to clear the vasculature from blood after the injection, and the difference between the levels in the injected and noninjected sides (as detected by ICP-MS) implies that the nanoparticles are not located in residual blood within cerebral vessels. This is confirmed by TEM images which show perfused blood vessels and nanoparticles in endothelium, neurons and glia. The nanoparticle distribution in the brain varied depending on what part of the brain was analyzed; cerebellum had lower levels in comparison with the contralateral side of the brain (Figure 2). The differences may partly be explained by different levels of vascularization of these areas of the brain. More surprising was the observation that the silver-enhanced coronal sections (Figure 3A) indicated local variation in the levels of nanoparticles in different areas of the ipsilateral side of the brain. We considered the possibility that the injection itself might have disrupted the barrier in some areas. However, immuno-histochemistry revealed comparable blood vessel integrity in ipsilateral and contralateral sides of the brain (Figure 1) and electron micrographs showed no evidence of barrier disruption or endothelial damage (Figures 3 & 5 & Supplementary Figure 5). Another explanation for

this variation may be intravascular streaming that follows infusion into the carotid artery [19]. Intravascular streaming causes variability in drug distribution in the brain and can lead to local toxicity [19,20]. It is the most likely explanation for the uneven distribution of gold nanoparticles that we have observed in the cortex.

We observed the glucose-coated gold nanoparticles in the cytosol of the brain endothelium, suggesting that they can enter the brain endothelium by passive transport. This observation is in accordance with our study *in vitro* [10], which suggested that glucose-coated nanoparticles primarily moved across human brain endothelium through the cytosol, crossing the apical and basal plasma membranes. There are several models of this passive transport mechanism that have tried to explain this phenomenon [21–23]. It is possible that the initial interaction of the nanoparticles with the cell membrane may also be affected by the endothelial glycocalyx and membrane proteins. In our previous study [10] we found that the endothelial Glut-1 transporter is not involved in the uptake of nanoparticles as inhibition of the transporter did not affect nanoparticle transmigration. The glucose is attached to a carbon linker which extends from the nanoparticle in a way that makes it unrecognizable to the transporter [11,24]. Therefore, the function of glucose is to stabilize the
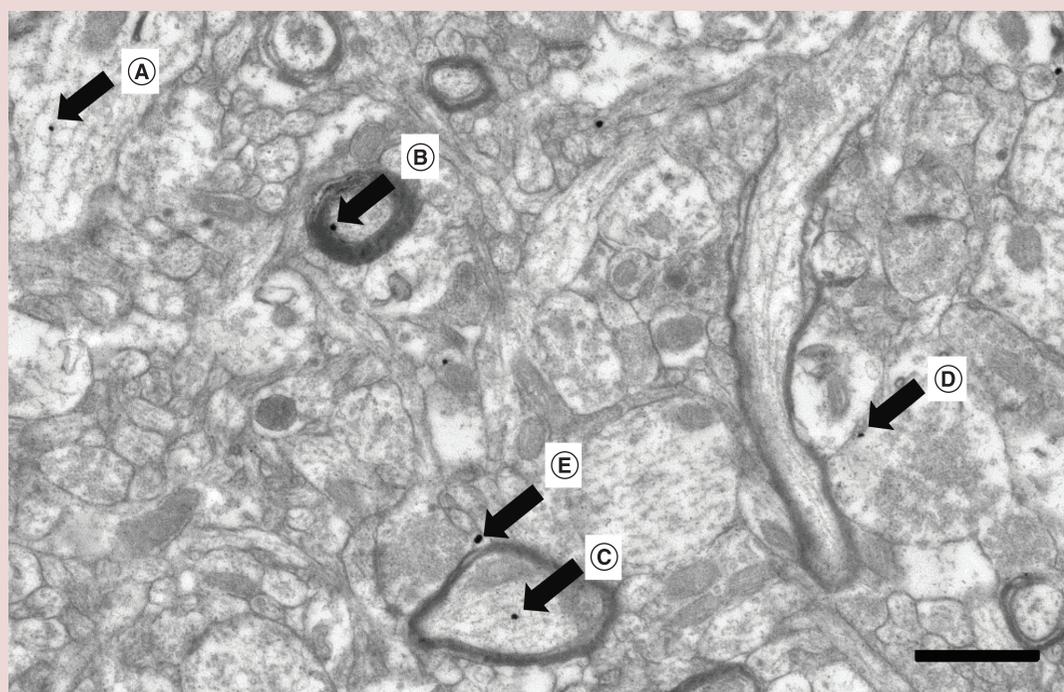

Figure 4. Glucose-coated gold nanoparticles in brain parenchyma. Nanoparticles localized in myelinated axons **(B & C)**, unmyelinated axons/dendrites **(A & D)** and in the extracellular space **(E)**. Scale bar: 1 μm.

nanoparticle and provide it with neutral charge (more details on characterization of the nanoparticles are given in the Supplementary Material).

Larger gold nanoparticles (80 and 13 nm) have been shown to enter cerebral cortex by electron microscopy [5,6], however, detailed ultrastructural analysis especially for small nanoparticles, has not been done previously. Our glucose-coated gold nanoparticles entered neurons and glial cells of the cortex (Figure 6 & Supplementary Figure 3). In comparison with the published studies [5,6], the nanoparticles were able to enter the nucleus as well as the cytosol of the cells which may be inherent to their size, but further studies *in vitro* would be necessary to uncover the mecha-

nism of nuclear transport. The route the nanoparticles may take into the nucleus would involve either crossing the double nuclear membrane or moving through the nuclear pore. The nuclear pore complex (size about 120 nm) allows passive diffusion of molecules of up to 40 kDa [25] and our nanoparticles (mean molecular mass of 27 kDa) would fit this criterion. Nuclear localization of nanoparticles also suggests a potential use for radiotherapy or a nuclear gene therapy.

The distance that the nanoparticles had moved in the brain was over 10 μm. This corresponds with the high rate of movement that we observed previously *in vitro* [10]. It implies that the tightly-packed
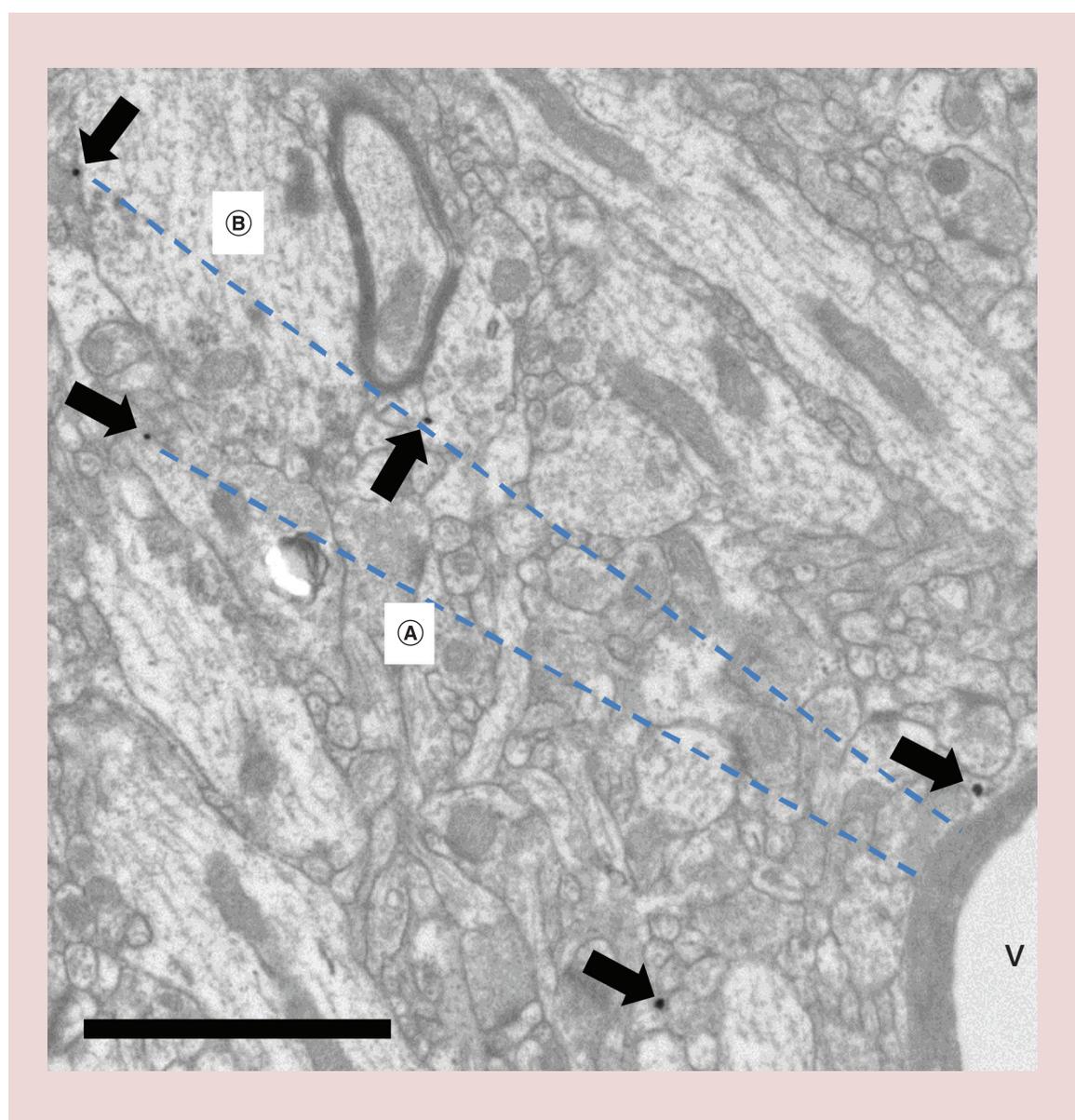

Figure 5. Distance traveled by glucose-coated gold nanoparticles in cortex. The nanoparticles (arrows) located at various distance (dashed line) from the blood vessel (v); examples: **(A)** 5 μm, **(B)** 7 μm. Scale bar: 2 μm.
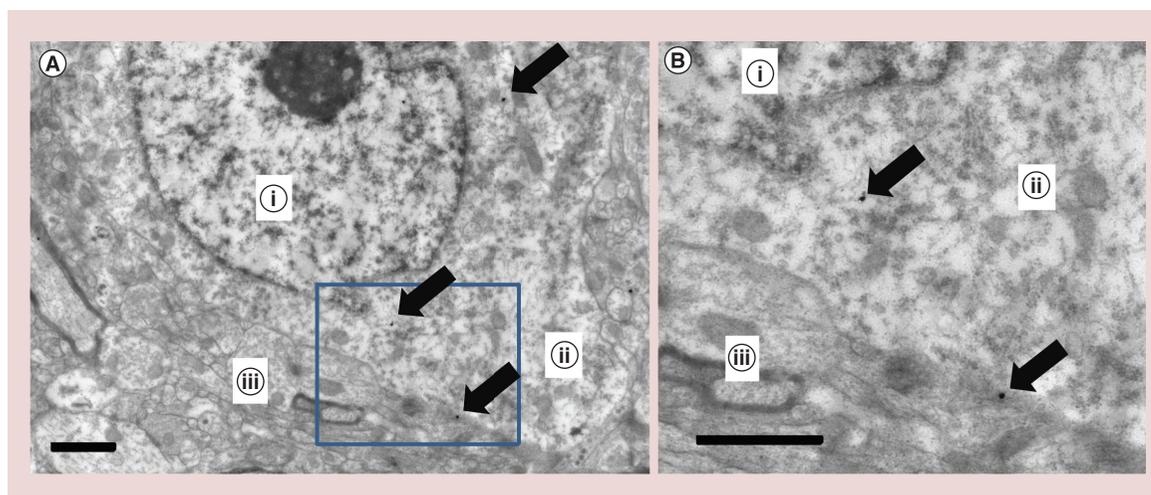
Figure 6. Gold nanoparticles in a neuronal cell body. (A) Nanoparticles (arrows) located in the cytosol **(Aii & Bii)** of a neuronal cell. The rectangular inset is enlarged **(B). (Ai & Bi)** nucleus of a neuron, **(Aiii & Biii)** brain parenchyma. Scale bar: 1 μm.

cells of the brain cortex do not present a substantial barrier to these nanoparticles. The nanoparticles were located in high numbers intracellularly in comparison with extracellular spaces, which suggested direct movement between cells. The distance the nanoparticles traveled implies that they are not moving by diffusion in the tortuous extracellular spaces. It is estimated that all cells of the brain are within 50 μm of a capillary [26]. This distance corresponds with the density of vessels that we observed in the electron micrographs, where the mean distance of blood vessels in the 2D space of the sections was measured as 50.6 ± 25.5 μm, based on 106 measurements (Supplementary Figures 6 & 7). Could the nanoparticles (Figure 5 & Supplementary Figure 5) have moved to their position from a microvessel that is not seen in the images? This would require a much higher density of microvessels in the cortex than is observed here or reported previously [26]. Each section viewed on the electron microscope is 80 nm thick. Assuming that the rates of movement demonstrated *in vivo* are correct, it implies that nanoparticles of this type could permeate all areas of the cortex within 50 min. The movement of the nanoparticles through the brain parenchyma might be facilitated by the perivascular spaces, between endothelium and astrocyte foot process, or the 'glymphatic system' [27], which may be responsible for removing molecules from the brain such as amyloid-β [28]. However, the nanoparticles were not specifically observed in these areas.

This study points out the potential of glucose-coated gold nanoparticles for carrying a therapeutic substance into the brain cortex. Due to their large surface area that can hold up to 40 covalently bound ligands, the amount that they may be able to deliver might be ther-

apeutically useful. In comparison to free drug molecules, the nanoparticles may also improve the drug efficiency by providing a depot for small drug release, or by orientating clusters of biomolecule ligands to act on cell surface receptors.

Conclusion

Nanoparticle-based therapeutic delivery into the cortex may be relevant to delivery of treatment for neurological conditions. Also, knowledge of nanocarrier behavior is crucial for improving the design of nanoparticles for targeting cells of the brain and optimizing transport and release of a potential cargo. Future studies with more time points would reveal the time and speed with which the nanoparticles are cleared from the brain. To conclude, we believe that the type of investigation shown here is essential for the development of any nanoparticle-based therapy, including the use of nanoparticles as drug carriers, therapeutic agents themselves or for imaging.

Future perspective

The major developments in disease treatments for the last 10 years have been in biological agents, all of which are excluded from the brain by the blood–brain barrier. There are several promising approaches for treatment of neurological conditions, including use of siRNA and cytokines; the challenge for researchers is to develop a way of transporting these agents into the brain.

First, a suitable carrier needs to be developed (as demonstrated in this paper), then it must be loaded with a biomolecule, that does not affect the transport properties across brain endothelium. Once the carrier has entered the brain it should release its therapeutic cargo and then be cleared, either by transcytosis/dif-

fusion/excretion or by biological breakdown. Over the next decade, we believe that the combined chemical and biological problems will be solved, so that imaging agents and therapeutic molecules can be delivered into the brain. Nanomedicine presents the most promising way forward in this area.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Glucose-coated gold nanoparticles (<5 nm) entered the brain within 10-min of intracarotid injection.
- The nanoparticle concentration within the brain is comparable to that in liver and lung, but much lower than in the kidney.
- Within the brain cortex, the nanoparticles were observed in cytosol of endothelial cells, implying a possible passive transport across the brain endothelium.
- In the cortical parenchyma, the nanoparticles localized within the cytosol and nucleus of both neurons and glial cells, pointing to their potential use for delivery of therapeutic agents as well as use for radiotherapy.
- The nanoparticles were observed more than 10 μm from the nearest capillary, suggesting that they may be able to move at more than 1 μm per minute within the cortex.

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