COMMUNICATION

Targeted delivery of DNA-Au nanoparticles across the blood-brain barrier using focused ultrasound

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Abstract: Nanoparticles have been widely studied as versatile platforms for *in vivo* imaging and therapy. However, their use to image and/or treat the brain is limited as they are often unable to cross the blood-brain barrier (BBB). To overcome this problem, herein we report the use of focused ultrasound *in vivo* to successfully deliver DNA-coated gold nanoparticles to specific locations in the brains of mice.

Functionalised metal nanoparticles that are able to co-deliver multiple drugs and/or imaging agents to a target location have attracted great interest in recent years. In particular, spherical nanoparticles coated with a dense layer of oligonucleotides, first pioneered by Mirkin *et al.*, have emerged as promising nanoplatforms for *in vivo* applications.[1] It has been shown that these nanoparticles can spontaneously enter over 50 different cell types without the need for an external transfecting agent, have low immunogenicity in cells, good stability and have shown no toxicity so far.[1–5] In addition to this, they are highly versatile, allowing them to be easily tailored for a wide range of applications. A variety of core materials can be used to prepare them, ranging from iron oxide nanoparticles to quantum dots, and almost any oligonucleotide sequence can be attached to the particle surface.[1]

However, due to their size, the majority of functionalised nanoparticles show limited accumulation in the brain due to their inability to cross the blood-brain barrier (BBB).[6] This limits their potential application for the imaging and treatment of a range of diseases such as Alzheimer's disease, Parkinson's disease and brain tumours. As of now, the only way to disrupt the BBB in a non-invasive, transient and targeted manner is with focused ultrasound and microbubbles.[7] In this method, microbubbles and the drug of choice are first injected intravenously into the bloodstream. The target region is then exposed to ultrasound, which drives the microbubbles into radial oscillation.[8] By exerting mechanical stress on the blood vessel walls, an increase in BBB permeability is observed, resulting in enhanced drug delivery.[7,9–11] The exact biological mechanisms through which this occurs is still under investigation, but it has been linked to a widening of the tight junctions between endothelial cells, increased vesicle activity and a suppression of the drug efflux pumps located at the surface of endothelial cells.[12,13] To date, focused ultrasound-mediated BBB disruption has been successfully used to deliver a wide range of small molecules into the brains of rodents and non-human primates.[10,14–17] In contrast, there are significantly fewer examples where nanoparticles have been successfully delivered across the BBB *in vivo* using focused ultrasound.[18–21] A reason for this is that the dependency of focused ultrasound-mediated delivery on nanoparticle diameter has not been previously investigated, making it difficult to design nanoparticles for this application: larger nanoparticles allow for higher payloads of drug to be delivered to the target location per particle, but may not be delivered as efficiently across the BBB using this technology compared to smaller nanoparticles.

With the aim to address this, herein we report the synthesis of a range of DNA-coated gold nanoparticles (DNA-Au NPs) of different sizes and show that they can be successfully delivered to specific brain locations across the BBB in mice using focused ultrasound (Figure 1). The motivation to carry out this study is to establish a methodology for the future use of nanoparticles as delivery vehicles for delivering drugs and/or imaging agents to specific locations in the brain. The size-dependency of focused

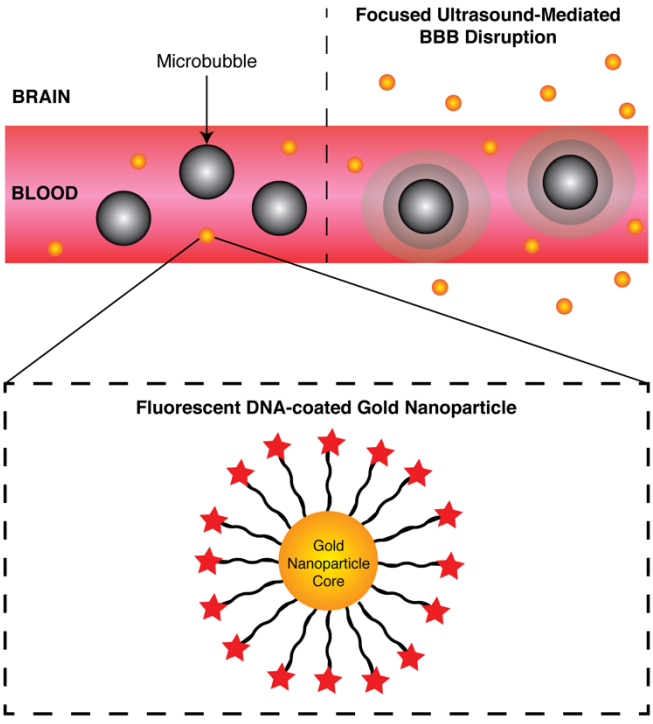


Figure 1. Schematic representation showing the use of microbubbles and focused ultrasound to deliver DNA-coated gold nanoparticles across the BBB.

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ultrasound-mediated nanoparticle delivery across the BBB has also been investigated in this study.

Three sets of Au NPs of different sizes were synthesised *via* a seeded growth protocol involving the reduction of $HAuCl_4$ with sodium citrate and traces of tannic acid (Figure 2a).[22] Au NPs were characterised using dynamic light scattering (DLS), transmission electron microscopy (TEM) and UV-Vis spectroscopy. DLS measurements and TEM images showed that the Au NPs were quasi-spherical in shape and narrowly dispersed, with core diameters of 6.3 ± 0.7 nm, 9.5 ± 1.4 nm and 14.2 ± 1.2 nm (Figure 2b,c and Table S1). The presence of the characteristic surface plasmon resonance (SPR) band at ~515 nm was observed in the absorption spectra of all Au NPs synthesised (Figure S2). Moreover, the absorbance and wavelength of the SPR band was seen to increase with increasing NP size as expected.

Table 1. Measured TEM diameters (d_{TEM}), hydrodynamic diameters (d_h) and zeta potentials (ζ) of synthesised Cy5-DNA-Au NPs.

	d_{TEM}/nm	d_h/nm	ζ /mV
A	6.3 ± 0.7	21.6 ± 3.3	-42.5 ± 3.1
B	9.5 ± 1.4	23.3 ± 2.6	-35.0 ± 3.5
C	14.2 ± 1.2	25.2 ± 1.9	-21.1 ± 1.6

Synthesised Au NPs were functionalised with Cy5-labelled oligonucleotides to give Cy5-DNA-Au NPs **A, B** and **C**. As previously described by Li *et al.*, Tween 20 was first added to stabilise the Au NPs and prevent their aggregation in high ionic strength environments.[23] Thiolated oligonucleotides were then added to the reaction mixture in the presence of salt to minimise electrostatic repulsions between oligonucleotide strands. A 25 base oligonucleotide sequence was chosen for this study as this is the typical length of oligonucleotides used for gene therapies.

DLS measurements showed that the synthesised Cy5-DNA-Au NPs had hydrodynamic diameters of 21.6 ± 3.3 nm (**A**), 23.3 ± 2.6 nm (**B**) and 25.2 ± 1.9 nm (**C**) respectively (Table 1). As expected, changes in the hydrodynamic diameters and zeta potentials were observed after functionalisation with oligonucleotides. Moreover, the zeta potentials were found to vary between Cy5-DNA-Au NPs **A**, **B** and **C** as the oligonucleotide loading was adjusted in each case so that the overall fluorescence intensities were similar to allow for comparison after delivery across the BBB (Figure S3).

The feasibility of delivering Cy5-DNA-Au NPs **A-C** into the brain *in vivo* using ultrasound pulses was then assessed. Wild type C57LB/6 mice were first injected with SonoVue© microbubbles, followed by Cy5-DNA-Au NPs **A**, **B** or **C** (Figure 3). The left thalamus of the brain was sonicated using ultrasound pulses (frequency: 1 MHz; peak-negative pressure: 530 kPa; pulse length: 10 ms; pulse repetition frequency: 0.5 Hz; sonication duration: 250 s), whilst the right thalamus acted as the non-

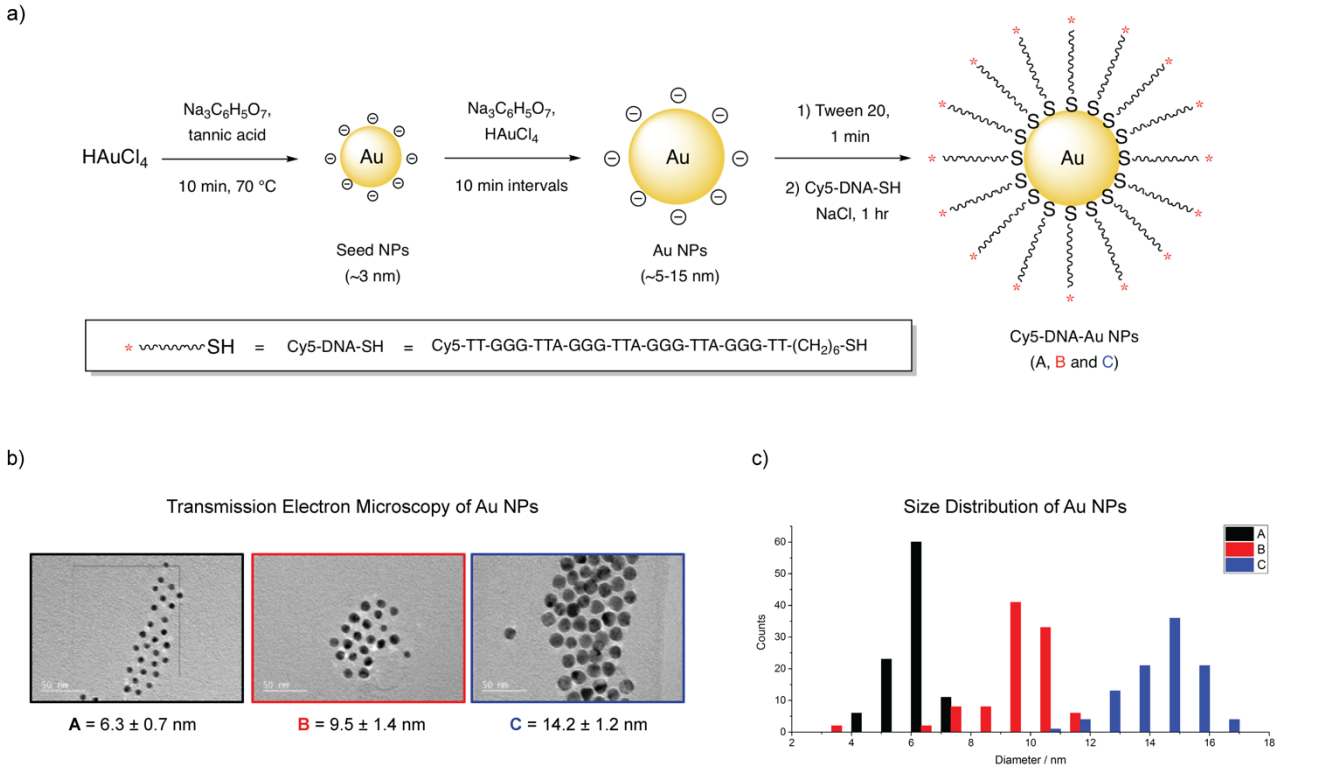


Figure 2. a) Three sets of Au NPs of core diameters 6.3 ± 0.7 nm, 9.5 ± 1.4 nm and 14.2 ± 1.2 nm were synthesised *via* a seeded growth approach. Synthesised Au NPs were functionalised with Cy5-labelled oligonucleotides to give Cy5-DNA-Au NPs **A-C**. b) Representative transmission electron microscopy images of Au NPs **A-C**. Nanoparticles were observed to be quasi-spherical and narrowly dispersed. c) Size distribution of Au NPs determined from transmission electron microscopy images. A total of 100 NPs per set were counted using ImageJ.

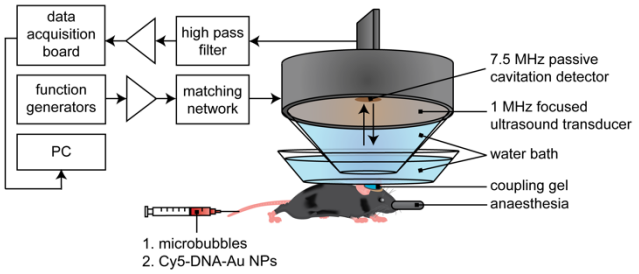


Figure 3. Experimental setup for focused ultrasound-mediated BBB disruption. Microbubbles are injected intravenously, followed by Cy5-DNA-Au NPs **A**, **B** or **C**. The left thalamus is sonicated using ultrasound pulses (frequency: 1 MHz; peak-negative pressure: 530 kPa; pulse length: 10 ms; pulse repetition frequency: 0.5 Hz; sonication duration: 250 s), whilst the right thalamus acts as the control.

sonicated control. Following sonication, the brain was extracted, sectioned into 30 µm horizontal sections and then imaged using brightfield and fluorescence microscopy. Evidence of successful BBB disruption in the left thalamus was observed in all experiments conducted.

Due to the characteristic red colour of the Au NP cores, their presence in the left thalamus could be detected in brightfield images (Figure 4). Fluorescence signal from the attached Cy5-labelled oligonucleotides was found at the exact same locations, indicating that the Cy5-DNA-Au NPs remain intact as they cross the BBB (Figure 4). To the best of our knowledge, this is the first time that DNA-coated gold nanoparticles have been delivered across the BBB *in vivo* using focused ultrasound. Moreover, in contrast to prior work by Jensen *et al.* in which DNA-Au NPs were reported to be able to cross the intact BBB in wild type mice by themselves, no evidence of Cy5-DNA-Au NPs **A-C** was detected in the right thalamus (control) in our experiments (Figure 5a).[6] It should be noted, however, that a lack of fluorescence signal does not indicate that there are no NPs present; instead, it might indicate that there are not enough NPs present to generate a detectable signal. The data presented here therefore shows that focused ultrasound and microbubbles can be used to significantly enhance the delivery of Cy5-DNA-Au NPs across the BBB; furthermore, it allows the directed delivery of nanoparticles to specific locations within the brain.

To investigate the effect of varying NP diameter on the efficiency of delivery using focused ultrasound-mediated BBB disruption, the normalised optical density – the average fluorescence intensity in the targeted region normalised by the control region – was calculated from the fluorescence images (Figure 5). A two-sided Student's *t*-test was performed to assess statistical significance. Our results show that there is a size-dependency associated with the delivery of Cy5-DNA-Au NPs across the BBB using focused ultrasound, with the smallest NPs tested in this study (**A**) being delivered across the BBB six times more efficiently than the largest NPs tested (**C**). As proposed in the past, the presence of a size-dependency suggests that the enhanced BBB permeability caused by focused ultrasound-mediated BBB disruption is primarily due to enhanced paracellular transport rather than increased vesicular transport as vesicles are much larger than the majority of therapeutic agents.[24,25] It should also be noted that the difference in diameter between Cy5-DNA-Au NPs **A** and **C** is small (less than 5 nm), suggesting that NP diameter is an important design criterion to take into account if one wishes to deliver NPs into the brain using focused ultrasound: whilst larger NPs offer a greater surface area for the attachment of therapeutic and diagnostic agents, our results show that they are delivered less efficiently than smaller NPs using the same ultrasound parameters.

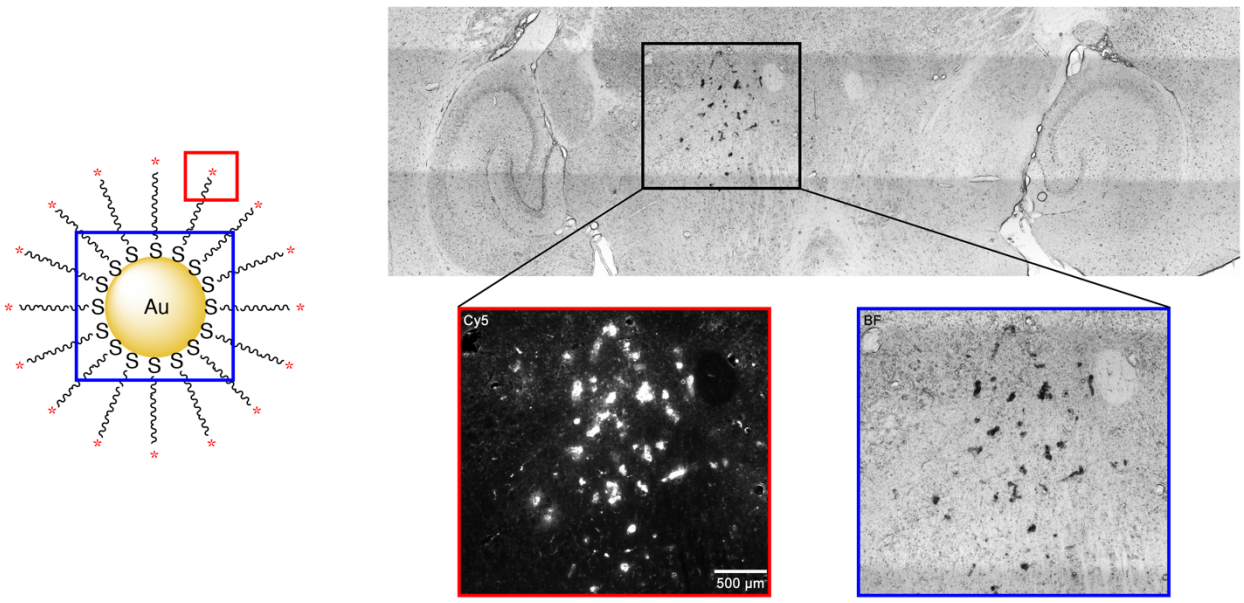


Figure 4. Cy5-DNA-Au NPs **A-C** were successfully delivered to the left thalamus of the brain using focused ultrasound. Fluorescence signal corresponding to the attached Cy5-labelled oligonucleotides (red; Cy5 channel) was found at the exact same locations as signal from the Au NP cores (blue; brightfield), indicating that the Cy5-DNA-Au NPs remain intact as they cross the BBB *via* focused ultrasound-mediated BBB disruption.

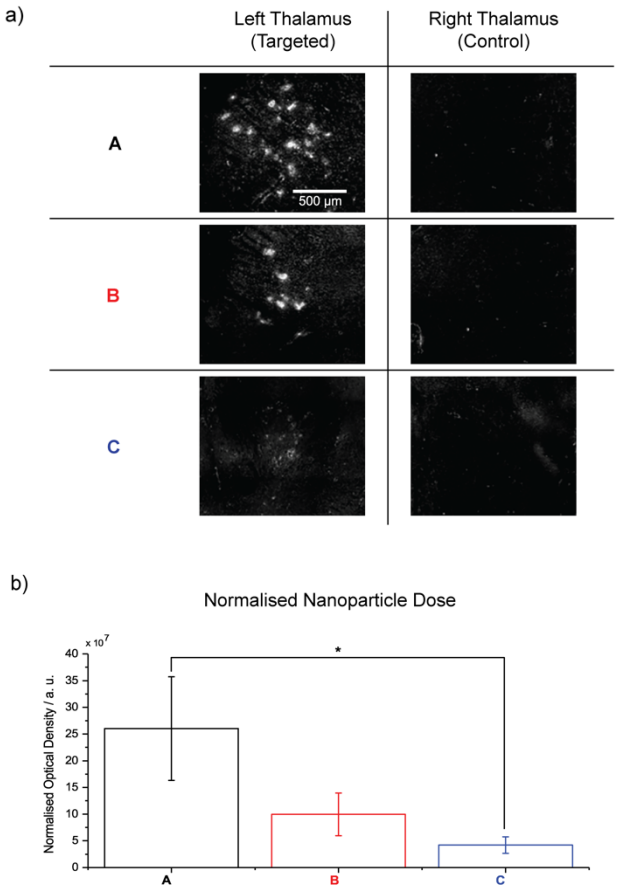


Figure 5. a) Representative fluorescence images from the delivery of Cy5-DNA-Au NPs **A-C** across the BBB using focused ultrasound. A spot-like distribution of fluorescence is observed in the left thalamus, whilst no fluorescence signal is detected in the right thalamus. b) Normalised optical density was calculated from fluorescence images as a measure of the nanoparticle dose delivered across the BBB (n = 5 for each set of NPs). A size dependency is observed, with the smallest NPs synthesised (**A**) being delivered six times more efficiently across the BBB using focused ultrasound than the largest NPs (**C**) ($p < 0.05$).

To assess the safety of the focused ultrasound-mediated BBB disruption conducted in this study, brain sections covering the entirety of the focal volume were stained with haematoxylin and eosin (H&E). For these experiments, a Texas Red-labelled 3 kDa dextran was injected as a marker of BBB opening and the brain was sonicated using the same ultrasound parameters as above. Following sonication, mice were immediately sacrificed and brains were extracted for histological analysis. In agreement with prior studies, minimal tissue effects were observed, with just a few sites of minor red blood cell extravasation identified in the targeted region (Figure S4).[26]

In summary, we have shown that DNA-Au NPs can be successfully delivered across the BBB using focused ultrasound in a non-invasive and targeted manner. Moreover, a size-dependency was observed, with smaller NPs being delivered more efficiently across the BBB using this technology than larger NPs.

Acknowledgements

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Keywords: Ultrasound • Blood-brain barrier • Nanoparticles • Brain • DNA

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COMMUNICATION

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