The influence of blood on targeted microbubbles

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The ability to successfully target the delivery of drugs and other therapeutic molecules has been a key goal of biomedical research for many decades. Despite highly promising in vitro results, however, successful translation of targeted drug delivery into clinical use has been extremely limited. This study investigates the significance of the characteristics of whole blood, which are rarely accounted for in vitro assays, as a possible explanation for the poor correlation between in vitro and in vivo experiments. It is shown using two separate model systems employing either biochemical or magnetic targeting that blood causes a substantial reduction in targeting efficiency relative to saline under the same flow conditions. This finding has important implications for the design of targeted drug delivery systems and the assays used in their development.

1. Introduction

The concept of targeted drug delivery was originally proposed in the literature by Paul Ehrlich (1854–1915) who suggested that if malignant tissue could be selectively targeted then a toxin could also be selectively delivered in a manner akin to a ‘magic bullet’ [1]. The central principle of drug targeting is that the distribution of the drug within the body is manipulated so that the majority interacts exclusively with the target tissue [2] thereby minimizing the required dose and the risk of unwanted side effects. Numerous targeting strategies have been developed, including antibody and other biomimetic approaches [3,4], electrostatic [5], acoustic [6,7] and magnetic methods [8,9]. Despite highly promising in vitro studies, successful translation of these strategies into clinical use has been extremely limited, largely due to poor targeting efficiency in vivo [10,11]. As a result, the cost effectiveness of developing targeted therapies has been called into question [10].

A wide range of micro- and nanoscale particles are used as vehicles for targeted drug delivery. These include solid metallic or polymeric particles, liquid-filled vesicles or liposomes and gas-filled microbubbles [12–14]. In addition to providing a substrate for incorporating the drug, targeting species may be attached to the particle surface and/or they may be functionalized to make them responsive to other methods of targeting, e.g. electrostatic or magnetic. A typical in vitro targeting assay involves incubation of the particles with a monolayer or suspension of cells, followed by flow cytometry to quantify adherence [15,16]. If successful, this is usually followed directly by testing in vivo, usually in a murine model [17–19]. Few in vitro studies examine the influence of flow upon the targeting efficiency of solid or liquid particles, and those that do, use low shear rates [20]. There have been some recent studies of targeted microbubbles in flow phantoms [21,22] at more physiologically relevant shear rates, and these have demonstrated the importance of this quantity, but it is still neglected in the majority of studies.

Similarly, the characteristics of blood when compared with water or saline are rarely accounted for in in vitro studies. This is despite the fact that it is well known that the rheology of blood and water differ significantly [23]. Blood is a particulate suspension, containing approximately 40% volume of particles mainly consisting of deformable red blood cells (erythrocytes). Macroscopically this makes blood highly non-Newtonian, and in small vessels (<0.5 mm) its apparent viscosity reduces with increasing shear rate. This Fahraeus–Lindqvist effect is due to the fact that at high shear rates the disc-shaped erythrocytes...
The experimental apparatus consisted of an optically transparent cellulose tube, 200 μm inner diameter, suspended in a water bath at room temperature. The flow velocity and hence wall shear rate in the tube was controlled using a high precision syringe pump (see the electronic supplementary material, SI). Phospholipid-coated gas microbubbles were selected as a model drug delivery vehicle since they can be readily visualized in vivo. For this part of the experiment, the tube was coated with avidin by first immersing it in a 7.68 \( \times 10^{-8} \) mol ml \(^{-1} \) solution of DSPC, DSPE-PEG(2000) and biotinylated by the incorporation of DSPE-PEG(2000)–biotin. Magnetic microbubbles were prepared in the same way, but using DSPC with the addition of a hydrocarbon suspension of 15 nm spherical magnetite nanoparticles. Targeting was achieved by positioning a N45 NdFeB permanent magnet 1 mm from the tube in the water bath producing a field strength of 0.375 T and gradient of 0.43 pN mm \(^{-1} \) at the tube wall.

Microbubbles were suspended in either PBS or heparinized whole porcine blood and injected into the tube at a flow rate of 10 \( \mu l \) min \(^{-1} \). The size distribution, concentration and volume of the microbubble suspensions were kept constant throughout the study as indicated in figures 1 and 2. The flow of microbubbles was observed using a water immersion 40× objective lens positioned above the central section of the tube and recorded at 30 fps. The number of microbubbles retained in each case over a period of 4 min was determined through image analysis (see the electronic supplementary material, SI). The experiment was repeated three times for each set of conditions.

2. Results and discussion

The experimental apparatus consisted of an optically transparent cellulose tube, 200 μm inner diameter, suspended in a water bath at room temperature. The flow velocity and hence wall shear rate in the tube was controlled using a high precision syringe pump (see the electronic supplementary material, SI). Phospholipid-coated gas microbubbles were selected as a model drug delivery vehicle since they can be readily visualized in vivo. For this part of the experiment, the tube was coated with avidin by first immersing it in a 7.68 \( \times 10^{-8} \) mol ml \(^{-1} \) solution of DSPC, DSPE-PEG(2000) and biotinylated by the incorporation of DSPE-PEG(2000)–biotin. Magnetic microbubbles were prepared in the same way, but using DSPC with the addition of a hydrocarbon suspension of 15 nm spherical magnetite nanoparticles. Targeting was achieved by positioning a N45 NdFeB permanent magnet 1 mm from the tube in the water bath producing a field strength of 0.375 T and gradient of 0.43 pN mm \(^{-1} \) at the tube wall.

Figure 1. Characterization and analysis of biotinylated microbubble targeting: (a) microbubble size distribution, inset shows optical image of biotinylated microbubbles (scale bar 40 μm), (b) biotinylated microbubbles targeted (i.e. retained against flow) to the upper surface of a 200 μm diameter avidin-coated cellulose tube in PBS after approximately 2 min, (c) number of microbubbles targeted in blood and PBS to the upper surface of avidin-coated and -uncoated tubes at the same flow rate and (d) biotinylated microbubbles targeted to the upper surface of an avidin-coated tube in blood after approximately 2 min. Error bars in (c) indicate 1 s.d. No microbubbles were retained against flow in the uncoated tubes in the presence of blood. (Online version in colour.)
Figure 2. Characterization of magnetic microbubble targeting: (a) microbubble size distribution, inset shows optical image of magnetic microbubbles (scale bar 40 μm), (b) magnetic microbubbles targeted (i.e. retained against flow) at the surface of a 200 μm diameter cellulose tube in PBS after approximately 2 min, (c) number of magnetic microbubbles retained in blood (cf. electronic supplementary material) and PBS at the same flow rate and (d) image of magnetic microbubble targeting in a 200 μm tube in blood after approximately 2 min. Error bars in (c) indicate 1 s.d. Double-headed arrows in (b,d) indicate the maximum width of the microbubble cloud formed (the cloud is too dense for individual microbubbles to be discerned). The magnet was positioned at the side of the tube corresponding to the lower portion of the image. (Online version in colour.)

Figure 1 shows the results obtained with the biotinylated microbubbles at a flow rate of 10 μl min⁻¹, corresponding to a shear rate of 212 s⁻¹. In PBS, a small number of microbubbles were seen to adhere to the top of the tube when it was not coated with avidin (figure 1c). This effect has been observed in previous studies [31] and attributed to the irregular surface of the cellulose wall. When the experiment was repeated in blood, however, no microbubbles were trapped on the tube wall (figure 1b). When the tube was coated with avidin, a much greater number of microbubbles (<200) was retained at the tube wall in PBS, as would be expected (figure 1b). Crucially though this number was substantially reduced, by approximately 90%, in the presence of blood (figure 1d) with fewer than 10 microbubbles being retained over 2 min (figure 1c).

A similar effect was observed with the magnetic microbubbles (figure 2). Over the same period and at the same flow and shear rate, microbubbles were seen to accumulate at the wall of the tube at the location of the magnet (figure 2b). A much larger number of microbubbles were retained than in the case of biotinylated microbubbles, but again a large reduction was observed upon substituting the suspending liquid for blood (approx. 70%) (figure 2c,d). For both types of microbubble, the standard deviation in the number of microbubbles retained was also greater in blood. The fact that these two different methods of targeting suffered substantial reductions in efficiency in the presence of blood demonstrates the importance of using appropriate models when developing targeting strategies. It also supports the hypothesis that this factor is responsible for the drop in targeting efficiency reported between in vitro and in vivo studies.

The data shown in figures 1 and 2 correspond in terms of flow rates and vessel diameters to arterioles/venules [23] and while the focus of this study was the effect of substituting blood for saline on targeting efficiency, the influence of vessel diameter and flow rate upon targeting efficiency are also extremely important. Additional studies were therefore conducted to examine targeting in blood and saline under other flow conditions (tube diameters of 1.6 and 3 mm and flow rates from 0.2 to 0.75 ml s⁻¹). For this part of the work, only magnetic targeting was investigated since the larger quantities of microbubbles retained enabled more reliable measurements. The principle of the experiment remained the same, but for investigating larger vessels it was also more practical to use ultrasound to image the flow and microbubble retention. Details of the experiments are included in the electronic supplementary material and the results are summarized in table 1. As may be seen, a similar effect was observed, with a significant decline in the number of microbubbles retained (as indicated by the change in ultrasound image intensity) upon substituting blood for saline.

As mentioned above, Munn et al. [24] reported that the binding frequency of leucocytes increased with increasing haematocrit (i.e. volume fraction of erythrocytes) and attributed this to the increasing probability of cell collisions that could drive leucocytes towards the vessel wall. It seems likely that collision with erythrocytes is an important factor in this study also, but in this case it appears to have had a negative impact upon targeting under all flow conditions investigated. Potential mechanisms are easy to identify: the presence of cells in the suspension will both impede microbubble motion, reducing the number of microbubbles that can accumulate in a given time period; and, in the case of biotinylated microbubbles, potentially shield target sites, preventing adhesion. Collisions could also potentially detach adherent microbubbles. Further evidence that the particulate nature of blood is likely to have been responsible for the reduction in targeting is shown in
The discrepancy with the results obtained with leucocytes is nevertheless somewhat surprising. The shear rate in the 200 \( \mu \text{m} \) tubes was above that at which a plasma-rich layer at the wall of the tube would be expected to form (more than 100 \( \text{s}^{-1} \) [23]) and the buoyant nature of microbubbles could be expected to assist with marginalization to the wall. The average size of the microbubbles in this experiment was smaller than that of leucocytes [23] (1–2 \( \mu \text{m} \) versus 8–12 \( \mu \text{m} \)) and recent studies on polymer nanoparticles have indicated that nanoscale targeting is inefficient in flowing blood relative to microscale targeting [26,27]. The relationship between particle size and probability of marginalization, however, is not clear and there are several other size-related factors to consider that will also affect targeting ability. For example, at equal concentration, larger microparticles will experience greater drag forces and have a greater probability of collision with erythrocytes; on the other hand, they will also be able to carry a larger number of targeting sites and/or functional nanoparticles. The relative importance of these factors remains an open question.

It was not possible to quantify targeting in smaller tubes in this study, but the impact of erythrocyte interaction is likely to be more significant in even narrower vessels where the probability of collision will be higher, although set against this is the fact that the flow rate will be lower. In the narrowest capillaries, in which deformation of erythrocytes themselves is observed due to confinement by the vessel walls [23], permanent adhesion of microscale particles would be extremely unlikely. No aggregation of either microbubbles or erythrocytes was observed in the experiments, but this is another factor to consider (particularly since the blood in this study was heparinized) which has been shown to be important with certain types of nanoparticle or adenovirus [31]. Additional factors that were not addressed in this study but may also influence targeting efficiency include the pulsatile nature of blood flow, although this is less pronounced in smaller vessels [23]; the viscoelastic properties of the vessel wall and the influence of temperature on the physical properties of the liquids. These would not be expected to have a significant impact on the collision and shielding mechanisms proposed above, however.

### 3. Conclusion

In conclusion, targeting drugs to specific sites within the body has enormous potential for increasing the efficacy of a treatment while minimizing harmful side effects. The results of this study indicate that the current methods used to assess different strategies for targeted drug delivery may fail to mimic the most important features of the in vivo environment.

In particular, failure to account for the characteristics of blood may explain the significant reduction in the efficiency of targeted drug delivery between in vitro and in vivo testing and consequently the limited translation of these techniques into clinical use. The results obtained here suggest that it is the particulate nature of blood that is responsible for the observed effects, most probably due to collisions between particles and red blood cells impeding translation. Other contributory factors cannot be conclusively ruled out however. This finding has important implications for the design of targeted drug delivery systems and the methods used for testing during their development.

**Data accessibility.** All data and methods are reported within this paper and the electronic supplementary material.

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