

# Ultrasound and Microbubbles Promote the Retention of Fluorescent Compounds in the Small Intestine

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**Abstract**—Focused ultrasound (US) is a novel means to increase the passage of medication through the wall of the small intestine. The purpose of this study was to determine whether US and microbubbles (MBs) can facilitate delivery of macromolecular therapeutic agents across the intestinal epithelium *in vitro* and *in vivo*. *In vitro* experiments involved delivery of compounds across a cell monolayer, namely Caco-2 cells cultured on ThinCert filters. The cells were cultured for a minimum of 3 weeks to mimic the polarised intestinal epithelium. A suspension of dextran with or without MBs, prepared in growth medium, was introduced into the apical chamber of the ThinCert with a syringe pump through a channel in the centre of a miniature focused US transducer (4 MHz, 1 MPa PNP). Each *in vivo* experiment involved a tethered endoscopic capsule with an US transducer and a delivery channel inserted into the small intestine of a terminally anaesthetised pig via a surgical stoma. The amount of fluorescent dextran delivered across the Caco-2 monolayer when employing US, MBs and dextran was higher than the amount delivered with dextran alone. With this approach, fluorescent marking of the wall of the small intestine was achieved *in vivo* by applying US and MBs. Our work indicates that US has potential for application in targeted treatment of gastrointestinal disease and oral drug delivery.

**Keywords**—therapeutic ultrasound; targeted drug delivery; microbubbles; capsules; pre-clinical; porcine; animal models;

## I. INTRODUCTION

The intestinal mucosa acts as a selective barrier to permeation of material. Molecules < 500 Da can usually pass the barrier provided they fulfil other physicochemical requirements, but macromolecular biotherapeutics cannot cross it. This limits the delivery of biologics to injections, which can be associated with administration-related injuries and often require administration by a healthcare professional. However, the oral route is the drug administration route best accepted by the patient. Focused ultrasound (US) is under active research as a means for enhancing tissue permeability and intestinal absorption of macromolecules [1]. An ingestible capsule, termed a smart capsule, incorporating an US transducer has the potential to offer a method for oral delivery of drugs to the small intestine. An US capsule could both protect the drug payload against the destructive action of low stomach pH and facilitate delivery of a drug once it reaches the intestine.

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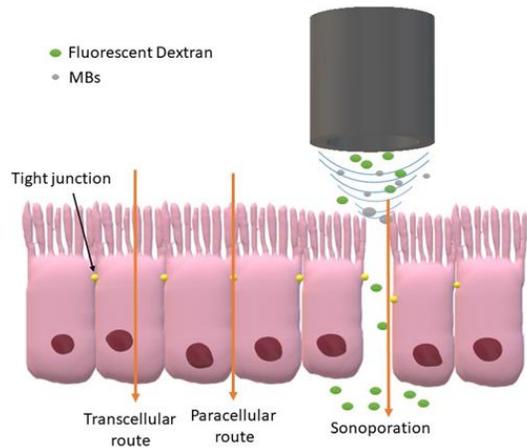


Fig. 1 Enterocytes lining the lumen of the small intestine. Drug molecules are considered to cross this layer by means of one or a combination of the following routes: transcellular, paracellular or through sonoporation.

Preliminary data has previously suggested that microbubbles (MBs) and US can facilitate the passage of large molecules [2]. There are three main mechanisms by which this may be possible: transcellularly i.e. through cells; paracellularly i.e. through intercellular spaces; and/or by sonoporation i.e. through formation of pores in cell membranes [3] as illustrated in Fig. 1. Sonoporation can induce bioeffects, such as spatiotemporal changes of intracellular calcium concentration ( $[Ca^{2+}]$ ), which can affect endothelial cell tight junctions and disrupt cell barrier permeability [4,5].

The overall aim of the experiments reported here was to determine the potential of US and MBs used together as a tool for minimally-invasive delivery of macromolecular therapeutic agents across an epithelial layer and into the wall of the small intestine. The objectives were to determine (1) if delivery of model drugs through a cell monolayer *in vitro* is promoted by US and MBs; and (2) whether US and MBs facilitate delivery of fluorescent compounds to the porcine small intestine *in vivo*.

## II. METHODS

### A. In vitro experiments

To investigate delivery across cell monolayers, FDA approved human epithelial colorectal adenocarcinoma cells (Caco-2 cells, ATCC, Rockville, USA) seeded on ThinCert filters (Greiner Bio-One, Kremunster, Austria) were allowed to fully differentiate and polarise for a minimum of 3 weeks; this enabled the formation of a monolayer with cellular junctions and microvilli similar to small intestine enterocytes [6]. The passage number of cells was in the range 50 – 60. All monolayers reached a transepithelial electrical resistance (TEER) of 800 – 1700  $\Omega$  following differentiation.

On the day of the experiment, cell medium was exchanged for Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich Corporation, MO, USA) and the cells were placed in an incubator at 38°C for 30 min. They were then incubated at room temperature for 1 hour prior to starting the experiments.

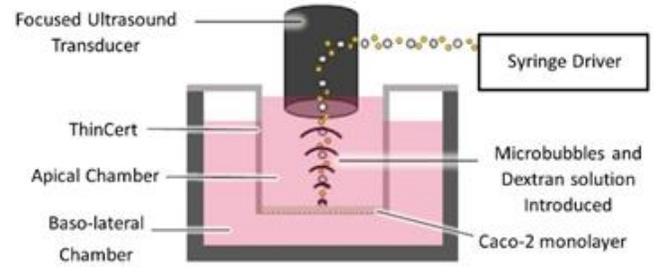


Fig.2. Focused US was applied to a cell monolayer using a bespoke ultrasonic transducer with a central delivery channel. The apical chamber's initial volume was 1.2 ml, whereas the baso-lateral chamber's initial volume was 1.5 ml

A suspension of 0.2 mg/ml of 4 kDa dextran fluorescently-labelled with fluorescein isothiocyanate (FITC, Sigma-Aldrich Corporation, MO, USA) +/- 50% MBs ( $2 - 5 \times 10^8$  MBs/ml, 2 – 8  $\mu$ m average diameter, SonoVue, Bracco, UK) prepared in HBSS was introduced into a multiwell plate divided by the ThinCerts carrying cells into apical and basal chambers (Fig. 2). Dextran and MBs were delivered with a syringe pump (NE-1000, New Era Pump Systems Inc., USA) through a channel in the centre of a miniature focused US transducer (4 MHz, 1 MPa PNP, 13 mm focal length, 1.79 mm<sup>2</sup> cross-sectional focal area). US was delivered for 6 min, with the MB suspension delivered throughout the middle 2 min (minutes 3 and 4) at 0.1 ml/min. Fluid in both the apical and baso-lateral chamber was sampled and the amount of fluorescent dextran was measured with a plate reader.

Statistical analysis was performed using a paired parametric t-test. Statistical significance was defined as  $P < 0.05$ . Calculations were performed in GraphPad Prism 7. The number of replicates (n) is provided in the relevant figure legend.

### B. In vivo experiments

To study delivery *in vivo*, a tethered endoscopic capsule with an US transducer and a delivery channel (Fig. 3), as

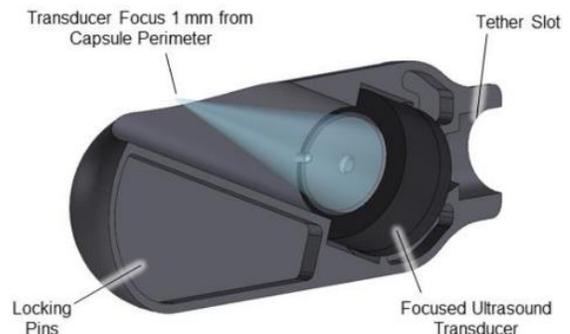


Fig.3. Diagrammatic cross-section of the tethered capsule. The focal point of the transducer was 1 cm away from the wall of the capsule.

### III. RESULTS

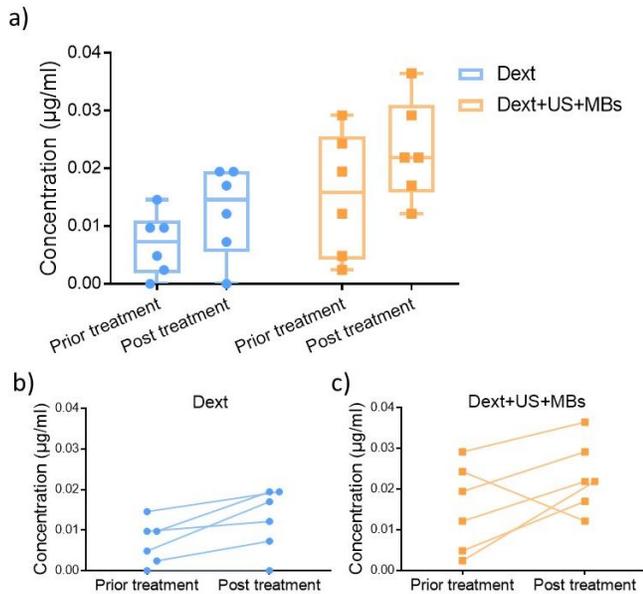


Fig.4. (a) US + MBs facilitates delivery of dextran through a Caco-2 monolayer ( $P=0.067$ ; two-tailed paired parametric t-test). Box-and-whisker plots show the median values (center line), the 25-75th percentiles (box) and the minimum and maximum values (whiskers), for all data points;  $n = 6$ . (b,c) Interleaved bars show the increase in dextran concentration in each sample, suggesting an increase in all but one cases. Dext = Dextran.

previously reported [7], was inserted through a surgical stoma into the small intestines of four terminally anaesthetised Landrace X female pigs. The pigs were 3 - 6 months old and their weights were in the range 40 – 50 kg. Each pig was fasted for 12 hours prior to stoma creation and its small intestine was cleaned by flushing saline through the stoma. Pig models were used because of the similarity of their GI tract to the human GI tract in terms of physiology and histological structure [8]. This study was conducted under the Home Office (UK) Procedure Project Licence PF5151DAF in accordance with the Animal (Scientific Procedures) Act 1986.

5% CdSeS/ZnS quantum dots (QDs, 6 nm average diameter, Sigma, UK) were delivered under the protocol described above. QD retention in excised tissue was verified with UV imaging at 365 nm.

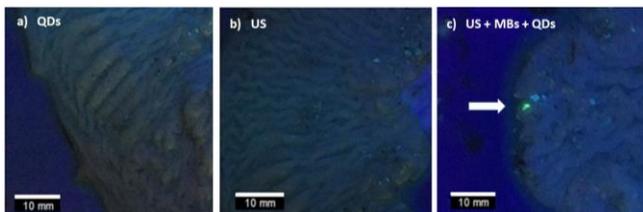


Fig.5. (a) Fluorescent QDs did not lodge into tissue (a) when they were released in the absence or (b) presence of US alone, but (c) they were retained when delivered combined with insonation and MBs.

#### A. Effect of insonation on dextran permeability in vitro

Although the fluorescence intensity values were low, insonation and MBs promoted delivery of fluorescent dextran across a Caco-2 monolayer in 5 out of 6 cases and did so more significantly than when dextran alone was applied to the apical chamber (0.007 and 0.005 µg/ml respectively), Fig. 4). Although this trend is encouraging, the average difference between the two situations was small and not significant (paired parametric t-test comparing the effect of application of dextran alone to dextran with US and MBs ( $t=2.2$ ,  $df=10$ ,  $P=0.067$ ), Fig. 4 (a)) and more experiments are required to validate this finding. In addition, initial dextran concentration values were lower in samples containing dextran alone (Fig. 4), than in those receiving dextran, US and MBs. This variation further illustrates the need for additional experiments.

#### B. Effect of insonation on QDs permeability in vivo

Seven separate sample were obtained from four different pigs. Two of these samples were obtained from a pig where, upon removal of the capsule, debris was found lodged in the delivery channel of the transducer. This created a physical blockage, blocking any transport from the capsule. Hence, the corresponding two sample were excluded from the analysis. Results showed that US alone and QDs alone did not mark the lumen of the small intestine with fluorescence. However, a combination of US, MBs and QDs facilitated visible retention of QDs in the intestinal wall, Fig. 5. US with MBs marked the small intestine in 4 out of 5 samples, corresponding to 80% of cases. In the unsuccessful sample no QD marking was observed.

### IV. CONCLUSIONS AND FUTURE WORK

This paper reports preliminary results for use of US as a method for targeted drug delivery in the small intestine. We found that US successfully enhanced the delivery of fluorescent model drugs with different molecular weights *in vitro* and *in vivo*. The results suggest that focused US and MBs could enhance epithelial translocation of macromolecules. We also showed that a capsule, of a size that allows ingestion, with a focused US transducer can mark tissue fluorescently *in vivo*. [9] used a similar setup and reported US and MBs enhance QDs marking of the intestinal wall *ex vivo*. Our work indicates potential applications in targeted treatment of gastrointestinal disease and oral drug delivery.

Future work will examine the range of molecular weights and quantity of fluorescent agents that US can successfully facilitate to cross the intestinal epithelium and where the agents reside. Furthermore, improvements in US transducer miniaturisation will allow the encapsulation of plane transducers with larger active areas, thus exposing a larger part of the lumen to US, with the potential to enhance drug delivery.

## ACKNOWLEDGMENTS

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