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Acoustic Cluster Therapy (ACT) – A novel concept for ultrasound mediated, targeted drug delivery

Per Sontum[a,*], Svein Kvåle[a], Andrew John Healey[a], Roald Skurtveit[b], Rira Watanabe[c], Manabu Matsumura[c], Jonny Østensen[d]

[a] Phoenix Solutions AS, PO Box 4741, N-0421 Oslo, Norway
[b] Institute for Energy Technology, Instituttveien 18, 2007 Kjeller, Norway
[c] Daiichi Sankyo Co., 3-5-1, Nihonbashi Honcho, Chuo-ku, Tokyo, 103-8426 Japan
[d] Inven2 AS, PO Box 1061 Blindern, N-0316 Oslo, Norway

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ABSTRACT

A novel approach for ultrasound (US) mediated drug delivery – Acoustic Cluster Therapy (ACT) – is proposed, and basic characteristics of the ACT formulation are elucidated. The concept comprises administration of free flowing clusters of negatively charged microbubbles and positively charged microdroplets. The clusters are activated within the target pathology by diagnostic US, undergo an ensuing liquid-to-gas phase shift and transiently deposit 20–30 μm large bubbles in the microvasculature, occluding blood flow for ~5–10 min. Further application of US will induce biomechanical effects that increases the vascular permeability, leading to a locally enhanced extravasation of components from the vascular compartment (e.g. released or co-administered drugs). Methodologies are detailed for determination of vital in-vitro characteristics of the ACT compound; cluster concentration and size distribution. It is shown how these attributes can be engineered through various formulation parameters, and their significance as predictors of biological behaviour, such as deposit characteristics, is demonstrated by US imaging in a dog model. Furthermore, in-vivo properties of the activated ACT bubbles are studied by intravital microscopy in a rat model, confirming the postulated behaviour of the concept.

1. Introduction

A prerequisite for successful therapy with a medicinal drug is that the active substance reaches its target pathology and that toxicity to healthy tissue and non-targeted organs is limited. This fundamental necessity limits the effective use of a large number of drug molecules with low therapeutic index, and has led to a massive hunt for concepts that enable localised release or enhanced uptake of active ingredients within the target pathology.

A wide variety of approaches for localised drug delivery or enhanced uptake have been explored over the last couple of decades (Devranjan and Jain, 2015), but transition to drug products and clinical practice has been very limited. In recent years, several concepts for ultrasound (US) mediated drug delivery have been investigated, some with quite encouraging results (Unga and Hashida, 2014; Tsusui et al., 2004; Heath Martin and Dayton, 2013). Most of the these concepts explore the use of regular US contrast microbubbles such as Sonovue™ or Optison™, either loaded with or co-injected with various active ingredients. Insonation of the target pathology containing microbubbles in vascular compartments leads to a variety of biomechanical effects that enhance extravasation and distribution of drug molecules to target tissue (Lentacker et al., 2014; Kooiman et al., 2014). Co-injection of Gemcitabine and Sonovue, with localised US insonation for a hypothesised enhanced drug uptake and therapeutic effect during treatment of pancreatic cancer (PDAC) is currently being explored in clinical trials (Kotopoulis et al., 2013). A similar approach is being investigated for treatment of Glioblastoma in humans (Carpentier et al., 2014). Whereas various drug delivery approaches exploring the use of contrast microbubbles have shown quite some promise, their effectiveness is hampered by several issues. Microbubbles have a very limited loading capacity and, being small and free flowing, they display limited contact with the

* Corresponding author.
E-mail addresses: per.sontum@phoenixsolutions.no, psontum@gmail.com (P. Sontum), svein.kvale@phoenixsolutions.no (S. Kvåle), andrew.healey@phoenixsolutions.no (A.J. Healey), roald.skurtveit@ife.no (R. Skurtveit), watanabe.rira.vi@daiichisankyo.co.jp (R. Watanabe), matsu4jf@gmail.com (M. Matsumura), jonny.ostensen@inven2.com (J. Østensen).

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endothelial wall, reducing the level and range of the biomechanical effects. In addition, these approaches typically utilize mechanical indexes (MI) that induce inertial cavitation, with ensuing potential safety aspects.

A related technology, acoustic droplet vaporisation (ADV), generate microbubbles in situ from administered nano sized (<200 nm) oil droplets (Sheeran and Dayton, 2014; Lin and Pitt, 2013). This concept relies on the enhanced permeability and retention (EPR) effect, which extravasate the administered nano-droplets to tissue compartments before US mediated vaporisation and generation of microbubbles. However, ADV concepts also suffer from several issues limiting their effectiveness and transition to clinical use. Most importantly, these nano-droplets require very high US intensities in order to phase shift; a Mechanical Index (MI) > 2 is typically required, and their stability after the phase shift event is very limited due to rapid re-condensation. In addition, in many cases the EPR effect level is limited and not capable of delivering an adequate amount of droplets to the targeted tissue.

As noted, even though huge resources have been spent over the last two decades in finding functional concepts, the current result of all these efforts is a very limited transition to clinical practice. In truth, the idea of targeted drug delivery currently remains essentially unresolved in medical practice.

The authors propose a new concept enabling targeted delivery and/or localized enhanced uptake; Acoustic Cluster Therapy (ACT) (Sontum et al., 2014). In brief, the concept comprises mixing negatively charged microbubbles with positively charged micro-droplets, with the ensuing formation of microbubble/microdroplet clusters from the electrostatic attraction between the two components. Critical aspects of the microdroplet oil component is that it display a relatively low boiling point (e.g. <50 °C), with an ensuing high vapour pressure at body temperature, and that the solubility in aqueous matrices (e.g. blood) is low (e.g. $<1 \times 10^{-4}$ M). Suitable compounds hence included partially or fully fluorated or halogenated, low boiling point oils. The ACT™ Cluster Dispersion for injection may be co-administered with a regular medicament (e.g. chemotherapeutic) to induce locally enhanced uptake of the systemically injected drug. Alternatively, the microdroplet may be loaded with a significant amount of a lipophilic, medicinally active ingredient, for local release in the targeted pathology. The clusters are small enough to be free flowing in the microvasculature after *i.v.* injection. When insonated with US (activated), the microbubble transfers energy to the microdroplet and acts as a vaporisation 'seed' initiating vaporisation of the oil droplet. Hence, the cluster construct enables the vaporisation process to occur at much lower acoustic powers than in the absence of a microbubble. The compound may be activated within the targeted pathology by regular, low MI medical US insonation (e.g. 1–10 MHz, MI < 0.4). Upon US activation, the oil component of the microdroplets instantly vaporise and subsequent inwards diffusion of blood gases produce a 20–30 μm large bubble that transiently deposit in the local microvasculature, stopping the blood flow for some minutes. Upon activation, the drug loaded into the microdroplets is released to the local blood compartment or expressed at the surface of the large, activated bubble. From the transient occlusion of the vessel, immediate wash out is avoided and the drug is kept locally at high concentration for a significant period of time. Post activation, low frequency (e.g. 0.3–1 MHz), low MI US is applied to induce controlled volume oscillations of the large, deposited bubbles and ensuing biomechanical effects to allow for extravasation of drug molecules to the targeted tissue. The concept is visualized in Fig. 1.

The proposed concept differs from microbubble and ADV approaches at several levels. Most importantly:

- The liquid-to-vapour phase transition of the microdroplet component is effectively enabled by low MI US (<0.4) at standard medical imaging frequencies.
- Post activation, the concept comprises a large bubble that transiently deposit in the microvasculature, occluding blood flow for 5–10 min and preventing a rapid wash out of drug.
- The activated bubble is in direct contact with the endothelial wall over a substantial segment length.
- Post activation, applying low frequency and low MI US insonation the absolute volume displacement from the activated

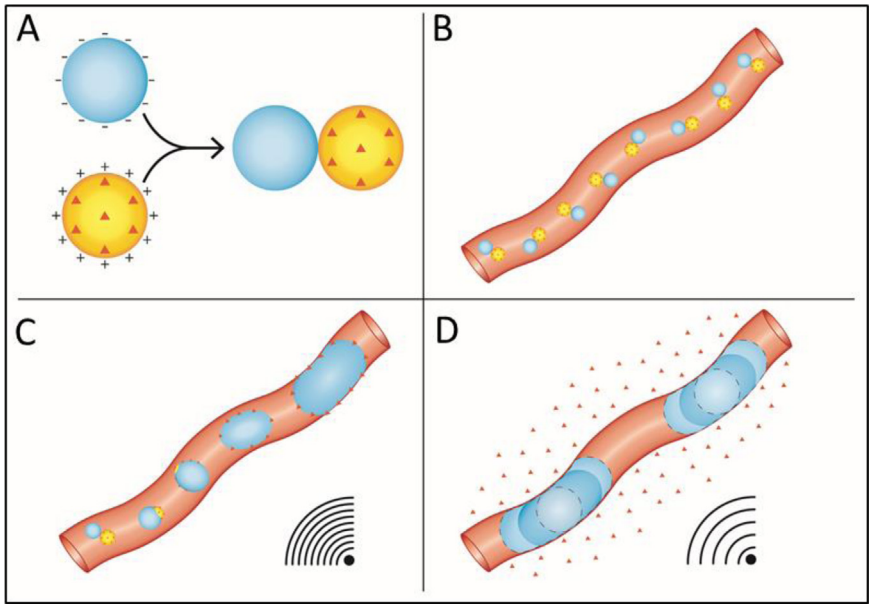

Fig. 1. Acoustic Cluster Therapy (ACT) for ultrasound (US) mediated drug delivery. Frame A: negatively charged microbubbles and positively charged microdroplets, optionally loaded with a therapeutic agent (▲), are mixed to form microbubble/microdroplet clusters. Frame B: After i.v. injection, the clusters are free flowing in the microvasculature. Frame C: local insonation of target pathology with low MI, regular medical imaging US activates the clusters, inducing a liquid-to-gas phase shift and the formation of 20–30 μm bubbles, trapped in the local capillary bed, transiently occluding blood flow. Frame D: application of low frequency, low MI US induce volume oscillations for generation of biomechanical effects that enhance extravastion and distribution of drug in target tissue.

bubble oscillations will be almost three orders of magnitude larger than with regular ultrasound contrast microbubbles.
- The ACT concept offers the possibility of loading the entire volume of a micron sized oil droplet with an active pharmaceutical ingredient.

In summary, the unique attributes of the ACT concepts offer a series of advantages compared to other approaches for US mediated drug delivery, which may lead to a significant improvement in clinical utility. The objectives of the current paper are:

- To describe important physicochemical characteristics of the ACT compound and how the formulation may be engineered and optimized.
- To show the relevance of the measured characteristics as predictors of biological behaviour and to demonstrate some of the postulated in-vivo attributes.

The current paper also provides details of formulation and characteristics of compounds investigated in Healey et al. (2015) and Van Wamel et al. (2015); parallel papers with further elucidation of in-vitro/in-vivo characteristics using a custom made sonometry apparatus (Healey et al., 2015) and results from preclinical pilots studying the potential of the ACT concept for drug delivery (Van Wamel et al., 2015).

2. Materials and methods

2.1. Materials

2.1.1. Non-loaded ACT formulations

For evaluation of the general characteristics of the ACT concept, several US imaging studies were performed using non-loaded ACT formulations; i.e. compounds without a molecular (drug) load dissolved into the microdroplet oil component.

The ACT compound is prepared by mixing a composition of negatively charged microbubbles (C1) with a composition of positively charged microdroplets (C2). The microbubble composition explored in the current paper consisted of Sonazoid™, an US contrast agent manufactured by GE Healthcare (Sontum, 2008). Sonazoid comprises perfluorobutane (PFB) microbubbles stabilised with a hydrogenated egg phosphateidylserine–sodium (HEPS–Na) phospholipid membrane, embedded in a lyophilized sucrose matrix. After reconstitution, Sonazoid contains approx. 8 μl or 1100 million microbubbles/ml with a volume weighted mean diameter of approx. 2.6 μm. The microbubbles in Sonazoid display a negatively charged surface, with a zeta potential of approx. −70 mV. The microdroplet composition explored in the current paper consisted of perfluoromethylcyclopentane (PFMCP) (F2Chemicals Ltd., UK) microdroplets stabilised with a distearoylphosphateidylcholine (DSPC) (Lipoid AG, Switzerland) phospholipid membrane added various amounts of stearylamine (SA) (Tokyo Chemical Institute Co., Ltd., Japan): a positively charged surfactant. The emulsion component was manufactured in-house by Phoenix Solutions AS. In brief, microdroplets were formed by high-speed rotor/stator homogenisation of PFMCP with an aqueous dispersion of DSPC/SA as described in (Kvåle, 1998). The resulting raw emulsion was then fractionated with procedures similar to those described in (Kvåle et al., 1996) to produce well-defined populations of microdroplets, and then filled to 5 ml glass vials. The ACT compound was prepared by reconstituting a vial of Sonazoid™ (C1) with 2 ml of the C2 emulsion component.

The formation of microbubble/microdroplet clusters upon mixing the two components is strongly influenced by a series of formulation attributes notably: the level of electrostatic attraction between the microbubbles and microdroplets, governed by the difference in their surface charge, i.e. a function of the amount of SA in the microdroplet membrane; the ratio between microbubbles and microdroplets; the composition of the aqueous matrix (e.g. ionic or buffer strength, pH); and the size of the microdroplets. The concentration and size of clusters in the ACT compound can hence be engineered through controlled variation of these attributes. Samples investigated within the current paper contained microdroplets with volume weighted mean diameters from approx. 2 to 4 μm, diluted in TRIS (2-amino-2-hydroxymethyl-propane-1,3-diol) (Merck KGaA, Germany) buffer in the range of 1–10 mM. Each milliliter of the C2 emulsion contained 300–1100 million or 2.5–5.5 μl microdroplets. The content of positive surfactant (SA) in the stabilising membrane was varied between 1.5 and 5.5% mol SA/mol DSPC and the pH in the final ACT compound ranged from 6.3 to 6.7.

2.1.2. Loaded ACT formulations

In order to study the ACT concept in a loaded version; i.e. where a molecular load is dissolved into the microdroplets, as PFMCP is a very poor solvent for most all solutes, the composition of the oil component needed to be optimized in order to facilitate a sufficient loading capacity. A range of solvent mixtures were screened for (1) their ability to dissolve optical imaging marker molecules: DiR (1,1′-Dioctadecyl-3,3,3′,3′Tetramethylindotricarbocyanine Iodide, Thermo Fisher Scientific Inc.) and: Nile Red (9-diethylamino-5-benzo[α]-phenoxazinone, Sigma–Aldrich Co.) while (2) keeping the physical properties of the ACT concept i.e. the ability to form stable clusters which can be activated in a similar manner as when using pure PFMCP. A suitable combination was found in a 1:1:1 by volume mix of PFMCP, trichlorofluoropropane (Matrix Scientific, USA) and trichloromethane (Merck KGaA, Germany). In this solvent mixture the solubility of DiR was >10 mg/ml (formulation used in Van Wamel et al. (2015)) and the solubility of Nile Red was >5 mg/ml. ACT compounds manufactured with this formulation also retained their basic physicochemical attributes with regards to clustering and US activation.

2.2. Methods

2.2.1. In-vitro characterisation

2.2.1.1. Coulter counting. Coulter analysis of the microbubble/microdroplet components and the ACT compound was performed using a Coulter Multisizer III (Beckman Coulter Inc.) instrument set up with a 50 μm aperture and a measuring range of 1–30 μm. A suitable aliquot of the sample was dispersed and homogenized in Isoton® II electrolyte (Beckman Coulter Inc.) prior to analysis. Primary responses were number and volume concentration and number and volume size distributions.

2.2.1.2. Flow particle image analysis. Flow particle image analysis (FPIA) is a fully automated microscopy and image analysis technique. In brief, a small aliquot of the analyte (i.e. microbubbles, microdroplets or the combined ACT compound) is diluted/dispersed in a particle free aqueous diluent (water for injection (WFI) or phosphate buffered saline (PBS)) and homogenized by continuous stirring. A known portion of the diluted sample is then drawn through a measuring cell in the instrument where a fixed set of micrographs are taken by a CCD camera with a stroboscopic light source. The particles in each frame are automatically isolated and analyzed by the image analysis software, and a variety of morphological parameters are calculated for each particle, as is the total particle concentration. In addition, the instrument provides a representative selection of micrographs for different size classes; < 5 μm, 5–10 μm, 10–20 μm and 20–40 μm together with concentration observed in

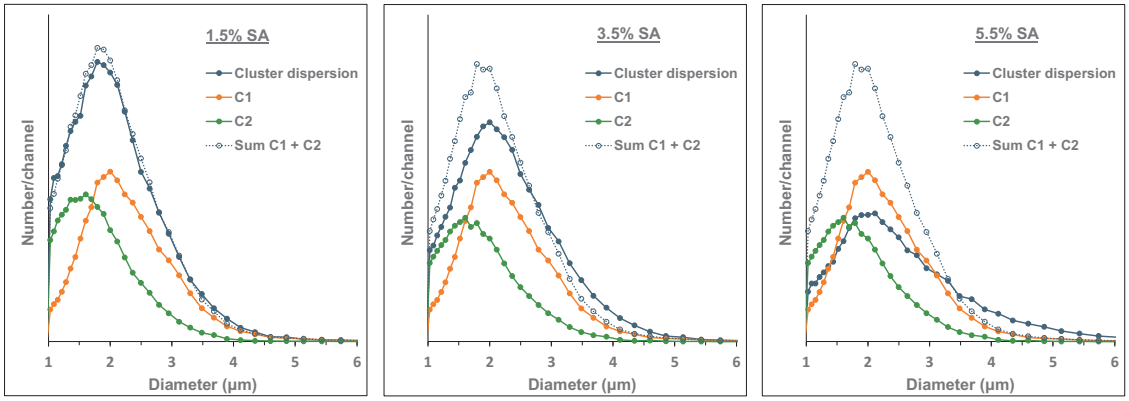

Fig. 2. Results from Coulter counter analyses on the Sonazoid™ microbubble component (C1), the microdroplet component (C2), sum of the individual components assuming no interactions (C1 + C2) and the ACT compound after mixing (ACT). Comparison of three levels of electrostatic attraction between the microbubbles and the microdroplets from variable amounts of stearylamine (SA) in the microdroplet membrane; Low 1.5% SA (left), Medium 3% SA (middle) and High 5% SA (right).

each class. For the reported analyses a Sysmex 2100 FPIA instrument (Malvern Instruments Ltd.) set up with a High Power Field (20×) and measuring range 0.7–40 μm was utilized. A suitable sample volume was diluted in water (WFI) and homogenized by continues stirring throughout the analysis. The cluster concentration and their circular equivalent diameter were reported as primary parameters for evaluation.

2.2.1.3. Microscopy/image analysis. As an alternative to the FPIA analysis a more manual microscopy technique coupled with an image analysis software was employed. For this purpose, a Malvern Morphology G3 system (Malvern Instruments Ltd.) with a 20× objective and a measuring range of 1.8–100 μm was utilized. In brief, a small aliquot of the analyte (i.e. microbubbles, microdroplets or the ACT compound) was diluted/dispersed in a particle free aqueous diluent (e.g. WFI or PBS) and homogenized. The diluted sample was then introduced into a microscopy channel slide (IBIDI 0.4 μ-slide, IBIDI GmBh), with a known channel height of 400 μm and placed under the microscope. The instrument automatically scans a preset area of the slide and a fixed set of micrographs are taken by a CCD camera. The particles in each frame are automatically isolated and analyzed by the image analysis software, and a variety of morphological parameters are calculated for each particle. The total number of particles is reported and from the known scan area and known channel height, the concentration of particles in the analyte can be calculated. As for the FPIA analysis, cluster concentration and the circular equivalent diameter was reported. Micrographs of all particles detected can be displayed and evaluated by manual, visual inspection. Hence, all clusters can be isolated from e.g. free microbubbles and a full cluster size distribution was constructed for the clusters in each sample.

This analysis was also applied to determine the ability of the clusters to be activated by US insonation, and the characteristics of the ensuing population of large, activated bubbles. After analysis of cluster size and content, the IBIDI slide was immersed in a 37 °C water bath and the sample was insonated for 10 s using an ATL HDI 5000 US scanner (ATL Ultrasound Inc.), with a P3-2 transducer at a nominal MI of 0.5. After activation, the sample was reanalyzed.

2.2.1.4. Sonometry. A custom-made instrumentation and software for characterisation of the ACT compound after US activation in an in-vitro measuring cell has also been utilized. This technique is described in detail by Healey et al. (2015). In brief, a small aliquot of ACT sample is diluted in WFI at 37 °C and with a controlled gas saturation at 85%. The sample is then activated by US insonation for 10 s using an ATL HDI 5000 US scanner, with a P3-2 transducer at a nominal MI of 0.5. Primary response parameters from this Sonometry analysis were activated volume per microdroplet volume and activated bubble size vs. time after activation.

2.2.1.5. Fluorescence microscopy. An ACT composition made with microdroplets loaded with 5 mg/ml Nile Red dye was prepared as detailed above. The sample was diluted in water, placed in a microscopy well and activated using a Vscan US scanner (GE Healthcare).

Images of the activated compound were acquired using a Leica TCS SP8 confocal microscope. The objective used was a HCX IRAPO L 25× water immersion objective with a numerical aperture of 0.95. The fluorescent dye was excited at 539 nm by a tunable white light laser. Emission in the range 570–670 nm was detected by a hybrid detector (HyD). The laser speed used was 400 Hz and pinhole diameter was set to 1 AU. Transmission images were acquired simultaneously and could be overlaid with the fluorescence images. 3D images of the sample were acquired by moving the objective stepwise in the *z*-direction.

2.2.1.6. Zeta potential. The zeta potential of microdroplet formulations with variable amount of SA in the stabilising membrane was measured using a Malvern ZetaSizer 3000HS. A suitable sample volume was diluted in 10 mM NaCl (aq.) and measurements were performed at a pH of 7.

2.2.2. In-vivo procedures

2.2.2.1. Ultrasound imaging of dog myocardium. All animal procedures were approved by The Norwegian Animal Research Authority (NARA). The animal (mongrel or mixed breed dog, 20–25 kg) arrived on the morning of the experiment day. Anesthesia was induced and kept with pentobarbital and fentanyl. A mid-line

Table 1
Results from flow particle image analysis: concentration (millions/ml) of microbubble/microdroplet clusters in various size classes in ACT compound at three levels of inter-particle, electrostatic attraction from variable amounts of stearylamine (SA%) in the microdroplet membrane. Average and SD from three samples.

SA%	Cluster concentration (millions/ml) in size classes			
	<5 μm	5–10 μm	10–20 μm	20–40 μm
1.5	6.5 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.5	112.6 ± 7.6	3.8 ± 0.4	0.1 ± 0.2	0.0 ± 0.0
5.5	84.7 ± 5.3	14.0 ± 1.0	2.3 ± 0.6	0.1 ± 0.1

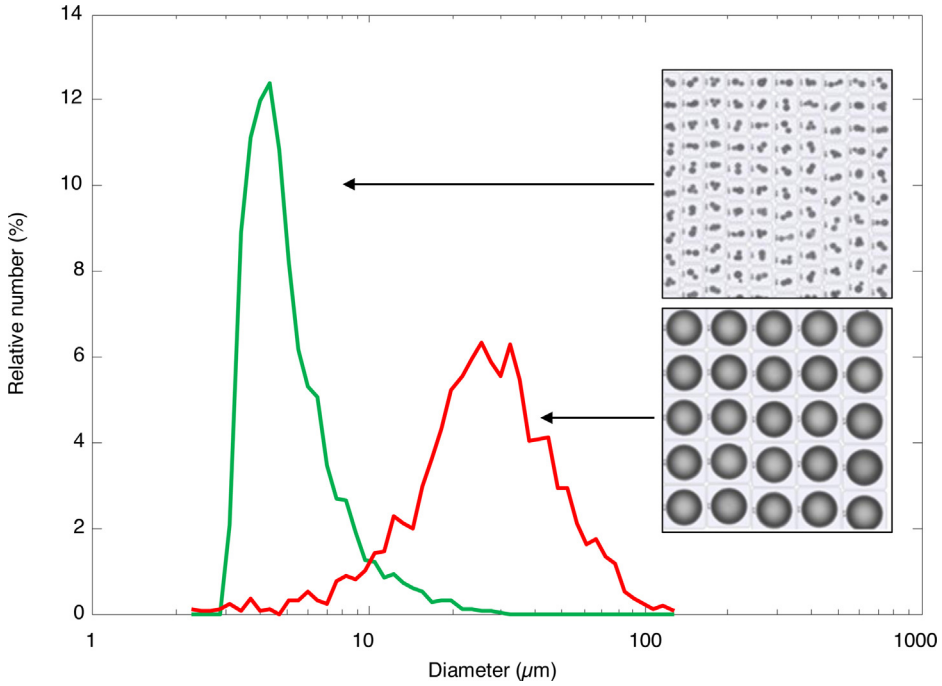

Fig. 3. Results from microscopy/image analysis: microbubble/microdroplet cluster (left hand) in ACT compound for injection and bubbles in activated ACT compound (right hand) size distributions with representative micrographs.

sternotomy was performed, and the anterior pericardium was removed. The heart was suspended in a pericardial cradle to avoid compression of the atria and veins. A 0.8 mm Venflon™ cannula was inserted in the right cephalic vein proximal to the elbow joint for injections of test substances. A midline, mid-papillary short axis view of the heart was imaged by an ATL HDI-5000 scanner. A P3-2 transducer was used, the scanner was operated in conventional fundamental B-mode with two focal zones, at the highest frame rate and maximum output power (MI ≈ 1.0). A 30 mm soft silicone rubber pad was used between the transducer surface and the epicardium. The depth of the image was adjusted to the smallest value that included the whole heart. A dynamic range of 50 dB was used. A pair of digital images from end-diastole and end-systole was stored at each specified point in time. The scanner was left continuously running, except brief periods of cine-loop recalls for storing the images. Prior to each injection, a new vial of Sonazoid™ was reconstituted with 2 ml of microdroplet emulsion (Section 2.1). The desired dose of ACT compound (200 μl) was withdrawn and diluted to 2.5 ml with 50 mg/ml TRIS-buffered mannitol (10 mM, pH 7,4). The dose administered was equivalent to 10 μl/kg b.w, equivalent to 0.025–0.055 μl DSPC/PFMCP microdroplets and 0.08 μl HEPS-Na/PFB microbubbles per kg. b. w. As controls, microbubbles (Sonazoid™) only and microdroplets only were also studied. Injections were made via the right cephalic vein, and the resulting myocardial contrast effect was quantified at 90 s and 3, 5, 7, 9 and 11 min. A baseline reading was performed before each injection. At least 20 min was allowed between injections to reduce potential carry-over effects. Myocardial contrast effect was read from a large region of interest in the anterior myocardium. The contrast effect at 90 s was used as the primary measure of the efficacy for each injection. Contrast intensity values were reported in dB.

2.2.2.2. Intravital microscopy. All animal procedures were approved by the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan. Male Wistar rats, 10–15 weeks of age at time of dosing, were used. General anaesthesia was administered and maintained with i.v. and i.m. pentobarbital sodium. The rats were intubated, and the tail vein or carotid vein was cannulised for administration. The ACT compound was administered at a dose of 1 ml/kg b.w. equivalent to 4 μl/kg b. w. microdroplets and 8 μl/kg b.w. microbubbles. The abdomen was opened by means of a vertical midline incision, the rats were then placed in the lateral position on a plastic plate incorporating a round window of cover glass, and the exteriorized mesenteries were placed on the cover glass window. The spread mesenteries were perfused with Krebs-Ringer buffer at 37 °C. Ultrasound was applied directly onto the exteriorised mesentery under the objective lens of the microscope. A Siemens Elegra ultrasound scanner (Siemens AG) equipped with a linear 7.5L40 transducer was used for ultrasound exposure. Output power was set at maximum corresponding to an MI value of 1.2. Sonar gel was

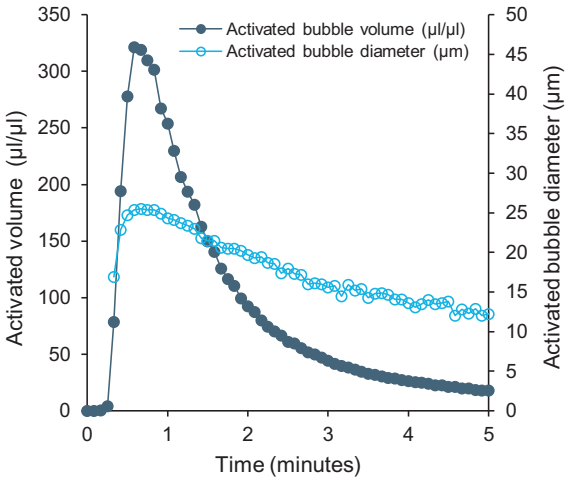

Fig. 4. Results from in-vitro Sonometry analysis: activated bubble volume and diameter as a function of time after US activation in the in-vitro measuring cell (sample activated at 15 s).

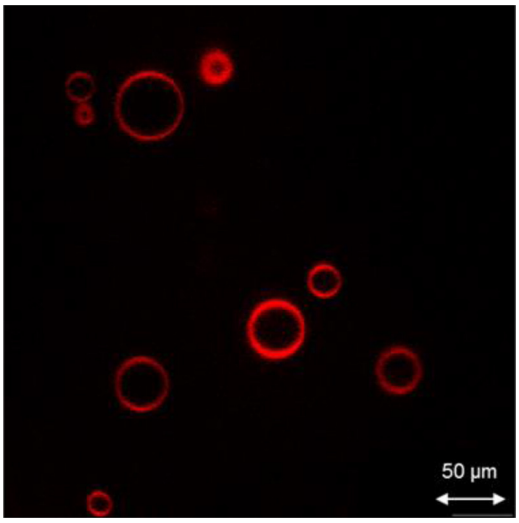

Fig. 5. Results from fluorescence microscopy: typical micrograph (intersections from *z*-stacking) from fluorescence microscopy on activated ACT compound loaded with Nile Red dye.

applied between ultrasound transducer and the mesentery. Images were generated using a Nikon ST-O microscope (Nikon Corp.) with a water immersion, 20× objective and recorded by a DXC-151A (Sony Electronics Inc.) CCD camera.

3. Results and discussion

3.1. ACT in-vitro characteristics

A principal feature of the proposed ACT concept is the formation of microbubble/microdroplet clusters upon combining a microbubble component and a microdroplet emulsion. As will be shown, these clusters are responsible for the formation of large, activated bubbles in-vivo upon US insonation. Analytical methodologies to quantify and characterise clusters in the preparation for administration are hence imperative tools to control and understand the ACT compound and its biological behaviour. Four different analytical tools have been explored for this purpose; Coulter counting, flow particle image analysis (FPIA), microscopy/image analysis and sonometry (Healey et al., 2015).

The number and size of clusters formed upon mixing the negatively charged microbubbles with the positively charged microdroplets is strongly influenced by a variety of formulation parameters (Section 2.1), the level of surface charge in particular. Fig. 2 shows results from Coulter analysis of the individual microbubble and microdroplet components and the resulting ACT compound after mixing, at three levels of electrostatic attraction from varying the content of SA in the microdroplet membrane from 1.5% mol/mol to 3.5% mol/mol to 5.5% mol/mol, keeping all other formulation attributes identical. The zeta potential of the microdroplets in these three formulations was measured to 23 ± 1 mV, 35 ± 3 mV and 43 ± 2 mV, respectively. For comparison, the theoretical sum of the two components, i.e. assuming no interaction between the individual microbubbles in C1 and the microdroplets in C2, is also shown. As two or more microbubbles/microdroplets in a cluster are counted as a single particle by the Coulter instrument, the formation of clusters upon combining the two components will lead to (1) a reduction in the total number of particles in the system and (2) a shift towards larger sizes. As can be noted from Fig. 2, at low electrostatic attraction (1.5% SA) there is no clear evidence of cluster formation – the concentration and size of particles in the ACT compound is comparable to the sum of the individual components. Increasing the electrostatic attraction by incorporating 3.5% SA, the formulation shows a slight, but significant decrease in concentration and an increase in large end tailing, demonstrating the formation of a significant population of microbubble/microdroplet clusters. The 5.5% SA formulation shows even stronger evidence of clustering, with a marked decrease in number concentration and a clear shift towards a stronger large end tailing.

The Coulter analysis does not, per se, discriminate between microbubbles, microdroplets or clusters; all entities are counted and sized as “a particle”. In order to differentiate and characterize the clusters specifically, microscopy techniques are necessary. The results from FPIA analysis (Table 1) on the same samples as shown in Fig. 2 confirm the effect of the level of electrostatic attraction on the formation of clusters. At 1.5% SA only a few clusters smaller than 5 μm are observed, at 3.5% SA a significant concentration of small (<5 μm) and some medium sized (5–10 μm) clusters are observed, and at 5.5% the concentration of small clusters decrease again, due to the formation of a significant amount of medium and large sized (>10 μm) clusters.

Further characterization of the clusters in the ACT compound, and their ability to be activated by ultrasound insonation, was performed by microscopy/image analysis. Fig. 3 shows the size distributions of clusters in a typical ACT compound with 3% SA, together with a selection of representative micrographs, compared to equivalent data after US activation of the same sample. As can be observed, the clusters are contained in a population between some 3 and 10 μm, with a median diameter of approx. 4.5 μm. The clusters predominantly contained two individual particles (one bubble and one droplet); for a typical sample, full image analysis

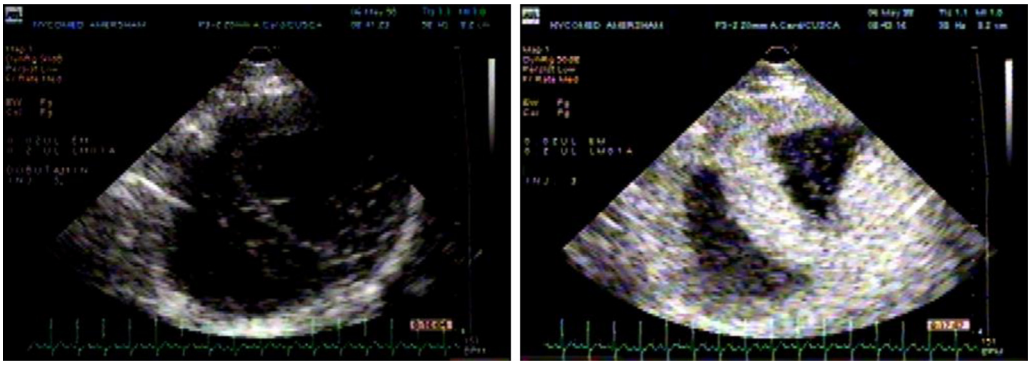

Fig. 6. US imaging of dog heart. Baseline B-mode image (left) and 90 s after administration and US activation of ACT compound.

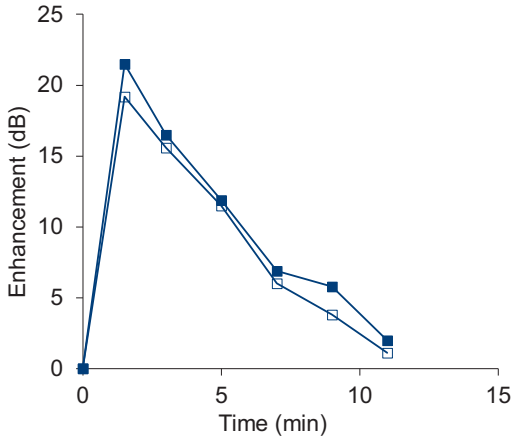

Fig. 7. US imaging: time–intensity curves for myocardial contrast in dog heart after injection of ACT compound and activation in the heart chamber (results from two injections).

showed 51% doublets, 19% triplets, 10% quartets, 7% quintets and 5% sextets. Hence, less than 8% of the clusters generated contained more than six individual particles and less than 5% of the clusters were larger than 10 μm. For a typical ACT™ formulation, the microbubble to microdroplet ratio is approx. 2:1. Due to this excess of microbubbles, most all of the multi-particle clusters contained a single emulsion droplet surrounded by two or more microbubbles. After activation, a population of large bubbles, contained between some 15 and 50 μm and with a median diameter of approx. 30 μm, was observed. No evidence of non-activated clusters, free emulsion droplets or microbubbles could be observed after US insonation.

Further elucidation of the characteristics of the activated ACT system can be achieved by using the Sonometry analysis detailed in Healey et al. (2015). Fig. 4 shows the total gas volume fraction and the number mean diameters of bubbles, as a function of time after activation, for the same sample as in Fig. 3. As can be noted, the gas volume fraction increase instantly upon insonation, reaching a peak after approx. 30 s with more than 300 μl/μl microdroplets. The gas fraction then display a close to exponential decay with a half-life of approx. 50 s in this system. The median diameter of the activated bubbles display similar dynamics, reaching a peak of some 25 μm, before slowly diminishing. Based on the model presented in (Healey et al., 2015) a lower bound for the activation efficacy in this set-up is approx. 91%. i.e. 91% of the oil volume vaporize during US activation.

The fate upon activation, of a hydrophobic molecular loading in the droplet component was investigated by fluorescence microscopy of an ACT sample loaded with 5 mg/ml Nile Red in the emulsion component. A typical micrograph of the activated system is shown in Fig. 5. As can be noted, after US insonation, the Nile Red is located at the gas/liquid interphase of the large activated bubbles.

3.2. ACT in-vivo characteristics

3.2.1. US imaging

Upon activation in-vivo, it is postulated that the activated bubbles will lodge in local microvasculature, immediately downstream of the activation site and that the number of lodged bubbles should be proportional to the perfusion of the tissue. Due to their size, the activated bubbles should show copious backscatter even in a linear imaging mode and the ACT compound should hence work as a "deposit tracer" contrast agent for B-mode US imaging. In order to study the in-vivo characteristics of the ACT concept, myocardial contrast efficacy of a series of ACT compounds with variable cluster characteristics was determined in an open chest dog model. Fig. 6 shows typical US images of baseline and 90 s post administration and activation of the ACT. As can be noted, no contrast is observed in the heart chambers after 90 s, whereas the backscatter signal from the myocardial tissue is strong, demonstrating the presence of trapped ACT bubbles.

Fig. 7 shows typical time–intensity curves for ACT compound. As can be observed, the ACT compound shows copious backscatter even in linear B-mode, peaking at or before 90s before decaying back to base-line after approx. 10 min. Sonazoid™ on the other hand shows insignificant contrast in this imaging mode, as expected (results not shown). The backscatter enhancement from conventional medical ultrasound contrast agent such as Sonazoid is negligible when imaging in linear B-mode, and typically at noise level when imaging the myocardium 90 s after injection. These compounds require specific contrast imaging modes for greater enhancement. Injecting the microdroplet component only also showed no measurable myocardial contrast after 90 s.

Fig. 8 shows the measured myocardial contrast enhancement at 90 s vs. the concentration of clusters between 3 and 10 μm in the administered ACT compounds from FPIA analysis and vs. the peak

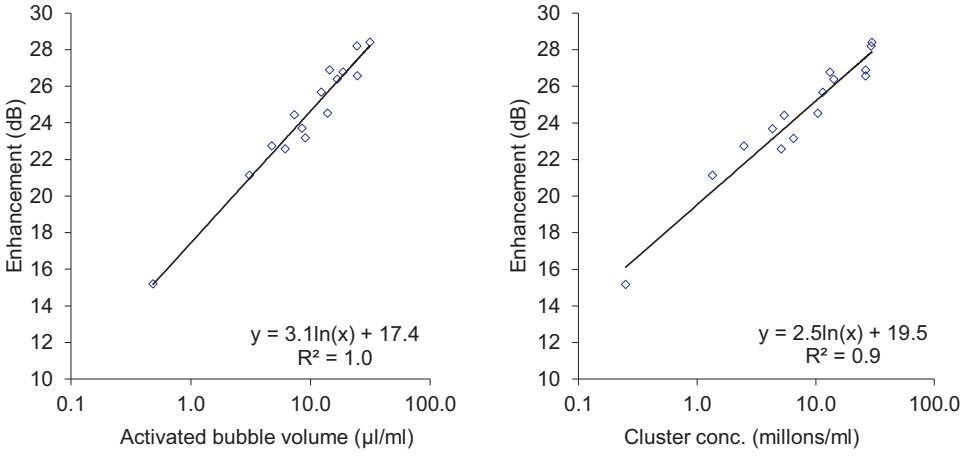

Fig. 8. US imaging: contrast enhancement in dog myocardial wall vs. concentration of clusters in administrated compound (left) and activated bubble volume from sonometry analysis (right).

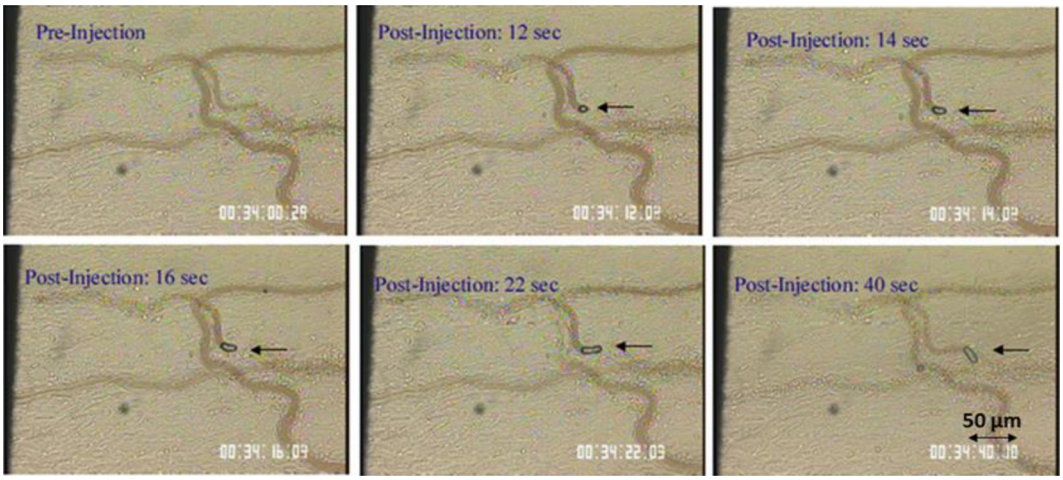

Fig. 9. Intravital microscopy of rat mesentery microvasculature. Pre-injection of ACT compound (upper left) followed by time series after administration and US activation, depicting an activated ACT bubble (indicated by black arrows) as it appears, grows and moves intermittently down the micro vessel.

activated gas volume from sonometry analysis on the same samples. As can be observed relatively precise correlations between myocardial enhancement and these in-vitro characteristics are observed, proving that the microbubble/microdroplet clusters are responsible for the observed contrast and hence the relevance of these parameters as predictors of biological performance.

3.2.2. Intravital microscopy

Typical micrographs from intravital microscopy are shown in Fig. 9. Three animals were investigated with US insonation and two animals were investigated without any US application. Upon US insonation, large, activated bubbles were observed after all injections. Activated bubbles were not observed without application of ultrasound. The growth phase of the activated phase shift bubbles could be observed in real-time. The nucleus of the activated bubble grew within a few seconds along with occlusion of the micro vascular blood flow. No expansion of the micro vessels was observed. All activated bubbles lodged in the micro vessels and transiently blocked blood flow. All activated bubbles were non-spherical and appeared ellipsoidal in shape, forming against a section of the micro vessel. The size of the activated bubbles in-vivo were consistent with results from in-vitro characterisation and with results from the extracorporeal evaluation of in-vivo size reported in Healey et al. (2015). The activated bubbles advanced intermittently down the micro vessels. After a period of time the bubbles shrank gradually until they were small enough to dislodge and blood flow was restored. Typical residence time was approx. 5–10 min.

4. Conclusions

In this paper, a novel approach for US mediated drug delivery – Acoustic Cluster Therapy (ACT) – is proposed, and the basic characteristics of the ACT formulation concept have been demonstrated. Upon reconstitution of a negatively charged microbubble component with a positively charged microdroplet component, small (~4 μm) microbubble/microdroplet clusters are formed. A molecular (drug) payload may optionally be included in the microdroplet component. US insonation (activation) of these clusters with low MI, regular medical imaging US induce a gas-to-liquid phase shift of the droplet component and the formation of ~20–30 μm large ACT bubbles. If a molecular payload is included in the formulation, after activation this will be expressed at the liquid–gas interphase of the ACT bubbles. The clusters are free-flowing in-vivo and can be locally activated by US insonation. Upon activation, the large ACT bubbles deposit in the local microvasculature, transiently occluding blood flow for a few minutes, before they shrink/dissolve and dislodge, and blood flow is restored. After activation, the ACT bubbles produce copious backscatter even in a linear imaging mode and the ACT compound hence work as a very effective "deposit tracer" contrast agent for B-mode US imaging, opening for theranostic attributes.

It is postulated that further insonation with low MI, low frequency US will induce biomechanical effects that locally increase the vascular permeability, leading to a locally enhanced extravasation of components in the vascular compartment (e.g. released or co-administered drugs). These attributes of the ACT concept are further studied and characterized in Van Wamel et al. (2015).

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