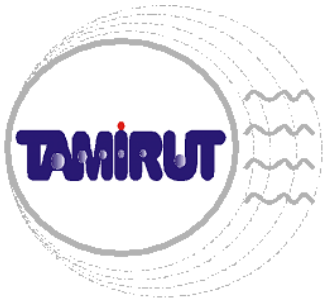


Project Nr. NMP4-CT-2005-016382

Project acronym: TAMIRUT

Project full name: A new bio-sensor concept for medical diagnosis: TArgeted MIcro-bubbles and Remote Ultrasound Transduction

Instrument type: STREP

Priority: 2&3 – IST & NMP

Publishable final activity report

Period covered: *from 1 October 2005 to 31 December 2008*

Date of preparation: *15 March 2009*

Coordinator organisation name: *ESAOTE S.p.A.*

Start date of the project: *1 October 2005*

Duration: *36 months + 3 months extension*

Further general information on report

Deliverable name: *D32P – Publishable final activity report*
Deliverable code: TAMIRUT-016382-D32P-R1.0
Author (Company): *A. Nencioni (Esaote)*

DOCUMENT HISTORY

Release	***Date***	***Reason of change***	***Status***	***Distribution***
1.0	15/03/2009	First Release	Draft	Internal

Project Co-ordinator Reference

Organisation: *Esaote S.p.A., Via di Caciolle 15, 50127 Firenze, Italia*
Responsible person: *Alessandro Nencioni*
Phone: *+39 055 4229281*
Fax: *+39 055 4229432*
E-mail: *alessandro.nencioni@esaote.com*

Table Of Contents

Section 1 - Project execution

1.1 Project objectives

The overall objective of TAMIRUT is to develop an innovative bio-sensor concept devoted to advanced medical diagnosis, in which the biological material is carried by targeted micro-bubbles injected inside the body and the transducer is remote. The remote transducer operates on the basis of the ultrasound response of such micro-bubbles, gathered and processed by an improved version of an ultrasound medical scanner (UMS). Targeted micro-bubbles (similar to the generic micro-bubbles composing the contrast agents actually used in medical echography) are designed to bind only to a desired target region: a bioconjugate ligand is attached to micro-bubble shells producing the adhesion of them to specific molecular signatures. The project goal is to exploit targeted micro-bubbles as bio-sensors producing measurements that will significantly complement the ultrasound molecular imaging and improve the medical diagnosis potential.

Among many potential medical applications, the case study that will be addressed in the TAMIRUT project concerns the early detection, the assessment of the micro-vessel density, and the correct staging and grading of the prostate cancer. Such tasks will be dramatically improved by developing appropriate targeted micro-bubbles (i.e., bubbles able to bind on the endothelium of the neo-vessels of such a cancer) and measuring their local concentrations by means of the novel bio-sensor. In addition, the repetition over time of such a measurement could help in assessing the disease evolution, especially regarding the angiogenesis aspects.

The main objective of this project will be pursued by a multidisciplinary, strictly joint development of all the bio-sensor components, taking into account the need to remotely interrogate targeted micro-bubbles. In turn, micro-bubbles and bioconjugate ligands will be refined and tuned taking into account the desires of the medical community in relation to the introduction of new bio-sensor and addressed case study.

The main TAMIRUT expected output is the prototype of the described bio-sensor composed by the following components, each of which will constitute a partial result of the TAMIRUT project:

(1) **A Targeted UCAs** produced through advanced micro-bubble engineering, with ligand molecules having the efficacy to target specific endothelial markers (i.e., molecular signatures) which are over-expressed in the selected case study (prostate cancer).

(2) **Signal Processing Procedures** able to correctly excite the targeted micro-bubbles, detect and separate the echoes produced by the adherent micro-bubbles, derive the concentration of them for each resolution cell and with a defined tolerance.

(3) **An Ultrasound Medical Scanner** characterized by a great flexibility and a high computational power, able to accurately transmit waveforms of arbitrary shape, update the scanning scheme in real-time, and allowing, at the same time, traditional and harmonic imaging.

(4) **An Ultrasound Probe** able to linearly transmit and receive over an ultra-wide frequency band in order to assess (without any modification) the harmonic components due to the bubble non-linear scattering.

1.2 Contractors involved

The consortium was for a large part built on an already existing basis of co-operation in the field of ultrasound contrast imaging among **Esaote (I)**, a leading European ultrasound

systems manufacturer, and **Bracco Research (CH)**, one of the world's leading research centers in the field of micro-bubbles as diagnostic agents for ultrasound imaging.

This experimented partnership was here reinforced by the essential contribution of two SMEs as very expert industrial providers in the two specialist fields of probe construction, **Vermon (F)** one of the leading ultrasound probe manufacturer in the world, and of signal processing, **SignalGeneriX (CY)**.

The consortium was completed by contribution of scientific institutions with world-wide recognized expertise in their respective fields, like the **Fraunhofer IBMT (D)** in the field of bioanalytical chemistry and biotechnology, the **Department of Applied Physics of University of Twente (NL)** and the **Dept. of Biomedical Engineering of Erasmus Medical Center (NL)** in the field of physics of fluids for studying and modelling targeted micro-bubbles response to ultrasound excitation, the **Department of Biophysical and Electronic Engineering of University of Genova (I)** in the field of signal processing procedures and the **Dept. of Radiology of University of Innsbruck (A)** with its specialists of extensive experience in diagnosis and biomolecular research of prostate cancer, having the main role to specify and to evaluate the potential of the novel bio-sensor concept in medical diagnosis applications..

1.3 Work performed and results

First year objectives of the project was dedicate to completion of phase of assessment of User Requirements and Bio-sensor Specifications and of part of Scientific and Technological Implementation related to get more clear basic knowledge of mechanisms bound to the medical and biochemical aspects of the addressed case study and to prepare the ground for studying the components necessary for the biosensor (targeted microbubble, signal processing, improved UMS). .

About assessment of User Requirements and Bio-sensor Specifications a collection of the overall medical needs and the consequent technical/scientific specifications of all bio-sensor components to face the prostate cancer case-study was organized.

This actions left open the final selection among PSMA and tissue factor as possible **endothelial markers** (i.e., molecular signatures) which are over-expressed in the selected case study of prostate cancer and that it could match the requisite for the researched biosensor characteristics. For each of them there are **antibodies** that potentially function as ligands The final choice will be made on the ground of more extensive verification of their respective efficiency for the purpose of case study.

Following this guidelines, the scientific and technological implementation of the different biosensor components was started. Objective achieved at end of period P1 was to get more clear basic knowledge of mechanisms bound to the medical and biochemical aspects of the addressed case study and to prepare the ground for protoyping the components necessary for the biosensor (targeted microbubble, signal processing, improved UMS).

Modellisation researches gave the basic knowledge necessary to proceed with further experiments on the characterization of the possible behaviour the population of the targeted micro-bubble for the biosensor and from this to the implementation of a first statistical model and of the related software routines was derived.

The development of a flexible simulation tool able to reproduce the whole ultrasound chain tuned in correlation with bubble statistical model was implemented to preliminary study, assess and compare the performance of different signal processing schemes, including transmission, processing of echoes and concentration estimation. The obtained results represents a major progress towards the achievement of an effective signal processing procedure able to correctly excite the targeted micro-bubbles, detect and separate the echoes produced by the adherent micro-bubbles.

A further mainstream of activity was those addressed to acquired more knowledge about the possible **endothelial markers** (i.e., molecular signatures) which are over-expressed in the selected case study, the efficacy of the possible ligand molecules to target such markers and the research of a micro-bubble model able to bind selected antibody for producing the targeted micro-bubble. This work done completes the background required for next six

months period to select the right antibody to be coupled onto micro-bubbles and to generate targeted bubbles for prostate cancer imaging.

Started also the study of the echographic scanner and the ultrasound probe with the characteristic defined during the work of assessment of User Requirements and Bio-sensor Specifications.

Consortium can now start the protyping of the biosensor component that is the objective for the end of period 2, before to proceed with the study of their assembling and evaluation of resulting biosensor in period P3.

After to have successfully reached at end of 1st year the objectives of the project dedicate to completion of user requirements and bio-sensor specifications and of scientific and technological implementation), during this 2nd year we get production and validation of the main components (targeted UCA, probe, scanner, signal processing software) of the novel bio-sensor system. The 3rd and last year of the project it was used to integrate all components in a test set up for the in vitro and in vivo final test of project proof of concept..

In particular we perform a deep and extensive exploration of the physical concepts involved in the bubble modelling and the routines adopted to simulate the bubble response, giving origin to models used to derive the signal processing structure (in particular through developed simulation tool) and to assess the most probable (acoustic) characteristics for the targeted micro-bubbles structure under development. Thanks to the relevant analysis performed, TAMIRUT partners concluded that, from specifications stated for the possible UCA, it is not easy detectable a free bubble from an adherent bubble in a bubble population. Nevertheless it remain open the chance to use the wash out curve to detect presence of adherent bubbles population respect to a free bubbles population only. It is a less immediate but still valid method to demonstrate the effectiveness of TAMIRUT objective achievement: if targeted microbubbles echo will persist for long time means they adhere to the target and then the diagnosis of prostate cancer is positive otherwise their echo will vanish shortly and the diagnosis is negative. A similar point was considered more than sufficient whitin the purpose of TAMIRUT.

As consequence of it also the signal processing was adapted to the new situation to being able to detect the targeted microbubbles developed by BRACCO and IBMT, but compatible with the features and potentialities of the TAMIRUT's probe and scanner.

A stochastic method of synthesis able to produce an insonification beam having a quasi-constant pressure profile over a large depth interval has been devised and studied. It represents a tool that, if necessary, properly works for both single pulse emission and multiple pulses emission, disregarding the related processing algorithms.

The processing of the received signals, many different signal processing options, with a different degree of innovation and originality, have been considered. At the same time, the signal processing options that are can be used jointly with the refined ultrasound scanner and are compatible with the characteristics of the developed probe have been selected. At the end, a short list of signal processing options have been arranged that can be adopted for and integrated in the biosensor prototype, providing satisfactory performance in term of enhancement of the micro-bubble response against the tissue response.

A solution based on the regression implemented by a support vector machine (SVM) has been developed and tested using simulated data for the measurement of the bubble concentration. Despite the measurement of the bubble concentration revealed to be an original and very difficult task, the accuracy is quite in line with the needs of the envisaged medical application, although in absolute terms it is not very high.

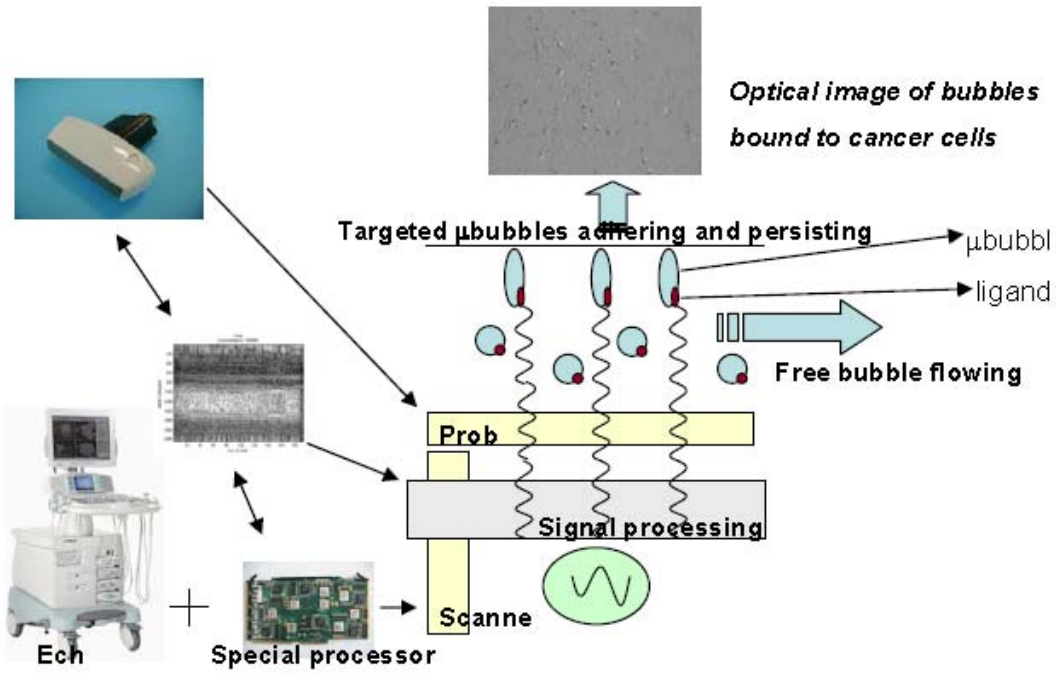
In parallel to signal processing was studied and implemented the improved UMS (scanner and probe) showing specific functionalities concerning ultrasound waveform generation, transmission setting, signal grabbing and storage, memory space, computational power, and post-processing opportunities.

The improved scanner is derived from a basic high-end echographic platform from Esaote integrated with specific processing power to give to this platform the necessary high computational capability to run algorithms devised for the signal processing developed.

To tune/verify the algorithms with the necessary flexibility to analyse different signal processing options, the solution selected is an off line processing as the best way to be fast and flexible in testing evolving signal analysis of bio-sensor model without loosing capability of the system.

In last but not least fundamental we produced the refined ligand-bearing microbubbles and were selected and tested the possible antibodies for their possible use with the two targets previously chosen: PSMA and Tissue factor. Two methods for the formulation of ligand-bearing microbubbles have been developed. The first one, based on streptavidin-biotin interaction, allows testing of target antibodies on a small scale, the second method aims at producing the TAMIRUT ultrasound contrast agent on a larger scale. Two antibodies against Tissue factor were selected and tested during the period, #4509 from American Diagnostica and TF9-10H10 from Calbiochem. The antibody against PSMA selected is Abcam ab22335. From this individuated processes and solutions, microbubbles have been refined and are now available for further work with either anti-TF antibodies or anti-PSMA antibodies at their surface, the selected targeted UCAs as innovative bio-sensors of TAMIRUT are ready...

With the above results in hand we was ready to start the next phase of integration and lab test of proposed biosensor of which the applicative concept is depicted in the figure below..



The TAMIRUT biosensor concept

A specific laminar flow system necessary for in vitro testing was realised. This is a specially designed phantom within a specially designed test setup to be used to mimic the in-vivo setting respecting distances from probe to targeted area. The test setup also contains a flow-cell with defined surface area, designed as a simplified model of a real tumour, allowing for simultaneous microscopic and ultrasound assessment in real-time. Microscopic assessment allowed to compare the ultrasound responses obtained with the responses simulated by the modelling routines used as reference. An artificial tumour mimicking surface was produced by growing tumour cells for 2 days on a support polymer sheet (of Mylar^{TM}), which was inserted in the flow-cell before the actual in-vitro test. The challenge was to achieve a phantom design, which was both optically and acoustically transparent, producing minimum backscatter and was compatible with cancer cell culture conditions (the cells needed to firmly adhere to the surface of the polymer sheets and the expression of the target proteins had to be validated.)

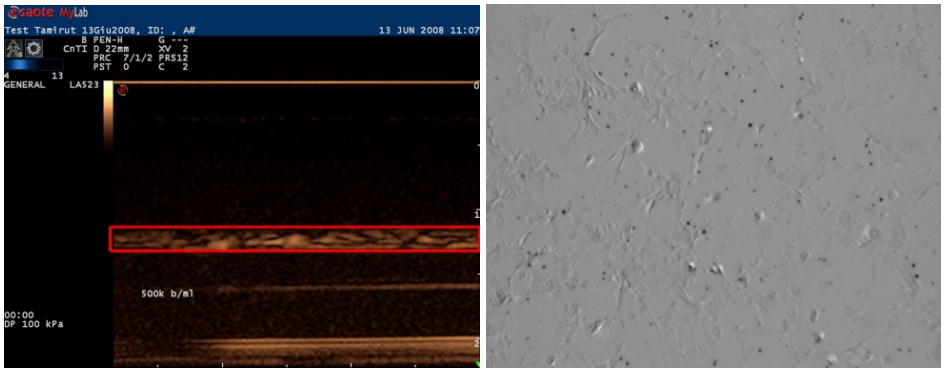
The signals acquired were used to check off-line the signal processing methods defined. The measurements of the bubble concentration obtained was compared with the ground-truth derived from the microscopic assessment. The "real" surface concentration of targeted microbubbles was obtained by manually counting all microbubbles attached to the surface within the field-of-view of the microscope camera ($1mm^2$). To simplify the counting, two images were subtracted. In the resulting difference images surface-attached microbubbles were easily visible as black spots on a grey, homogeneous background, where the silhouettes of cancer cells were almost vanished.

.

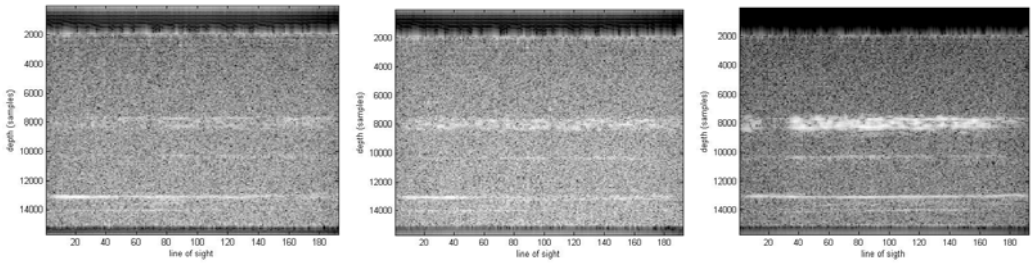
Experimental setup for in-vitro study (flow phantom attached to the microscope, ultrasound probe held in place with a stativ)..

The experimental setup above described allowed to investigate the bio-sensor accuracy, and specifically the concentration estimation accuracy, under well defined conditions. To maintain the goal established i.e. the maximum flexibility in installing on board of signal processing method from WP5, the strategy pursued is to arrange the required modifications to the transmission/receiving settings (i.e., transmission waveforms, beam-forming laws and transmission timings) embedded on the system and to have a proper off-line software processing to verify with no additional work or time delay the feasibility of potential alternative solution to be experimented.

With this apparatus we tested the binding of targeted microbubbles with anti-TF antibodies at their surface (prepared just before the experiments from streptavidin-bearing microbubbles BG6128 and biotin-bearing anti-TF antibodies) to the TF-expressing cancer cells U87-MG. Anti-PSMA bearing targeted microbubbles (and the related PSMA expressing cell line LNCaP) were not used in the in-vitro test, because LNCaP showed unsatisfactory adherence to the polymer support sheets and were washed away under flow conditions. For the objectives of WP6, namely to validate the ability of the scanner and the signal processing, to accurately estimate the concentration of surface attached targeted microbubbles, it was indifferent, which ligand and which target cancer cells were used, so all the tests were performed with anti-TF targeted microbubbles and TF-expressing cells of U87-MG only.



Ultrasound image of microbubbles in flow phantom (flow channel highlighted in red) and related optical image of microbubble bound to tumour cell.



Ultrasound images at low bubble concentration: 19 (left) , 54 (middle), 98 (right) bubbles per mm^2

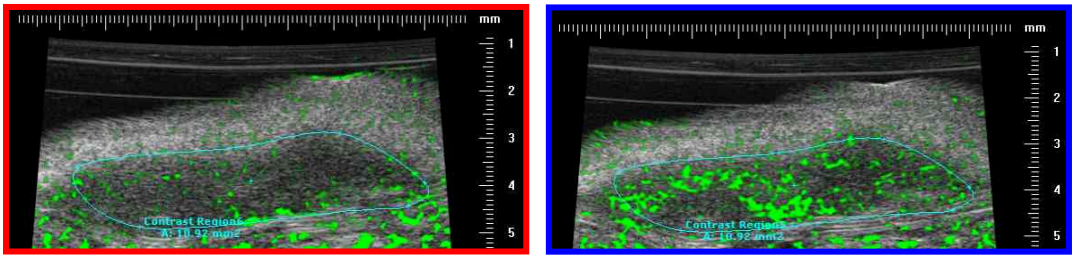
The results obtained during the test period in June 2008 allowed to positively assess the concentration estimation accuracy of the TAMIRUT scanner and to define the best suited signal processing method for this task.

Using only in vitro tests is not sufficient to evaluate the effective potential of targeted micro-bubbles as bio-sensor, because these tests cannot reproduce totally a realistic model of prostate cancer in physiological environment. The in vivo experiments on animal are necessary. In-vivo experiments were organized at Innsbruck Medical University in December 2008 using, instead to follow the protocol defined at the beginning of the project, a revised protocols with less animal but more concentrated activity, due to the short time available respect to those initially planned. In any case this adjustment was sufficient to evaluate the effective persistency and enhancing of echo signal in the area of tumor and also to evaluate the capability of developed biosensor to measure a concentration estimation

Male, athymic, nude mice (Balb-c, nu/nu) have been adopted to carry out in-vivo experiments. Tumour-growth induced by subcutaneous injection of prostate cancer cells (PC3, LnCaP, VCaP) in the rat model. Tumour establishment usually required about 6 weeks for LnCaP and 3-4 weeks for the other two models..All experiments was carried out under i.p. anaesthesia with 200µl Ketamin and 100µl Xylazin. The degree of severity never exceeded degree 2. Tumours was never allowed to exceed the diameter of 1 cm. When a tumour reached this size the animal were sacrificed by carbon dioxid inhalation. The targeted microbubble are delivered by i.v. access via tail vein of rat. The echographic examination is confirmed by histological analysis through dissection of the tumour (and preserved under formaline)

The number of mice used in the experiment was 11. 5 of these was used to test the anti-PSMA targeted micro-bubble PSMA. 6 of them was used to test the anti-TF targeted micro-bubble.

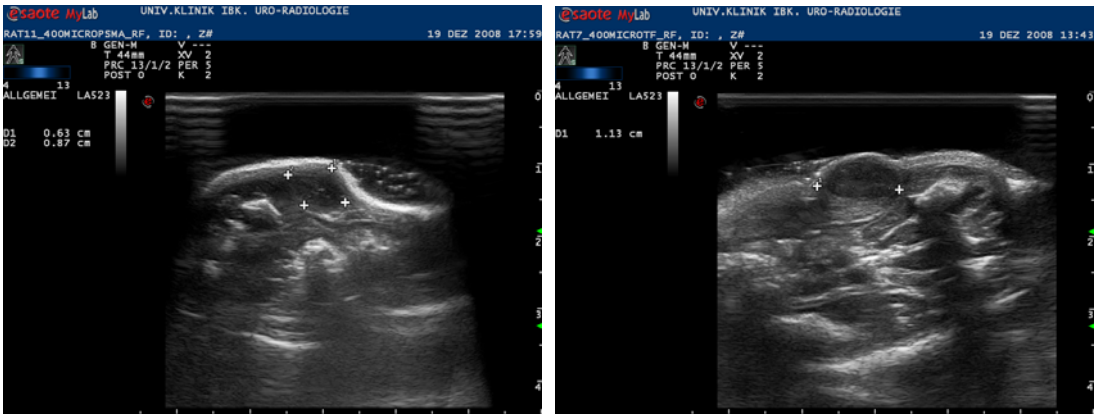
Image at 10 min of normal bubbles ***Image at 10 min of targeted bubbles***



Imaging of tumor enhanced by targeted micro-bubble infusion.

The first meaningful expected effect is that using the targeted micro-bubbles the signal is clearly enhanced and persists for some minutes meaning that they adhere to the targeted signature. This is not the case if we are going to use normal microbubble The off line processing indicate that the intensity of signal seems to show correlation to same extent with the contrast media concentrations.

This means the achievement of the TAMIRUT project objective.



Imaging of specific mice tumour enhanced by targeted micro-bubble infusion.

1.4 Project impact

Although experimentations on patients involving targeted-UCAs are not yet approved, the long-term philosophy of this project is implemented keeping in mind from now the (future) relevant medical applications for which the proposed bio-sensor represents a clear added-value: the development of novel ultrasound targeted micro-bubbles activated through remote ultrasound transduction is a very innovative approach to contribute in solving cancer diagnosis problems.

Cancer is a growing concern all over the world. Among the different kind of cancers, prostate cancer is the most frequent cancer among men accounting for 12% of all cancer cases overcoming also lung cancer accounting “only” for 10% of cases. Prostate cancer is still a rare event in men under 40’s but with a rate destined to increase with the age. It has been calculated that a man out of six is destined to experience prostate cancer clinically evident in its life. Most prostate cancers are multifocal, with synchronous involvement of multiple zones of the prostate, which may be due to clonal and nonclonal tumors. Unfortunately prostate cancer, for most part, is a silent disease for whom it is essential that specialist screening should be offered annually, beginning at age 50 years, to men who have at least a 10-year life expectancy and to younger men who are at high risk. PSA screening for prostate cancer

is very sensitive tumoral marker, but it presents poor specificity. Risky, painful and costly biopsies need to integrate suspected occurrences of cancer to exclude the relevant number of false positives generated by PSA. Unfortunately, by biopsy a not insignificant number of false negative is present, delaying the cancer detection (prostate biopsy is a statistical sampling, and not a whole organ examination therefore then there are chances to miss the target).

The feasibility of the proposed novel bio-sensor system will open the road to innovative, fast and minimally invasive diagnostic procedures to confirm first stage prostate cancer screening (Digit Rectal Exploration + PSA) in place of biopsy. Only an injection of targeted UCA, and a subsequent examination by the advanced UMS, are needed in order to achieve a better knowledge of the region to be examined, instead of the big amount of specimens to be taken with the biopsy procedure. Furthermore, the proposed bio-sensor will offer the possibility to examine the prostate as a whole, no area excluded, instead of considering only the restricted parts from where biopsy specimens are extracted. A small cancer can be missed from an examination by samples like biopsy. Sure this will grant a higher specificity to proposed bio-sensor. In addition, the process offers a simple, efficient and cost effective system also to monitor the results of the therapy.

The positive proof of concept obtained as results of this project confirms that this road is possible and the development of novel ultrasound targeted micro-bubbles activated through remote ultrasound transduction is a new way to contribute in solving cancer diagnosis problems.

Achieving the advances expected from the TAMIRUT project and demonstrating the feasibility of the novel approach, TAMIRUT partners potentially promote a new era of bio-sensors with wide capability of clinical applications. From the prostate cancer case-study is disclosing a very high potential of solutions for the diagnosis and the assessment of other cancer types and also other pathologies.

Such a result is foreseen to enhance European competitiveness when transferred from research into commercially successful products in the fields of ultrasound and targeted UCAs technologies. Participating industries could have the necessary know-how and expertise to do this but it should be obvious that, for a more robust and fast achievement of commercially exploitable outcomes, TAMIRUT participants can be only the core, pursuing the objective of this project, of all the potential interested stakeholders and that TAMIRUT is only a proof of concept demonstrating that the idea can give a solid result. The need for clinical trials and of the novel biosensor for prostate cancer with all related necessary technological optimisation and subsequent approval in humans means that the targeted microbubble agent is unlikely to be available for at least three/five years.

This means that in the follow up of the project, before to arrive to an effective industrially exploitable result, a larger extent of collaboration will be necessary at medical (validation of the bio-sensor inside the clinical environment as a new diagnostic tool for prostate cancer), scientific (new molecules and more refined structures for targeted micro-bubbles to make them more sensible and effective to remote ultrasound transducing in prostate cancer as new type suitable for a larger range of applications, modelling of the micro-bubble's behaviour for each application, etc.), technological (more and more specific signal processing, electronic solution for beamforming and for signal transduction, etc.), and industrial (manufacturing of components for the UMS and the targeted UCAs) level. This will allow to explore in deep, singularly or with an integration perspective, many very innovative aspects strictly related to the TAMIRUT project that, among the others, include::

- micro-bubble formulations for targeted drug delivery;
- molecular imaging of ultrasound-enhanced tissues;
- advanced method of medical signal processing;
- highly performing medical ultrasound scanners and probes.

The perspective goal is to generate an European network of excellence able to take advantages from the TAMIRUT bio-sensor, developing and clinically validating new applications, at the forefront of the world-wide innovation, in the diagnosis of cancers and

other diseases and in the related therapies carried out at molecular-level by the drug delivery concept.

Section 2 - Dissemination and use

2.1 Publishable result

Sensitive ultrasound and targeted contrast agents to reveal early-stage cancer

TAMIRUT is a research project that demonstrated an innovative bio-sensor concept devoted to advanced medical diagnosis, in which the biological material is carried by targeted micro-bubbles injected inside the body and the transducer is remote. The remote transducer operates on the basis of the ultrasound response of such micro-bubbles, gathered and processed by an improved version of an ultrasound medical scanner (UMS). Targeted micro-bubbles (similar to the generic micro-bubbles composing the contrast agents actually used in medical echography) are designed to bind only to a desired target region: a bioconjugate ligand is attached to micro-bubble shells producing the adhesion of them to specific molecular signatures.

Such innovative bio-sensor could help medical professionals visualise tiny quantities of pathological tissue in patients and the could localise tumours in their very earliest stages of development and help doctors begin treatments much earlier, giving patients a much better chance of survival. The approach uses medical ultrasound, a safe technology most commonly used for pre-natal visualisation of the foetus and the imaging of other soft tissues. To improve the sensitivity of this imaging technique, an ultrasound imaging system may sometimes inject a so-called contrast agent into patients, which greatly increases the scattering of the acoustic waves back to the probe. The developed micro bubble medium for this biosensor are specifically targeted to bind to specific molecular signature, expressed by a predefined type of cancer, present in the blood vessel. Specific signal processing capabilities integrated in an enhanced ultrasound equipment detect where these micro bubbles adhere to target cells, and reveal the presence of early-stage tumours.

Among many potential medical applications, the case study that was addressed in the TAMIRUT project concerns the early detection of the prostate cancer.

Cancer is a growing concern all over the world. Prostate cancer is the most frequent cancer among men accounting for 12% of all cancer cases overcoming also lung cancer accounting “only” for 10% of cases. Prostate cancer is still a rare event in men under 40’s but with a rate destined to increase with the age. It has been calculated that a man out of six is destined to experience prostate cancer clinically evident in its life. Of cases of prostate cancer, 70% arise in the peripheral zone, 15-20% arise in the central zone, and 10-15% arise in the transitional zone. Most prostate cancers are multifocal, with synchronous involvement of multiple zones of the prostate, which may be due to clonal and nonclonal tumors.

Unfortunately prostate cancer, for most part, is a silent disease for whom it is essential that specialist screening should be offered annually, beginning at age 50 years, to men who have at least a 10-year life expectancy and to younger men who are at high risk. PSA screening for prostate cancer is very sensitive tumoral marker, but it presents poor specificity. Risky, painful and costly biopsies need to integrate suspected occurrences of cancer to exclude the relevant number of false positives generated by PSA. Unfortunately, by biopsy a not insignificant number of false negative is present, delaying the cancer detection (prostate biopsy is a statistical sampling, and not a whole organ examination therefore then there are chances to miss the target).

The proposed bio-sensor goes in the direction to eliminate or strongly reduce this problem, offering a second degree of evaluation after PSA screening, sensitive and specific, able to examine the organ as a whole. Only an injection of targeted UCA, and a subsequent examination by the advanced UMS, are needed in order to achieve a better knowledge of the

region to be examined, instead of the big amount of specimens to be taken with the biopsy procedure. Furthermore, the proposed bio-sensor offers the possibility to examine the prostate as a whole, no area excluded, instead of considering only the restricted parts from where biopsy specimens are extracted. A small cancer can be missed from an examination by samples like biopsy. Sure this will grant a higher specificity to proposed bio-sensor. In addition, the process offers a simple, efficient and cost effective system also to monitor the results of the therapy.

The proposed new concept of bio-sensor system represents a highly complicated problem, which can be addressed only by duly combining know-how and skills owned by a variety of scientific and technological areas, putting together the necessary critical mass, and bringing together European-wide expertise and resources. Among the involved areas one can cite: molecular bioanalytical chemistry and bio-sensor technology, ultrasound micro-bubbles contrast agent production, advanced signal processing techniques, ultrasound probe construction, ultrasound scanner construction, micro-bubble/ultrasound signal interaction modelling techniques, as well as biomedical and clinical practice.

The project consortium started from an already existing basis of co-operation in the field of ultrasound contrast imaging among **Esaote (I)**, a leading European ultrasound systems manufacturer, and **Bracco Research (CH)**, one of the world's leading research centers in the field of micro-bubbles as diagnostic agents for ultrasound imaging. This experimented partnership was reinforced by the essential contribution of two SMEs as very expert industrial providers in the two specialist fields of probe construction, **Vermon (F)** one of the leading ultrasound probe manufacturer in the world, and of signal processing, **SignalGeneriX (CY)**.

The consortium was completed by contribution of scientific institutions with world-wide recognized expertise in their respective fields, like the **Fraunhofer IBMT (D)** in the field of bioanalytical chemistry and biotechnology, the **Department of Applied Physics of University of Twente (NL)** and the **Dept. of Biomedical Engineering of Erasmus Medical Center (NL)** in the field of physics of fluids for studying and modelling targeted micro-bubbles response to ultrasound excitation, the **Department of Biophysical and Electronic Engineering of University of Genova (I)** in the field of signal processing procedures and the **Dept. of Radiology of University of Innsbruck (A)** with its specialists of extensive experience in diagnosis and biomolecular research of prostate cancer.

To address the case study of a biosensor for early detection of the prostate cancer, a collection of the overall medical needs and the consequent technical/scientific specifications of all bio-sensor components to face the prostate cancer case-study was organized. This actions left to the final selection of PSMA and tissue factor as possible **endothelial markers** (i.e., molecular signatures) which are over-expressed in the selected case study of prostate cancer and that it could match the requisite for the researched biosensor characteristics.

Following this guidelines, the scientific and technological implementation of the following different biosensor components was started:

- **Targeted UCAs** having the efficacy to target specific endothelial markers (i.e., PSMA and tissue factor);
- **Signal Processing Procedures** able to detect the echoes produced by the adherent micro-bubbles and to derive the concentration of them;
- **Ultrasound Medical Scanner** characterized by a great flexibility and a high computational power to acquire and process the adherent micro-bubbles echoes.
- **An Ultrasound Probe** able to assess the harmonic components due to the bubble scattering.

A deep and extensive exploration of the physical concepts involved in the bubble modelling and the routines adopted to simulate the bubble response, gave origin to models used to derive the signal processing structure and to assess the most probable characteristics for the targeted micro-bubbles structure under development. Thanks to the relevant analysis performed, TAMIRUT partners concluded that, from specifications stated for the possible UCA, it is not easy detectable a free bubble from an adherent bubble in a bubble population. Nevertheless it remain open the chance to use the wash out curve to detect presence of

adherent bubbles population respect to a free bubbles population only. It is a less immediate but still valid method to demonstrate the effectiveness of TAMIRUT objective: if targeted microbubbles echo will persist for long time means they adhere to the target and then the diagnosis of prostate cancer is positive otherwise their echo will vanish shortly and the diagnosis is negative.

The processing of the received signals has been approached by two directions: (1) to process the echoes collected after the emission of just one pulse; (2) to process the signals obtained by the emission of a particular sequence or pulses and the pre-processing of the related echoes. For both directions, many different signal processing options, with a different degree of innovation and originality, have been considered. Owing to a comprehensive simulation tool, an analysis of the performances obtainable by the different options has been carried out, considering the effects of undesired phenomena like body motion, thermal noise, harmonic distortion, etc. The performance of each technique has been assessed by an objective metric and compared with those of the other techniques. At the same time, the signal processing options that are can be used jointly with the refined ultrasound scanner and are compatible with the characteristics of the developed probe have been selected. At the end, a short list of signal processing options have been arranged that can be adopted for and integrated in the biosensor prototype, providing satisfactory performance in term of enhancement of the micro-bubble response against the tissue response. According to the project outcomes above as coming out from modelling analysis, the rejection of the response of the free-circulating micro-bubbles will be carried out waiting until such free bubbles are far away from the considered region.

A solution based on the regression implemented by a support vector machine (SVM) has been developed and tested using simulated data for the measurement of the bubble concentration.

In parallel to signal processing was studied and implemented the improved UMS (scanner and probe) showing specific functionalities concerning ultrasound waveform generation, transmission setting, signal grabbing and storage, memory space, computational power, and post-processing opportunities. The improved scanner is derived from a basic high-end echographic platform from Esaote integrated with specific processing power to give to this platform the necessary high computational capability to run algorithms devised for the signal processing developed. To tune/verify the algorithms with the necessary flexibility to analyse different signal processing options, the solution selected is an off line processing as the best way to be fast and flexible in testing evolving signal analysis of bio-sensor model without loosing capability of the system. The obtained UMS probe is a prototype showing the required central frequency and geometry as well as specific functionalities concerning bandwidth and linearity destined to give the prototypal object for ultrasound transduction requested by the research.

In last but not least fundamental we implemented the refined ligand-bearing microbubbles and were selected and tested the possible antibodies for their possible use with the two targets previously chosen: PSMA and Tissue factor. Two methods for the formulation of ligand-bearing microbubbles have been developed. The first one, based on streptavidin-biotin interaction, allows testing of target antibodies on a small scale, the second method aims at producing the TAMIRUT ultrasound contrast agent on a larger scale. Two antibodies against Tissue factor were selected and tested during the period, #4509 from American Diagnostica and TF9-10H10 from Calbiochem. The antibody against PSMA selected is Abcam ab22335. From this individuated processes and solutions, microbubbles have been refined and made ready for the in vitro and in vivo final test of project proof of concept.

A specific laminar flow system necessary for in vitro testing was realised. This is a specially designed phantom within a specially designed test setup to be used to mimic the in-vivo setting respecting distances from probe to targeted area. The test setup also contains a flow-cell with defined surface area, designed as a simplified model of a real tumor, allowing for simultaneous microscopic and ultrasound assessment in real-time. Microscopic assessment allowed to compare the obtained ultrasound responses with the responses simulated by the modeling routines used as reference. An artificial tumor mimicking surface was produced by growing tumor cells for 2 days on a support polymer sheet (of Mylar^{TM}), which was inserted in the flow-cell before the actual in-vitro test. The challenge was to achieve a phantom design, which was both optically and acoustically transparent, producing minimum backscatter and

was compatible with cancer cell culture conditions (the cells needed to firmly adhere to the surface of the polymer sheets and the expression of the target proteins had to be validated.)

The signals acquired was used to check off-line the signal processing methods defined. The obtained measurements of the bubble concentration was compared with the ground-truth derived from the microscopic assessment. The "real" surface concentration of targeted microbubbles was obtained by manually counting all microbubbles attached to the surface within the field-of-view of the microscope camera ($1mm^2$). To simplify the counting, two images were subtracted. In the resulting difference images surface-attached microbubbles were easily visible as black spots on a grey, homogeneous background, where the silhouettes of cancer cells were almost vanished.

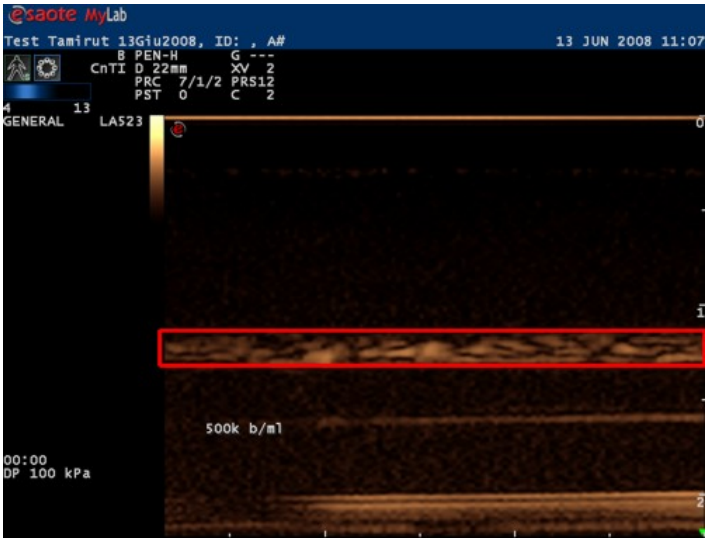
.

Experimental setup for in-vitro study (flow phantom attached to the microscope, ultrasound probe held in place with a stativ)..

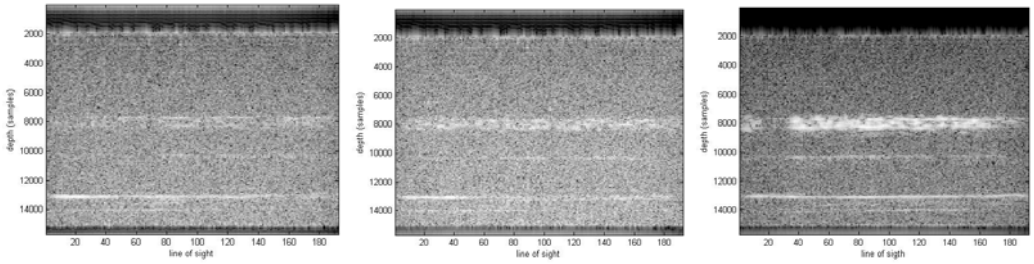
The experimental setup above described allowed to investigate the bio-sensor accuracy, and specifically the concentration estimation accuracy, under well defined conditions. To maintain the goal established i.e. the maximum flexibility in installing on board of signal processing method from WP5, the strategy pursued is to arrange the required modifications to the transmission/receiving settings (i.e., transmission waveforms, beam-forming laws and transmission timings) embedded on the system and to have a proper off-line software processing to verify with no additional work or time delay the feasibility of potential alternative solution to be experimented.

With this apparatus we tested the binding of targeted microbubbles with anti-TF antibodies at their surface (prepared just before the experiments from streptavidin-bearing microbubbles BG6128 and biotin-bearing anti-TF antibodies) to the TF-expressing cancer cells U87-MG. Anti-PSMA bearing targeted microbubbles (and the related PSMA expressing cell line LNCaP) were not used in the in-vitro test, because LNCaP showed unsatisfactory adherence to the polymer support sheets and were washed away under flow conditions. For the objectives of WP6, namely to validate the ability of the scanner and the signal processing, to accurately estimate the concentration of surface attached targeted microbubbles, it was indifferent, which ligand and which target cancer cells were used, so all the tests were performed with anti-TF targeted microbubbles and TF-expressing cells of U87-MG only.

The results obtained during the test period in June 2008 allowed to positively assess the concentration estimation accuracy of the TAMIRUT scanner and to define the best suited signal processing method for this task.



Ultrasound image of microbubbles in flow phantom (flow channel highlighted in red)



Ultrasound images at low bubble concentration: 19 (left) , 54 (middle), 98 (right) bubbles per mm^2

Using only in vitro tests is not sufficient to evaluate the effective potential of targeted micro-bubbles as bio-sensor, because these tests cannot reproduce totally a realistic model of prostate cancer in physiological environment. The in vivo experiments on animal are necessary. In-vivo experiments were organized at Innsbruck Medical University in December 2008 using, instead to follow the protocol defined at the beginning of the project, a revised protocols with less animal but more concentrated activity, due to the short time available respect to those initially planned. In any case this adjustment was sufficient to evaluate the effective persistency and enhancing of echo signal in the area of tumor and also to evaluate the capability of developed biosensor to measure a concentration estimation

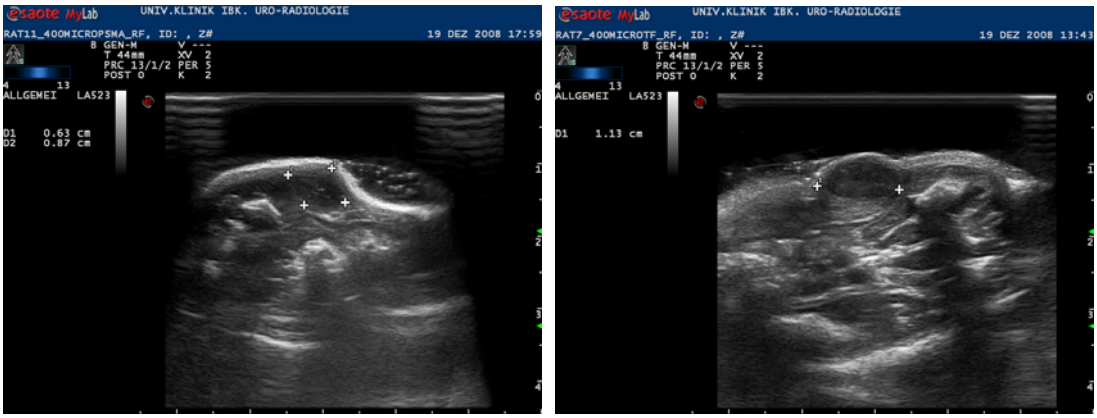
Male, athymic, nude mice (Balb-c, nu/nu) have been adopted to carry out in-vivo experiments. Tumour-growth induced by subcutaneous injection of prostate cancer cells (PC3, LnCaP, VCaP) in the rat model. Tumour establishment usually required about 6 weeks for LnCaP and 3-4 weeks for the other two models..All experiments was carried out under i.p. anaesthesia with 200µl Ketamin and 100µl Xylazin. The degree of severity never exceeded degree 2. Tumours was never allowed to exceed the diameter of 1 cm. When a tumour reached this size the animal were sacrificed by carbon dioxid inhalation. The targeted microbubble are delivered by i.v. access via tail vein of rat. The echographic examination is confirmed by histological analysis through dissection of the tumour (and preserved under formaline)

The number of mice used in the experiment was 11. 5 of these was used to test the anti-PSMA targeted micro-bubble PSMA. 6 of them was used to test the anti-TF targeted micro-bubble.

The first meaningful expected effect is that using the targeted micro-bubbles the signal is clearly enhanced and persists for some minutes meaning that they adhere to the targeted signature. This is not the case if we are going to use normal microbubble The off line processing indicate that the intensity of signal seems to show correlation to same extent with the contrast media concentrations.

.

This means the achievement of the TAMIRUT project objective.



Imaging of mice tumor enhanced by targeted micro-bubble infusion.

The positive proof of concept obtained as results of this project confirms that the development of novel ultrasound targeted micro-bubbles activated through remote ultrasound transduction is a new way to contribute in solving cancer diagnosis problems.

Achieving the advances expected from the TAMIRUT project and demonstrating the feasibility of the novel approach, TAMIRUT partners potentially promote a new era of bio-sensors with wide capability of clinical applications. From the prostate cancer case-study is disclosing a very high potential of solutions for the diagnosis and the assessment of other cancer types and also other pathologies.

The need for clinical trials and of the novel biosensor for prostate cancer with all related necessary technological optimisaton and subsequent approval in humans means that the targeted microbubble agent is unlikely to be available for at least three/five years.