OPTOBACTERIA Report Summary

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Final Report Summary - OPTOBACTERIA (Multianalyte automatic system for the detection of drug resistant bacteria.)

Executive Summary:
Optobacteria aims to solve some of the problems related to the early detection of beta-lactamases using a multidisciplinary approach. Combining a new highly sensitive technology (engineering approach based on optimized optical fiber system) with specific ligands for targeting ESBL (Extended Spectrum Beta Lactamases) a new method for the detection of drug resistance bacteria will be developed. Optobacteria addresses a specific scientific and technological problem and responds to a medical need that is adopted by the 4 SME from the 3 European countries participants through outsourcing research activities to 4 RTD performers.
The ALD is based on a new technology (opto electronic device) in which the appropriate ligand–probe is linked to an opto electronic surface and specifically recognizes (and binds or fishes out) proteins and biomolecules that are biomarkers of the resistant infections.
The proposed work will enable the SME participants to improve their competitive position in the field of automatic laboratory detector (ALD) market useful for providing a specific antibiogramlike report on infecting microbes resistant to commonly used antibiotics.

Project Context and Objectives:
The main goal of OPTOBACTERIA is the development of a Multianalyte automatic system for the detection of drug resistant bacteria. to achieve this target research and tecnical activities has been carried out. In the technical field the limits of what is technical feasible today have been reached, design and manufacturing a prototipe of ALD, Automatic Laboratory Detector, as well as the specific fiber glass biosensors. The scientific activities have been developed in the field of bioanchoring of ligands to the surface of biosensors, synthesis and identification of new ligands, validation of the inhibitor effect on beta-lactamases and clinical validation of ALD.
Taking into account that once processed the samples (about 1 hour of total processing) the time for detecting the presence of B-lactamases by the ALD is practically immediate, the time needed for a final result is between 7 and 25 hours. This means a significant advance in comparison with the classical methods which lasts 48-72 hours (24hincubation + 24h presumptive identification of resistance by automatic MIC or 24hincubation + 24h + isolation + 24 identification of resistance by disc-diffusion or automatic MIC).
The main advantages obtained as a result of the project are detailed below by technological work packages.
From WP1 Optical biosensor technology optimization, activities have been focused on the development of the bioactivated fiber optic probes to be functionalized by the bioreceptors. Two main products have been obtained toward the industrial exploitation:
o A software for the efficient design of LPG based optical transducers,
o A chemical functionalised optical transducer prototype working in reflection mode

Main advantages are, respectively:
o The availability of a design tool for LPG based optical transducer not already present on the market,
o An optimised optical platform (LPG working in reflection mode) ready to be implemented in several molecular recognition fields due to the efficient surface functionalization.
From WP2 Bioanchoring of ligands to the sensor surface, activities have been focused on the functionalization of the probe surface with ligands able to recognise betalactamases.

Main product is
o A specific probe to be used in EBSL detection
Main advantages provided by the activities are:
o The knowledge transferred to the company related to the bio anchoring techniques
o The availability of a probe sensitive to beta-lactamases that has been characterised in terms of expected sensing performances
From the technologies related to WP3 and WP4 the main products are:
o Product 1. Ligand panels for the molecular recognition of beta lactamases (BL, CTX-M-15, KPC-2, Amp-C, NDM-1 and VIM-1) using a full profile analysis through different technologies such as optical fiber probe, microarray systems and chips miniaturization. The ligands consist of a number of chemical compounds that have been selected through two methods: a) known ligands tested against BL proteins never tested before; b) virtual screening studies against the selected protein panel. The selection criteria for the protein panel are consistent with the ligands mode of binding. The ligands are immobilized on the sensing surfaces. The panel profile is reported in separate documents. Based on the accurate literature search available the said application is unique. Other than optical fiber application, other application can be foreseen as chip technology and plate reading technology for microarray technologies. The different ligands can also be associated in different shapes.
o Product 2. One single ligand, a boronic acid derivative. This molecule is unpublished and has a high affinity profile suitable for BL protein fishing out.
o Product 3. Linker chemistry for water soluble ligands. The linker is useful for the suitable modulation of the sensing signal of the ligand when binding the BL protein. The linker can be linked to the ligand to insert a distance between the probe surface and the recognition head of the ligand.
o Product 4. Detachment procedures. The detachment is useful for the regeneration of the optical probe and for every surface that can immobilize the BL proteins, based on the displacement method. (Collaboration with WP2 CNR IMCB)
o Product 5. Scaffolds for the development of further ligands with different profile and against new beta-lactmase.
o P6. A new software program able to identify small molecule ligands that can bind target proteins/enzymes (FLAPdock)

Main advantages are respectively:
o P1. The ligand panels consists in unique ligands sets (4 panels provided) and can be applied for the detection of at least 5 BL proteins among those tested:
CTX-M-15, KPC-2, Amp-C, NDM-1 and VIM-1. The advantage is that instead of using one single ligand to detect one protein, the multiple profile will allow the identification of the different protein isoforms.
o P2. The boronic acid compound and derivatives where the compound is derivatized in position 3 with alkylic chains. These molecules have not been published and could be patented by TYDOCK PHARMA. The use of this compound for molecular recognition of BL is highly recommended.
o P3. Linker chemistry for linking the BL recognizing function (compound) to the probe surface. The advantages is that the recognition element is not close to the probe surface, but at a certain optimal distance in order to maintain the optimal sensitivity with respect to the sensing surface and at the same time grant the fishing out of the protein from the biological sample.
o P4. The detachment method based on displacement strategy is based on the known methodology of the competition assays in which stronger binders displace a weak ligand bound to a protein. The innovation here is in the application of the method for the probe regeneration to save the time to prepare it again and is economically rewarding due to the use of the same probe.
o P5. Scaffolds for the development of further ligands with different profile and against new beta lactamase. These products are important for the implementation of the compound set that is provided as P1.
The scaffold are unique to be applied to the BL section cases.
o P6. An addition to the MD software portfolio improving competitiveness in the market, along with validation of approach and it's demonstrated effectiveness.
From WP5 Automatic laboratory detector assembling, activities have been focused on development of the automated robot able to manage multiple probes (8 probes simultaneously) for the infection tests. Main product is:
o The ALD system
Main advantages are,

The availability of a prototype of the automatic laboratory fully operating the probes in specific protocols with a dedicated software retrieving and elaborating the optical probes outputs in user friendly outputs. The developed system is very flexible and innovative.

Project Results:
WP1
Optical biosensor technology optimization
WP Leader: Andrea Cusano
The main tasks of WP1 reliedon the design and manufacturing of optical fiber probes working in reflection mode,highly sensitive to external refractive index variations and bearing functionalities suitable for the following coupling step with the biological recognition element.

Task 1.1 Reflection probes
In the first part of WP1, researchers from CeRICT have focused their efforts on the development of a suitable virtual environment (VE) for the design and simulation of nanoscale coated LPG transducers working in transition mode. This represented a key activity for the realization of LPG-based biosensors. Indeed there are several parameters that have to be carefully chosen to tailor the features of the LPG (e.g. grating pitch, length and the refractive index (RI) modulation depth), in order to provide the right specifications to manufacturers for the realization of optimized devices to be used in biosensing applications.
The developed VE has been realized with MATLAB® code and is characterized by a graphical user interface(a picture of which is reported in Figure 1.1.(a)) making its use “user-friendly”. By feeding the system with all the input parameters (among which the optical fiber characteristics, surrounding RI (SRI), number and optical and geometrical features of the coating overlays, LPG parameters and wavelength span) the transmittance spectrum of the target LPG transducers can be obtained, combined to further information about the excited cladding modes as well as other info about the simulation status and errors.The functional validation of the developed VE has been performed by the design of some examples of “known” LPG transducers, whose resonant dip wavelength positions and intensities have been experimentally characterized in laboratory measurements. Just as an example, in Figure1.1.(b), the comparison between numerical and experimental spectrum for a given LPG having a grating pitch Λ= 350 μm, length L= 4 cm and refractive index modulation Mod= 2.1•10-4 is shown. Obtained results evidenced the very good agreement between numerical prediction and experimental data.

Figure 1.1.

The same VE can be also used to perform a complete analysis either of bare or coated LPGs: e.g. the modal dispersion plot (reporting the resonant wavelengths versus the grating pitch or the SRI) can be obtained for all the selected cladding modes, as well as their SRI sensitivity vs. the SRI (or vs. the overlay thickness), or even the contour plot reporting the SRI sensitivity for all the selected modes vs. the overlay thickness and grating pitch. All these plots play a key role for the design of multilayer coated LPG transducers working in transition mode.
The realized tool has been successively used by CeRICT researchers for the design of optimized LPG transducers, the design process being carried out either on commercially available LPGs (i.e. with fixed LPG characteristics, such as grating pitch, length and RI modulation depth) and on customized LPGs, where all the parameters, including the LPG features, have been suitably designed. In the first case the LPG transducers optimization basically relied on the design of optimized thicknesses for the aPS layers that maximize the SRI sensitivity around a target external RI. In the second case, the design also included the definition of all the LPG features.

Once defined the design parameters, CeRICT and IMCB-CNR researchers cooperated for the development of optimized reflection type LPGs, thus enabling a more practical LPG interrogation in reflection instead of the typical transmission configuration. As schematically represented in Figure 1.2.(a), This requires the integration of a silver reflecting layer (acting as a mirror) at the end-face of the optical fiber just after the grating. A key aspect in this step relies on the identification of the precise LPG position within the fiber, that is of fundamental importance in order to cut the fiber just after the grating. To understand this concern, it is worth pointing out that, if the mirror is realized on the fiber facet, light passing through the LPG is reflected back (by the mirror) and passes through it for the second time. This means that, if the optical fiber containing the LPG is cut immediately after the grating, this will be equivalent to an LPG working in transmission configuration and having a double length, which also leads to the positive implication of an increased resonance visibility, as schematically represented in Figure 1.2.(b).

Figure 1.2.

Differently, if the optical fiber is cut not just after the grating, but at a distance L from it, the LPG will result in the cascade of two identical LPGs spaced by a distance d=2L. This would lead to the formation of interference fringes within the attenuation bands, similarly to those observed in a Mach–Zehnder interferometer (see Figure 1.3), that have to be avoided since they enormously increase the complexity of the LPG interrogation unit.

Figure 1.3.

In light of these considerations, a correct identification of the LPG position inside the optical fiber is mandatory. To solve this problem, we fixed the optical fiber straight on the working plane, covered the fiber region containing the LPG with a liquid having a refractive index similar to the fiber one (e.g. glycerol) and coupled the optical fiber with a pigtailed red (635nm) diode laser. As schematically described in Figure 1.4, light scattered by the grating comes out of the core into the cladding and, consequently, into the liquid with matched refractive index, thus allowing the visualization of the LPG starting position. By repeating the same procedure on the other side of the optical fiber the position of the LPG can be easily inferred.
Figure 1.4.

After having identified the LPG position, a high precision fiber cleaver is used to cut the optical fiber just after the grating, followed by the integration of an Ag reflecting layer (i.e. the mirror) on the terminal face of the cut optical fiber. To this aim, a silver mirror reaction (Tollen’s test) was adopted.
The further step towards the manufacturing of the first prototypes of optimized LPG transducers relied on the deposition of a high RI (HRI) polymeric layer (atactic polystyrene - aPS) onto the surface of the reflection type LPGs in order to tune the working point of the LPG in the transition (highly sensitive) region. To this aim, we used the dip-coating technique, a relatively simple and less time consuming method compared to electrostatic self assembly and Langmuir-Blodgett, that has been demonstrated to provide a lower roughness of the layer which in turn determines its higher optical quality. For the deposition of the ordinary aPS film (having a RI of 1.555), the fiber containing the LPG was first immersed into a PS solution and successively withdrawn with a controlled speed of 10 cm/min. The thickness of the deposited layers ranged from 250 to 320 nanometers, depending on the features of the used LPG.
The high quality (either in terms of smoothness and homogeneity) of the aPS deposited layers has been monitored by means of both optical microscope imaging (see Figure 1.5) and spectral measurements. Indeed the goodness of the deposition process is testified by the subsistence of the attenuation band visibility, since the presence of overlay defects determines a dramatic decrease of the LPG attenuation bands depth due to scattering losses. The effects of nanoscale HRI coatings on LPGs were also verified by means of SRI sensitivity characterizations, which confirmed a drastic modification of the devices sensitivity characteristics with respect to the bare ones, from the nonlinear monotone behavior of the bare LPG (with maximum sensitivity in correspondence of SRI close to the cladding RI) to a resonant-like behavior of the coated one. The sensitivity curves demonstrated a selective behavior, exhibiting the maximum value (i.e. the maximum sensitivity point) in correspondence of the transition index for each cladding mode (see Deliverable D1.2 for more details).

Task 1.2 Probes surface chemical functionalization
After the deposition of the HRI overlay, useful to significantly increase the transducer SRI sensitivity, the active LPG surface has to be chemically functionalized to bear functionalities (in our case carboxylic groups) suitable for the following coupling step with the biological recognition element (BRE). This scope could be reached, on the one hand, by acting on the HRI cladding (polymeric) overlay having by means of physical or wet chemical methods. On the other hand, by depositing an additional polymeric (ultra)thin film, either possessing the appropriate functionality or post-processable, onto the former aPS one. It is worth noting, however, that direct modification of the HRI polymeric overlay involves mostly wet chemical treatments, plasma treatments and UV irradiation, all methods that present some drawbacks for our purposes. In fact, chemical surface modification reactions are difficult to control and typically result in irregular etching, ionized gas treatments require costly and large infrastructures and increase surface roughness, UV light exposure (usually) alter the LPG characteristics as well as bulk polymer properties. Especially the increase in surface roughness discourages the use of these techniques because it can greatly reduce the optical quality of the overlay (essential condition to fully exploit the benefits of the LPGs working in transition mode). Moreover all these techniques produce a broad spectrum of functional groups without ensuring a high density of the desired functional

group.
On the other side the use of bulk-functionalized polymers as overlay could result in unsuitable bulk characteristics (refractive index, hydrophilicity, etc.). It was therefore straightforward the need to implement a multilayer strategy where the first overlay served to tune the working point of the device in the transition region while a second thinner overlay provided the specific functionality.
It is important at this point to highlight that the deposition of a second layer by dip coating implies the need for a solvent/nonsolvent strategy. In other words, the second layer had to be deposited from a solution whose solvents did not damage the underlying layer.
During task 1.2, IMCB researchers have investigated the possibility to deposit a thin layer of PMMA-co-MA on the aPS one by means of the dip coating technique. One important difficulty that was considered in this system is that aPS is an highly hydrophobic polymer while PMMA-co-MA has an hydrophilic nature, therefore the use of solvents with a higher polarity, with respect to chloroform, could prevent the good adhesion of the secondary layer and cause its slipping as well as de-wetting defects. The problem was solved by using a mixture of solvents containing a small amount of a mutual solvent for both polymers, in our case chloroform, and a major part of mutual nonsolvent, in our case isopropanol, in a volumetric ratio 1:3. In this way the integrity of the first layer was preserved and a good adhesion and uniformity of the secondary layer was ensured. The PMMA-co-MA was thus performed by immersing the aPS-coated LPG into a PMMA-co-MA solution in chloroform:isopropanol and successively withdrawing it with a controlled speed of 10 cm/min. Considering the relatively small amount (1.6%) of methacrylic acid in this PMMA co-polymer we assumed its refractive index to be the same as the homopolymer, i.e. 1.49.
Similarly to what made with the aPS-coated LPG, the double layer-coated one was characterized by means of optical microscope imaging (see Figure 1.5), spectral measurements and SRI sensitivity characterizations. The last ones, in particular, were performed in order to ascertain that the additional layer with lower refractive index does not have detrimental effects on the sensitivity characteristics of the final transducer.
Figure 1.5.

After having assessed all the manufacturing steps for the fabrications of multilayer coated reflection type LPG working in transition mode, the efforts of CeRICT and IMCB-CNR researchers have been devoted to the manufacturing of the LPG transducer prototypes. In particular, a huge number of chemically functionalized LPG transducers prototypes have been realized, either on commercially available or customized LPGs, based on the optimal design parameters obtained during the design stage. Realized probes have been supplied to IMCB-CNR for the successive bio-functionalization step (carried out in WP2).Realized probes have been characterized in terms of reflectance spectrum (see Figure 1.6.a) and SRI sensitivity (see Fig. 1.6.b) and have been validated (i.e. they were subjected to acceptance) based on the goodness of the match between numerical and experimental results.
The sensing characteristics of all the prototypes well matched those obtained numerically by the developed VE, and exhibited SRI sensitivity (in correspondence of an SRI=1.34) in the range ~ 1000-2000 nm/RIU, with sensitivity gains of about two orders of magnitude with respect to the bare LPG.
Figure 1.6.

In addition, as some kinds of instability of the multilayer coated LPG sensors response have been observed during the β-lactamase detection tests (carried out in WP2) and, in particular, when the LPG probes were immersed in phosphate buffer solutions at pH=7, the efforts of CeRICT and IMCB researchers have been focused to overcome this problem. In particular, two possible strategies have been identified:
1) the definition of a new protocol, that avoided the use of the phosphate buffer solutions at pH=7 (carried out in WP2 by IMCB in collaboration with CeRICT);
2) the optimization of the probes, making use of a more stable silicon nitride (Ni3Si4) overlay instead of the polymeric ones, that could be safely used also in the phosphate buffer solutions at pH=7 (carried out by CeRICT researchers).
Concerning this second strategy, a detailed numerical analysis has been carried out by CeRICT researchers and several Ni3Si4 coated LPG prototypes have been fabricated and characterized, either in term of their spectral response and SRI sensitivity.
However, it turned out that, in order to obtain a very high quality of the silicon nitride layer (needed to enable the LPG modal transition phenomenon) and, thus, to sufficiently improve the sensitivity of the Ni3Si4-coated transducers, a further time consuming optimization step had to be performed. Based on these considerations, and due to the fact that the first strategy (involving the definition of a new protocol that avoids the use of phosphate buffer solutions) demonstrated to be able to overcome the LPG probes instabilities, CeRICT and IMCB researchers decided to focus all their attention and efforts in performing the biological experiments exploiting the optimized protocol.

Conclusions
All the objectives of WP1 have been fully achieved and obtained results constitute an enormous progress beyond the state of the art in the field opening a plenty of potential market options.
In particular, the following clearly significant results have been obtained:
- a dedicated software able to fully simulate the operation of fiber optic biosensors based on long period gatings has been developed and successfully validated.
- the dedicated software constitutes a powerful tool to fully control the design of this kind of biosensors enabling the optimization of figures of merit.
- a fabrication procedure has been developed and optimized to fabricate optical biosensors based on long period gratings working in transition mode and reflection configuration.
- the fabrication route has been fully validated.
- a dedicated fabrication step has been developed to enable at the same time the maximum sensitivity and an active surface ready to be biofunctionalized for specific application.

Overall, no critical aspects have been encountered, all the activities have been completed and reported in the deliverables D1.1 ('Technical Report on the design and manufacturing of the probes'), D1.2 ('Prototypes of reflection type fiber optic probes based on LPGs') and D1.3 ('Technical report on the procedures of the chemical functionalization of the optical probes') which have been delivered on time.

Products
A huge number of chemically functionalized LPG transducers prototypes were realized, either on commercially available or customized LPGs, based on the optimal design parameters obtained during the design stage. Realized probes have been supplied to IMCB-CNR for the successive biofunctionalization step (carried out in WP2).

SME Interest
There is potential interest in the assessment of the development phases of reflection-type LPG-based transducers working in reflection mode. These devices, indeed, constitute the basic components for the development of label-free fiber optic biosensors.

Risk
The potential risks for this WP (as reported in the DOW) could be related to difficulties in efficient surface functionalization of the LPG transducers.

How we overcome
The chemical functionalization of LPG surface can be made, on the one hand, by acting on the HRI cladding (polymeric) overlay by means of physical or wet chemical methods. On the other hand, by depositing an additional polymeric (ultra)thin film, either possessing the appropriate functionality or post-processable, onto the former aPS one. As the former approach presents some drawbacks for our purposes (irregular surfaces, increase in surface roughness, broad spectrum of functional groups without ensuring a high density of the desired functional group) we implemented a multilayer strategy involving the deposition of a secondary ultra-thin layer of PMMA-co-MA on the aPS one by means of the dip coating technique. To do this, one important difficulty is that aPS is a highly hydrophobic polymer while PMMA-co-MA has a hydrophilic nature, therefore the use of solvents with a higher polarity, with respect to chloroform, could prevent the good adhesion of the secondary layer and cause its slipping as well as de-wetting defects. The problem was solved by using a mixture of solvents containing a small amount of a mutual solvent for both polymers, in our case chloroform, and a major part of mutual nonsolvent, in our case isopropanol, in a volumetric ratio 1:3. In this way the integrity of the first layer was preserved and a good adhesion and uniformity of the secondary layer was ensured.
Still concerning the risk management, some kinds of instability of the multilayer coated LPG sensors response had been observed during the β-lactamase detection tests (carried out in WP2) and, in particular, when the LPG probe was immersed in phosphate buffer solutions at pH=7. In order to overcome these issues, two possible strategies had been identified:
1) the definition of a new protocol, that avoided the use of the phosphate buffer solutions at pH=7 (carried out in WP2 by IMCB in collaboration with CeRICT);
2) the optimization of the probes, making use of a more stable silicon nitride (Ni3Si4) overlay instead of the polymeric

ones, that could be safely used also in the phosphate buffer solutions at pH=7 (carrie out by CeRICT researchers).
Concerning this second strategy, a detailed numerical analysis has been carried out by CeRICT researchers and several Ni3Si4 coated LPG prototypes have been fabricated and characterized, either in term of their spectral response and SRI sensitivity.
However, it turned out that, in order to obtain a very high quality of the silicon nitride layer (needed to enable the LPG modal transition phenomenon) and, thus, to sufficiently improve the sensitivity of the Ni3Si4-coated transducers, a further time consuming optimization step had to be performed. Based on these considerations, and due to the fact that the first strategy (involving the definition of a new protocol that avoids the use of phosphate buffer solutions) demonstrated to be able to overcome the LPG probes instabilities, CeRICT and IMCB researchers decided to focus all their attention and efforts in performing the biological experiments exploiting the optimized protocol.

WP2
Bioanchoring of ligands to the sensor surface
WP Leader: Anna Borriello
The Work Package 2, Bioanchoring of ligands to the surface, is devoted to the functionalization of the sensor surface with different ligands that arebiologically equivalent, but with different chemistry, and to the selection of the most suitable ligands (among those that are biologically equivalentproposed by WP3), for surface derivatizationand thefunctionalization of the surface with the ligands after identification of the linkage chemistry. In particular, standard 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydrosuccinimide (EDC/NHS) coupling chemistry was used to link the specific bioreceptors (NH2-ligand) on the surface of the coated LPGs. The protein detection is focused on the AmpC and NDM-1 β-lactamases,while ligands that competitively inhibit this kind of protein are boronic acids. Two selected ligands for the biofunctionalization LPG probe are: 3-aminophenylboronic acid (3-APBA) and 5-aminobenzo[b]thiophene-2-ylboronic acid (BZD).
In this document we report on the work done in WP2, and in particular: the manufacturing, characterization, chemical functionalization of the sensor surface, bioanchoring of selected ligands and β-lactamases attachment tests. The prototypes of optimized LPGs-based reflection probes have been supplied from CeRICT.

Reflection type LPG transducer
It was proposed a multilayer approach for the coating of the LPGs in order to independently tune the working point in transition region and achieve an efficient surface functionalization without incurring the problem of the attenuation bands fading. In particular we used atactic polystyrene (aPS) as primary HRI coating, while the secondary very thin layer of poly(methyl methacrylate)-co-methacrylic acid (PMMA-co-MA) was then deposited to provide a carboxyls-containing surface minimizing at the same time its impact on the optical design of the device. This approach allowed to obtain desired functional groups (carboxyls) on the surface of the device for a stable attachment of bioreceptors and minimal perturbation of the optical design.
The possibility to interrogate the LPGs in reflection configuration has been investigated as a mean to transform a LPGs-based device in a more practical probe, with the collaboration of CeRICT group. As schematically represented in Figure 1, this requires the integration of a selective mirror at the end of the optical fiber. To this aim, the optical fiber inside which the LPGs is written has to be properly cut, and the fiber end-face has to be coated by a reflecting metallic layer, acting as a mirror.
Figure 2.1.

After having identified the LPG position, a high precision fiber cleaver (Fujikura CT-30 Series) is used to cut the optical fiber just after the grating, followed by the integration of an Ag reflecting layer (i.e. the mirror) on the terminal face of the cut optical fiber by Tollen's test.
After that the LPGs Grating was coated using the Dip Coating Technique (DC) (see Figure 2.2) by means of a 9.5% (w/w) solution in chloroform of atactic polystyrene, whose bulk refractive index (RI) is about 1.59. This deposition technique consists mainly inimmersing the LPG into a solution of the polymer and then withdrawing it with a well-controlled speed. The DC was performed by means of an automated system at an extraction speed of 108 mm/min. The aPS coated LPGs was subsequently dip-coated into a 10% (w/w) polymethylmetacrylate-co-metacrylic acid (PMMA-co-MA)solution in chloroform:isopropanol (1:3).

Figure 2.2.

Figure 2.3 shows a scheme of the optoelectronic set-up used either for the acquisition of the LPG spectrum (before and after the deposition of each overlay) and for the SRIcharacterization of the final device. It mainly comprises a broadband light source (with bandwidth 1200-1700nm), a 2x1 directional coupler and an optical spectrum analyzer connected to a PC.

Figure 2.3.

In order to perform the experiments, after solvent evaporation the double coated LPG was fixed straight at controlled room temperature. The device was fed by a superluminescent light emitting diode, and spectra were recorded each 45 seconds from a computer controlled optical spectrum analyzer. In this way the wavelength shift of the attenuation band was followed during the tests.
Highly sensitive real-time monitoring of multiple affinity assays between ligand and β-lactamase was performed by following the shift of the LPGs attenuation bands.

Biological experiments
A typical biological experiment consists in: chemical functionalization (activation of carboxyl groups by reaction with EDC/NHS, ligand anchoring and protein attachment. In the initial part of the project work, the biological experiments were performed by the use of procedures which have been improved and optimized only during the last months.

The work done in the first year of WP2has been detailed reportedin Deliverable D2.3. Overall practical and chemical aspects, data analysis interpretation and critical issue related to the experiments have been discussed. In particular critical issues were concerned about some kinds of instabilities of the sensors response that we observed during some of the β-lactamase detection tests, mainly occurring when the LPG probe is immersed in phosphate buffer solutions at pH=7.
In order to overcome the aforementioned issue, two possible strategies had been identified:
1) the definition of a new protocol, that avoids the use of the phosphate buffer solutions at pH=7;

2) the optimization of the probes, making use of a more stable overlay (instead of the polymeric ones) that could be safely used also in the phosphate buffer solutions at pH=7 (discussed by CeRICT in Integration of D2.3).

During the β-lactamase detection tests, the immersion of the LPG probe in phosphate buffer solutions at pH=7 (Deliverable 2.3, section 4.f) leads to some kinds of instabilities of the sensors response. The sensorgram obtained during one of β-lactamase detection test has been reported (a-b, Figure 2.4). It clearly shows that the sensor response is almost stable during the EDC/NHS coupling chemistry and exhibits the behavior expected (and typically occurring) in this phase (sensorgram a, Figure 2.4). However it shows a relevant drift (the barycentral wavelength strongly decreases) as soon as the probe is immersed in the phosphate buffer solution at pH=7. The response variation continued for all the biological test, without reaching a plateau value, therefore superimposing the typical LPG response against either the 3-APBA attachment (red circles in sensorgram b, Figure 2.4) and β-lactamase attachment test (for details see Integration of D2.3).
Figure 2.4.

Due to the very high sensitivity of the LPG biosensor to refractive index and thickness variations occurring in the close proximity of its functionalized surface, the salt aggregates attachment on the surface is detected by the probe as a blue shift in the barycentral wavelength (similarly to what happen in case of attachment of the ligand/protein molecules). This means that, even if the LPG biosensor is still able to reveal the permanent binding of either ligand and β-lactamase on its surface, the concurrent response dynamic, occurring due to the salt deposition on the polymeric surface of the LPG, precludes a quantitative interpretation of the probe response.
The pH=7 solution is the most common physiological environment, thus a necessary condition for the stability of protein structure. In order to overcome the aforementioned issue, double-distilled water (DDW, pH=7 and n=1.333) have been used (instead of phosphate buffer solution) in all biological test.
One of the most reliable β-lactamase detection test by using double-distilled water have been reported (Figure 2.5) by using 3-aminophenylboronic acid (3-APBA) ligand 5.84 mM and β-lactamase (AmpC) 5.0 □M. In overall test the LPG probe has been tested in water for a long time. As it can be observed from the analysis of overall results, the stability of the functionalized probe is preserved although the high immersion times of biosensor in solutions.
It is important to underline thatthe starting protein is dissolved in phosphate buffer pH=7 whose salts are essential to guarantee a stable reconstitution procedure of dialyzed protein. Nevertheless, the response in the sensorgram

showed a continued stability, and this behavior suggests that a reduced amount of salts in a diluted double-water solution of protein (the phosphate buffer is only the third part of final solution) is exploitable to guarantee the stability of the protein and consequently that of system.

Figure 2.5.

Improvement of the experimental procedures
During the last year of work several aspects of the experimental procedures have been improved. Firstly, as mentioned before, the definitionofa new protocol based on the use of double-distilled water has been defined.Actually, in order to carry out the experiments in more reduced times,the protocol has been improved alsoby the use of premixed solutions (while in previous experiments the ligand and protein solutions were added to the simple double distilled water). Furthermore, for the biofunctionalization of sensor surface, has been defined a standard procedure based on standard immersion timesenstablished on the basis of average values obtained from experiments.
In a typical sensor biofunctionalization experiment, the LPG coated sensor is immersed in double-distilled water (DDW) to acclimate the system, until the stabilization of the optical signal. Subsequently 500 □L of EDC/NHS solution (freshly prepared in DDW) was added to reach respectively 0.05/0.03 M (1:1) concentrations in the final solution. After 45 minutes the sensor is immersed in clean DDW(for 30 minutes) to remove the reagent nonspecifically adsorbed on the sensor surface. Thus, the so functionalized probeis immersed in a ligand solution (3.38 mM) for 1 h, so itisextracted and immersed in clean DDW for a washing of 30 minutes (also in this case to remove the reagent nonspecifically adsorbed on the sensor surface). The ligand anchoringsteps(immersion in a fresh ligand solution followed by the washing in DDW) are repeated using a freshly prepared ligand solutionin the same conditions.
All steps of the biofunctionalization procedure are summarized in the Table 2.1.

Table 2.1.
The dynamic of biological experiments (i.e. sensorgram) is continuouslymonitored through an automated spectral acquisition.In the obtained sensorgramis reported thewavelength variationof LPGattenuation versus time.
Once the anchoring of ligand on the surface was checked, the biofunctionalized LPG has been submittedto the protein attachment tests for both selected classes of β-lactamases: AmpC and NDM-1. Furthermore, in order to explore possible nonspecific protein binding, blank tests for both proteins have been also performed on functionalized probes with only EDC/NHS (that is without anchored ligand).
As for biofunctionalization, the protein attachment procedure has been improved by the use of premixed solutions. The experiments were carried out exploring different ranges of concentrations: in order to investigate the sensor surface response,the presence of an excess of protein (tipically higher of0.5□M) has been used, while much lower concentrations to achieve the Limit of Detection (LOD).
The two β-lactamases have been tested on both biofunctionalized LPG with selected ligands (see Table 2.2).

Table 2.2.

In a typical protein attachment test, the biofunctionalized LPG is immersed inDDW to acclimate the system. After the stabilization of the optical signal, it is immersed in a β-lactamase solution at lower concentration and successively, when the optical signal reaches a quite stable value, the device is immersed in clean DDW for a washing to remove the reagent nonspecifically adsorbed on the sensor surface. This procedure (immersion in protein solution and successive washing) is repeated using increasing concentrations of protein solution. All steps of the generic protein attachment testare reported in the Table 2.3.

Table 2.3.

The protein solution is prepared by dilution with DDW of a starting solution in phosphate buffer solution (PBS) at pH=7, whose salts are essential to guarantee a stable reconstitution procedure of lyophilized protein.
In the following section we report the typical results obtained in the β-lactamase attachment tests carried out by using the general procedure described.
AmpC attachment tests
• AmpC β-lactamase attachment test on 3-APBA biofunctionalized probe
Firstly, the tests have been carried out using β-lactamase concentrations of 0.3 and 0.5μM. Successively, higher concentrations (1.5 and 5.0 □M) have been explored.
• AmpC β-lactamase attachment test on BZD biofunctionalized probe
Firstly the tests have been carried out using very low concentrations: 0.05, 0.1 and 0.3 μM. Successively, also higher concentrations (0.5, 1.0 and 5.0 □M) have been explored.
As mentioned before, nonspecific protein binding has been explored through blank tests performed on functionalized probe with only EDC/NHS, so without any anchored ligand. Actually, any attachment of protein was detected, as suggested by the irrelevant shifts in LPG resonance wavelength obtained also at very high concentrations.
NDM-1 attachment tests
• NDM-1 β-lactamase attachment test on 3-APBA biofunctionalized probe
The tests have been carried out using a range of very low concentrations: 0.15 and 0.1 and 0.3 μM.
• NDM-1 β-lactamase attachment test on BZD biofunctionalized probe
The tests have been carried out using low concentrations: 0.15 and 0.1 and 0.3 μM.
As mentioned before, nonspecific protein binding has been explored through blank tests performed on functionalized probe with only EDC/NHS, so without any anchored ligand. In this case, the obtained shifts are higher than those of the AmpC blank test.

Data analysis: calibration curves and LOD
The β-lactamase attachment tests have been carried out by using several probes, each one characterized by slightly different sensitivity. Therefore, in order to trace the sensors calibration curves without being affected by the different probe sensitivities, we defined a new observable (O) as the ratio: , where$\Delta\lambda_{concentration}$ isthe central wavelength shift of the LPG resonance due to the protein binding onto the optical sensor surface, and $\Delta\lambda_{PMMA}$is the central wavelength shift due to the PMMA-co-MA (external layer) deposition, during the optical fiber fabrication process. The fitting data of AmpC attachment tests led to calibration curves (Figure2.6.a) calculated as a function of the cumulative AmpC concentration.In particular, the three calibration curves are related to the tests with the probes functionalizated with both ligands and without any ligand. It can be seen that the curves present a linear behavior at low concentrations of AmpC,while tend to a saturation values for higher concentrations.
Also for NDM-1 data we report the calibration curves as a function of the cumulative concentration (Figure 2.6.b).
Figure 2.6.
In order to calculate the Limit of Detection (LOD), we considered the sensitivity calculated as the derivate of the observable (O) with respect to the cumulative protein concentration (C) in the linear region. A comparison of calculated LOD of β-lactamases for each ligand-probe is reported in the Table 2.4.
Table 2.4.

The analysis of data suggests that the detection of NDM-1 in sample mixture, using LPG probes biofunctionalized (with 3-APBA or BZD), has been obtained at lower concentrations of protein compared to the AmpC.

Risk
During the first year, as already reported in WP1, critical issues related to some kinds of instabilities of the sensors response in the attachment tests had been observed, mainly occurring during the immersion of LPG probe in phosphate buffer solutions at pH=7. This problem has been overcome by using a new protocol in double-distilled water (DDW, pH=7 and n=1.333), instead of phosphate buffer solution, in all biological tests. It is important to underline that the starting protein was dissolved in phosphate buffer pH=7 whose salts are essential to guarantee a stable reconstitution procedure of dialyzed protein (the pH=7 solution is the most common physiological environment, thus a necessary condition for the stability of protein structure). Nevertheless, the response in the sensorgram showed a continued stability, and this behavior suggested that a reduced amount of salts in a diluted double-water solution of protein (the phosphate buffer is only the third part of final solution) is exploitable to guarantee the stability of the protein and consequently of the system. After that CeRICT and IMCB researchers decided to focus all their attention and efforts in performing the biological experiments exploiting the optimized protocol.
The procedure of biofunctionalization of LPG probe was improved exploiting a protocol based on enstablished times of the steps. Furthermore all biological tests were performed by the use of premixed mixture of reagent (ligand/protein), leading to optimal results in reduced experiment times.
In this period the experiments are devoted to test the biofunctionalized sensor surface in complex mixture containing the selected beta-lactamases.
Products and SME interest
In line with the demands of Optosmart S.R.L. SME and in collaboration with CeRICT, the prototype of biosensors based on

fiber optic technology was produced, in particular fiber optic sensor active surfaces were chemically functionalized in order to support the specific bioreceptor; successively the surfaces were functionalized with the ligands and the limit of detection (LOD) investigated. The LOD is the specific parameter for resolution in the biosensing field.
In particular LPG probes functionalized with two different selected ligands were prepared. The LOD for both beta-lactamases was obtained by performing biological tests in simple matrix of proteins.
WP3
New ligands identification and synthesis
WP Leader: Maria Paola Costi
The main work performed in WP3 is related to ligand design/selection and synthesis.
Task 3.1 Identification of new ligands through computational techniques
Computational analyses were devoted to the identification of known and unknown compounds for targeting the selected serine- and metallo-beta lactamases, i.e. CTX-M-15, KPC-2, AmpC, VIM-1, NDM-1. Docking and virtual screening experiments were performed to first select know ligands immediately available to target both serine- and metallo-beta-lactamases, and then to identify new possible inhibitors. Also, alinker for 2-4 selected ligands to ensure a suitable binding to the probe surface and allow a better recognition of the BL in solution was also designed.
Sets of ligands usable for identify the aforementioned proteins in a multi-ligand approach were selected.
a) Selection of known ligands for targeting serine and metallo-beta lactamases.
Different ligands known to bind the aforementioned proteins and almost ready to be anchored on the ALD probe were selected. Boronic-based compounds already demonstrated to well inhibit AmpC and were extensively investigated by UNIMORE researchers. The X-ray structures of AmpC with 3-APBA, BZB and a derivative of BZD (PDB codes 4lv0, 1c3b, 2i72, respectively) also support the stability of the complexes and demonstrate that the compounds could be easily derivatized by attaching a linker. 3-APBA and BZD, in fact, present a primary amino moiety, which could be directly exploited for anchoring the ligands on the probe surface or used as anchoring point for the attachment of a linker. BZB could be as well, easily derivatized (Scheme 3.1).

Scheme 3.1.
Compounds 1 and 3 were sent to WP2 to be anchored onto the probe surface since they already bear a primary amino group. Other compounds have been identified to complete the ligand set profile.

b) Virtual screening for the identification of unknown ligands (details are reported in Deliverables 3.2, 3.3, 3.4).
In order to identify new scaffolds able to target the different ESBLs, the Specs database (www.specs.net) was screened against the selected β-lactamases. The original library of about 200.000 compounds was filtered according with the molecules LogP predicted by Moka, to retain compounds with LogP lower than 2, thus likely soluble for the subsequent in vitro analyses. Then, tautomers and protomers were built using again the Moka software. The final library contained about 27.000 compounds. After performing the first screening, the highest scored compounds (about 1% of the whole library) were re-docked into the target binding site with the best modality, taking more computational time but giving more reliable results. The higher the FLAP S-Score is, the higher should be the affinity of the molecule towards the target. The models of all compounds into the corresponding pocket were visually inspected. The most interesting molecules, according with the FLAP S-Score, the pose, the number of hydrogen bonds formed with the surrounding residues and the chemical diversity, were purchased and will be subsequently tested in experimental assays by WP4.
A total of 83 molecules were thus selected for CTX-M-15, KPC-2, IMP-1, NDM-1, and VIM-1. Molecules were also chosen in order to have a diverse chemical structure, thus increasing the possibility of giving peculiar responses according to the β-lactamase present in the samples. (see Deliverable 3.4 for further details).

The inhibition activity showed by the 83 compounds towards the different β-lactamases are graphically reported in Figure 3.1, where the color-code representation allows a clearer identification of the compounds specificity profile.
Of the 21 molecules specifically selected for CTX-M-15, 19 demonstrated to have a certain level of activity towards the target. In particular, three of them, OP57, OP61 and OP74 showed a calculated IC50 lower/equal than 100 μM. For KPC-2 even better results were obtained, since 14 over 14 selected compounds were active and 12 of them had a calculated IC50 lower than 100 μM. Of the 17 compounds selected to target VIM, 9 resulted actives and three of them (OP24, OP33, OP68) showed IC50 values lower/equal than 130 μM. Finally, for NDM-1, 8 over 23 compounds presented a certain leel of activity, with two of them (OP56 and OP65) having a calculated IC50 lower than 75 μM.
Some compounds (highlighted in blue in Table 3.1 In D3.4) showed poor solubility in solution, which could affect the absence of any inhibition activity towards the β-lactamase.
These results significantly overcome the standard virtual screening success rate, which, for random screenings, typically ranges from 0.1 to 0.5%.
No compound was specifically selected for targeting AmpC since boronic compounds, as 3-APBA, BZB and BZD (serine-BL controls), were known to inhibit this class C β-lactamase. Nevertheless several compounds showed calculated IC50 lower than 200 μM, with OP39 presenting an interesting specificity profile.

Figure 3.1.

The compounds showing the most peculiar profile and likely able to select the investigated proteins in a multi-ligand approach were selected and combined in three different sets. The known
AmpC inhibitor BZD, synthesized by UNIMORE, was also added to the sets. No molecule was, in fact, specifically select to target AmpC, given the presence of ligands able to target it with IC50 in the nanomolar range.
The sets of ligands are reported as follows in Figures 3.2-3.4.

Figure 3.2.

Figure 3.3.

Figure 3.4.

According to the results obtained with the first screening, different analogues were purchased at Sigma Aldrich and selected (re-synthesized) from Tydock in house database. All the compounds are derivatives of molecules OP02 and OP06 and were chosen because of their availability and of the possibility to chemically modifying them, for improving the affinity against the protein target and for the linker attachment (Figure 3.5). The choice was also performed taking into account the orientation assumed by the ligands after docking simulations in AmpC and KPC2. (see D3.4 with undisclosed structures).

Figure 3.5.

Compounds 7, 9, 15, 16 showing the highest inhibition activity towards KPC-2 and AmpC were selected and suggested as possible compounds to be anchored onto the optical probe. Compounds were also chosen according to the availability of an amino group (cpd 15) or of a nitro group easily reducible into an amino one (cpd 9). Compound 5, presenting one of the most different inhibition profile towards KPC-2 and AmpC, and being easily modifiable through the substitution of the bromine with an amino group, was also selected.
Given the promising results obtained from the second screening, we decided to expand the set of Ty MPC 34 and Ty MPC 46-like compounds (compounds 9 and 15 in Table 3.3, D3.4). This choice was, again, guided by the inhibition % estimated towards the targets but also by the possibility of chemically modify the molecules.
A larger list of 32 compounds was thus synthesized and tested against AmpC and KPC-2. All molecules present a naphthalic or a phthalic anhydride core (Figure 3.6). The analyses were performed in a structure-activity relationship perspective, to investigate the activity of the different substituent groups. Inhibition assays results are reported in D3.4 with undisclosed structures.

Figure 3.6.

All the compounds showed high activity level against AmpC and KPC-2, presenting IC50 values in a low micromolar range. This profile could be explained by the capability of the molecules to covalently attack the target, forming a covalent bond with the catalytyc serine, thus mimicking the standard antibiotics mechanism of action.
Most of the molecules belonging to the third library demonstrated to be poorly soluble in water. The solubility could be improved by performing chemical substitutions and/or modification of the most promising ligandsbest properties for chemical modifications and linker attachment.
The compounds more suitable to be functionalized for an amino group or linker attachment, i.e. Ty MPC 26, Ty MPC 32, Ty MPC 35, were chosen and suggested as possible new compounds able to target serine BL.
FINAL COMPOUNDS SELECTION
The following set of compounds, obtained by the first virtual screening showed the most interesting inhibition profile

and were selected to possibly functionalized the ALD optical probe upon chemical derivatization.
- Set1 (OP10, OP33, OP34, OP39, OP61, BZD).
- Set 2 (OP13, OP32, OP39, OP49, OP74, BZD).
- Set 3 (OP05, OP35, OP49, OP50, OP68, BZD).
From the second and third selection runs the following molecules demonstrated to have the best inhibition towards serine β-lactamases.
- 3-bromomethyl phtalic anhydride, Ty-MPC-43 (cpd 5 from second library).
- 2,3-naphtalenedicarboxylic anhydride (cpd 7 from second library).
- 4-[(3-nitro-phenylamino)-methyl]-isobenzofuran-1,3-dione, Ty-MPC5-46 (cpd 9 from second library).
- 6-amino-benxo[de]isochromene-1,3-dione, Ty-MPC5-34 (cpd 15 from second library).
- 2-benzoyloxy-2-Phenylmalono-Nitrile (cpd 16 from second library).
- Ty-MPC5-26 (cpd 11 from third library).
- Ty-MPC5-32 (cpd 17 from third library).
- Ty-MPC5-35 (cpd 19 from third library).
According to the experimental evidences and to the proposed covalent mechanism of reaction, these last molecules could be reasonably used to target the aforementioned serine β-lactamases with respect to metallo β-lactamases. Still we have to consider that in order to be anchored to the ALD optical prope, any molecule should have a primary amino moiety able to interact with the carboxylate groups functionalizing the probe surface. Also, the presence of a linker could help ameliorating the molecules solubility (the bioachoring process has to be performed in water solution) and could even improve the recognition of a specific BL.
Most of the selected molecules should thus be chemically modified in order to allow the bioanchoring and to ameliorate the protein binding.
MOLECULES READY FOR BIOANCHORING
The ligand ready for bioanchoring and sent to WP2 are:
1) 3-APBA, known inhibitor of AmpC; Scheme 6, compound 1.
2) BZD, known inhibitor of AmpC; Scheme 6, compound 2. Both 3-APBA and BZD interact with AmpC through the formation of a covalent intermediate.
3) Captopril, known inihibitor of MBL. The ligand L-captopril shows Kd values with respect to IMP-1 and NDM-1 of about 12.5 □M and 39 □M, respectively, and has been co-crystallized with NDM-1 (PDB code 4exs). The selection has been thus performed according with the crystallographic evidences and the several information found in literature, testifying the capability of sulfhydryl compound of binding MBLs. As reported by King et al., the sulfhydryl group exists as an anion, mimicking the catalytic hydroxide, because of the zincs effect which lowers the pKa of bound thiols groups by ~2 orders of magnitude. Scheme 6, compound 3. In order to be anchored to the ALD probe, captopril was derivatized by using 1,4-butanediamine to add a primary amino moiety onto the carboxylic group (Scheme 6, compound 3; see D3.5 for chemical synthesis details).
4) Compounds derived from AK-968/41924915 (OP68) and AK-968/41923225 after reduction of the nitro group to a primary free amine;Scheme 10, compounds 4 and 5. According to the enzimatic assays and to the observations previously reported these ligands could help in identifying CTX-M-15 and KPC-2 (see set 1). The presence of a nitro group, which could be easily reduced to an amine, make these compounds almost ready for the bioanchoring process.
5) Compound Ty-MPC5-46 after reduction of the nitro group to a primary free amine;Scheme 10, compound 6. According to the enzimatic assays previously reported these ligands should be able to recognize serine BL, in particular KPC-2 and AmpC, through the formation of a covalent intermediate.
Task 3.2 Synthesis of the selected ligands
The synthetic work was mainly focused on two different strategies:

a) Synthesis of benzo[b]thien-2-yl boronic acid derivatives
b) Introduction of a linker in known inhibitors of β-lactamases
c) Other synthetic approaches necessary for providing new products to the projects.
See D3.5 with undisclosed synthesis

Important comments on the overall results achieved and experimental design.
As reported in RW1, given the structural and pharmacophoric similarities of β-lactamases, and the consequent difficulties of identifying a single specific ligand for each target as initially reported in the project, consortium proposed to adopt a bioprofile approach instead of a one-ligand method. Thus the compounds were selected in order to have significantly different affinity with respect to the different proteins and to present specific bioprofiles, or fingerprints, able to identify a unique single target with respect to the others. In summary, the identification of the specific β-lactamases was carried on according with Figure 3.7.

Figure 3.7.
The fingerprints provided by in vitro analyses performed by WP4, i.e. enzymatc assays of the selected compounds towards the investigated β-lactamases, gave information about the inhibition activity shown by the ligands in solution. As pointed out in D2.1 and D4.4, there is no direct correlation between the inhibition activity estimated in vitro, expressed in terms of inhibition percentage, IC50 or Kd, and the Limit of detection (LOD) determined once the compounds are anchored on the ALD optical probe. It is thus difficult to predict a priori, which compounds should be anchored on the probe to better target the selected BL, only basing on in vitro experiments. The LOD, in fact, could be affected by:
i) the optical properties of the ligands and of the proteins, e.g. NDM-1 bearing two zinc ions in the binding site gave stronger signals with respect to AmpC upon binding to 3-APBA or BZD, despite the lower affinity estimated in solution.
ii) The reduced mobility of the ligands upon anchoring on the ALD probe surface. The anchoring implies a significant change of the binding energetics given by the reduction of the degrees of freedom and by the desolvation effects.
The compounds selection thus results to be an empirical process, rather then a computationally/experimentally driven prediction. Compounds have to be chosen after the estimation of the LOD upon bioanchoring on the optical probe surface, beacuse of the many effects influencing the protein/bioanchored-ligand recognition.
Nevertheless, the selected panels of compounds provide valuable tools for the identification of the aforementioned proteins not only in a ALD system, but possibly in plate-reading systems or in displacement assays using fluorescent probes, chps and microfluidic systems.
Risk
The risk for this work was that no new ligands would be identified by the virtual screening procedure and subsequent experimental testing.
The computational approach FLAPdock was developed and extensively validated against known experimental data on a range of targets so that the confidence of being able to distinguish effective ligands from ineffective ligands was increased. This strategy was effective since we achieved a 30-50% success rate after ligand selection of new hits; random selection is usually less than 0.5% for virtual screening.
Poor solubility was identified for some of the selected compounds. This negatively affected the in vitro tests and the anchoring of the compounds to the optical probe. A linker for improving the solubility was specifically designed, synthesized and attached to the selected compounds
Products
(1) ligands sets for BLs sensing;
(2)scaffolds for further ligands developemnt;
(3) one compound series has been identify that could be patented. (4) SOPs for protein detachments and probe regeneration
(5) linker chemistries.
SME interest
1. There is interest in the identified compounds (ligand sets) as ligands that can detect the various beta-lactamases in analytical applications such as the ALD that is the ultimate goal of this project. Once protected in terms of IP, the probes functionalized with these ligands could be commercialized along with the ALD.
2. There is interest in the novel compound with undisclosed structure, to develop for ligand optimization and patenting.
3. There is interest in the use of these identified compounds as antibiotics, however from a drug discovery perspective it is likely that many years of work would be required to optimize the compounds to turn them into effective drugs, therefore this aspect has quite a low probability of short term success. More investments are necessary.
4. The developed FLAPdock software is of immediate interest to Molecular Discovery, since this is (a) the core business expertise of the company, (b) it fits well within the FLAP product suite that Molecular Discovery is offering, (c) this project provides further evidence of the effectiveness of Molecular Discovery software approaches and (d) MD customers are already using the software in their drug discovery projects.
5. There is an interest in the SOP for detachment procedure because this would save time and costs.
6. There is interest in the scaffolds identification for further studies.
WP4
Validation of the inhibitor effect on β-lactamases
WP leader: Maria Paola Costi, Jesus Blazquez

WP4 was mainly involved into the biochemical characterization of beta-lactamases and into the validation of the

inhibitory effects of the ligands selected by WP3 towards the related targets.
The most clinically relevant beta-lactamases, i.e. CTX-M-15, KPC-2, NDM-1, VIM-1, VIM-13, were cloned in p-GEX-6PI and pCA24N expression vectors by IBIS researchers.
Proteins were purifiedthrough several steps of ion exchange chromatography followed by a final step of gel filtration, except for NDM-1 (Class-Bβ-lactamases), for which a Ni/Hys-TAG affinity chromatography was performed. Each β-lactamase protein was characterized from a kinetic point of view determining kcat and Km values against the main β-lactam antibiotics substrates (Cephalothin-Nitrocefin-Chromothin), obtaining for each of them a good correspondence level to values reported in literature. The ligands selected by WP3 were tested through medium throughput assays (Spectramax-Multiplate Reader, MOLECULAR DEVICES) at the specific wavelength of the employed β-lactam antibiotic substrate, using a saturating concentration (≈10-times Km value).
More details about the experimental procedures are reported hereafter in the paragraphs describing tasks 4.1, 4.2 and 4.3.

Tasks 4.1 IC50 determinations against beta-lactamases and other drug resistance biomarkers.
Task 4.2 Biochemical characterization: beta-Lactamase purification, detailed kinetics, and UV Differencespectroscopy.
Task 4.3 Cloning and purification of enzymes inferring bacterial resistance to inhibitors.

Initially, known ligands from Tydock Pharma databases were tested against a number of strains to identify their biological profile. The strains were prepared by Jesus Blázquez lab. The list of the strains is reported below.At the beginning of the projects the relevant BL from different strains were not available yet. Therefore to know which compounds should be selected for the detector functionalization, we directly tested them onto the strains. The obtained data represented a preliminary screening step whichhas been followed by the direct detection against the purified recombinant enzyme.
Beta-lactamase selected and corresponding crystallographic structures available in the Protein Data Bankat month 9.
CTX-M-14: 2 structures from Escherichia coli, one in the apo form (1ylt) and one complexed with an inhibitor (1ylz).
CTX-M-15: 2 structures from Escherichia coli recently released, one in the apo form (4hbt) and one complexed with an inhibitor (4hbu).
KPC-2: 7 structures from Klebsiella pneumoniae, one apo form (3dw0), three complexed with inhibitors (3rxx, 2ov5, 3rxw), two complexed with the B-lactamase Inhibitor Protein, one with a short deletion at the C-terminal (3c5a).
OXA-23: no structure.
OXA-24/40: 9 structures from Acinetobacter baumannii, two apo (3g4p, 2jc7), four complexed with inhibitors (3fyz, 3hbz, 3fv7, 3fzc) and two mutants (3pag, 3pae, 4f94).
OXA-48: 1 structure from Klebsiella pneumoniae in the apo form with a modified lysine (3hbr).
OXA-51: no structure.
IMP-1: 8 structures, three from Pseudomonas aeruginosa complexed with ligands (1dd6, 1jjt, 1jje), one apo form from Pseudomonas aeruginosa (1ddk), two from Serrata marcescens complexed with ligands (1vgn, 2doo), and two mutant always from Serrata marcescens (1wuo, 1wup).
VIM-2: 3 structures from Pseudomonas aeruginosa, one apo form with a reduced cysteine (1ko3), one with an oxidized cysteine (1ko2) and one with an inhibitor (2yz3).
VIM-4: 2 structures from Pseudomonas aeruginosa complexed with citrate (2whg and 2wrs).
NDM-1: 22 structures all from Klebsiella pneumoniae with the exception of one apo form from Escherichia coli (3s0z). The others are crystallized in the apo form (3pg4, 3zr9, 3spu, 3rkj, 3rkk), with inhibitors (3q6x, 4eyf, 4exs, 4exy, 4ey2, 4eyl, 4eyb, 3sbl, 3sfp, 4hl1, 4hl2, 4hky), with different metals rather then the catalytic zinc (4h0d, 3srx) or in a mutated form (4gyq, 4gyu).
Proteins cloning and purification.
B-lactamasecloning.Three different types of β-lactamases, CTX-M-15, KPC-2 and NDM-1 were cloned into pCA24N cloning vector (Fig. 4.1), and transformed into Escherichia coli DN5α. Positive clones, able to grow on LB plates supplemented with ampicillin 100 µg/ml and chloramphenicol 50 µg/ml, were re-checked by PCR, digestion and sequence analysis. PCR of the cloned β-lactamases gave positive results for the three clones. Digestion by SfiI, gave also the release of the fragment. It is important to highlight that the restriction sites for SfiI are only generated if the fragment is cloned in the right direction. Sequence analysis also confirmed the cloning.

Figure. 4.1.

CTX-M clones.Different types of CTX-M β-lactamases were also available for the study. The following beta-lactameses were cloned in the pBGS18 cloning vector:CTX-M-1, CTX-M-3, CTX-M-15, CTX-M-30, CTX-M-32, CTX-M-62.
MIC against bacterial strains expressing AmpC.
The activity of the boronic acids compounds was investigated against several cellular lines expressing different β-lactamases isoforms, obtained from the National Center for Biotechnology- Consejo Superior de Investigaciones Cientificas of Madrid (CSIC). The analyses were performed following the guidelines of the National Committee for Clinical Laboratory Standards (CLSI), using a ratio of 1:1 antibiotic/inhibitors..
In the first experiment, the MIC (Minimum Inhibitory Concentration) of compounds 3-propionic-benzo[b]thien-2-yl boronic acid (3-PBZB), 5-methyl-benzo[b]thien-2-yl boronic acid (5-Me-BZB), 7-carboxy-benzo[b]thien-2-yl boronic acid (7C-BZB) and the 5 bromo-methyl-benzo[b]thien-2-yl boronic acid (5Br-BZB), in combination with Ceftazidime (CAZ), was evaluated against 11 cellular lines overexpressing of AmpC-β-lactamases, using the benzo[b]thien-2-yl boronic acid as the reference.

Ligand selection among known boronic acid inhibitors.
Selection of boronic acid derivatives among known ligands was performed through:
- cell-based assay against more than 10 BL producing bacterial strains such as AmpC.
- protein-based assay in which the boronic acid derivatives have been tested against 4 proteins: CTX-M-15, KPC-2, AmpC, NDM1.
.
Biological evaluation of benzo[b]thien-2-yl boronic acid derivatives.
The activity of the boronic-acid compounds was investigated against several cellular lines expressing different β-lactamases isoforms, obtained from National Center for Biotechnology- Consejo Superior de Investigaciones Cientificas of Madrid (CSIC). The analyses were performed following the guidelines of the National Committee for Clinical Laboratory Standards (CLSI), using a ratio of 1:1 antibiotic/inhibitors.
In the first experiment, the MIC of compounds 3-propionic-benzo[b]thien-2-yl (Ty-BB07) boronic acid, 5-methyl-benzo[b]thien-2-yl boronic acid (Ty-BB25), 7-carboxy-benzo[b]thien-2-yl boronic acid (Ty-BB70) and the 5 bromo-methyl-benzo[b]thien-2-yl boronic acid (Ty-BB30), in combination with Ceftazidime (CAZ) was evaluated against 11 cellular lines with overexpression of AmpC-β-lactamases, using the benzo[b]thien-2-yl boronic acid (BzbTh-2B) as the reference.
The test of the compounds against the bacterial cells provides information regarding the ability of the compounds to enter the bacterial cells and inhibit cellular growth, i.e. the efficacy of the compounds against a specific protein that is the main target of the compound. The assay is conducted in a way that the BL inhibitor is added to the reacting solution containing the beta-lactam antibiotic (Ceftazidime). The strains tested are usually expressing high level of BL belonging to the BL set proposed in the project (see WP4) . The BL inhibitor effect in this context shows a specific effect.
AmpC represent the first recombinant protein that was used in the initial experiments. Almost all the boronic compounds are active against the AmpC overexpressing bacterial cells and showed also IC50 in the microMolar to nanoMolar range against the recombinant protein AmpC (data not shown).
As an example, in further analysis, the activity of compounds Ty-BB07, 3-[5-diformylaminomethyl-2-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-benzo[b]thiophen-3-yl]-propionic acid (Ty-BB78), 3-[5-Aminomethyl-2-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-benzo[b]thiophen-3-yl]-propionic acid (Ty-BB91), 7-amino benzo[b]thien-2-yl boronic acid (Ty-BB80) and N-BOC-7-amino benzo[b]thien-2-yl boronic acid (Ty-BB74) was evaluated against several bacterial strains expressing different isoforms of β-lactamases, in combination with Cefotaxime (CTX). The compounds BZBTh-2B and Ty-BB89 (5 methyl-amino-benzo[b]thien-2-yl boronic acid) were used as the reference.

The graph reported in Figure 4.2 shows the compound effect=compound profile. A large variation of the inhibitory effect of the compounds in combination with Cefotaxime against the three different strains can be observed. KPC-2 and CTX-M15 are the most sensitive to the boronic acid set.

Figure 4.2.

The same compounds were also evaluated in terms of inhibition effect against the same isolated BL-proteins.
In Figure 4.3 the IC50-graph shows a very similar correspondence between the MIC-values and IC50-values for the boronic acid set, in fact, in the same way, all the compounds able to reduce the MIC-value also present the best enzyme-kinetic profile.

Figure 4.3.

Figure 4.4.

The diagram reported in Figure 4.4 shows the IC50 against different BL and the corresponding effect on the bacterial cellsover-expressing the same proteins. It can be observed that the trend of the effect observed on the protein is very similar to that against the corresponding cells. This observation suggests that the compounds are able to enter the bacterial cells and specifically inhibit the BL protein into the cells.
We can use the entire bacterial cells as a model for testing the compounds in complex matrices similar to the one that will be used in the final clinical testing. In fact BL could be inside the bacterial cells or outside. However the bacteria cell membrane will be lysed and the cellular suspension will be used as sample for testing.
Finally, based on the above results, the compounds selected for the first trial corresponding to 2 known compounds available in our in house library are the 3-aminophenyl boronic acid and Ty-BB89 (5 methylamino-benzo[b]thien-2-yl boronic acid).

β-LACTAM PROTEINS PURIFICATION reported in WP4 Deliverables.
CLASS-A β-LACTAMASES:
• CTXM-15: Protein Production and Purification Procedure
• KPC-2: Protein Production and Purification Procedure
CLASS-B β-LACTAMASES:
• VIM-1: Protein Production and Purification Procedure
• NDM-1: Protein Production and Purification Procedure
CLASS-C β-LACTAMASES:

PURIFING RESINS-PREPARATION AND MAIN COLUMN-FEATURES
PROTEIN LYOPHILIZATION

PROTEIN IDENTITY CONFIRMATION BY MASS-SPECTROMETRY EVALUATION
β-LACTAMASE ENZYME SCREENING ASSAY

The OPTO-library including 83 compounds identified and selected from docking studies by WP3 was screened against all purified β-lactamase enzymes by spectrophotometric assay using a 96-well Multiplate-Reader (Spectramax-190, Molecular Devices). The compounds inhibition activity was tested at fixed concentration value equal to 100μM (except for VIM-1 β-lactamase, 200μM) performing three replicates for each compound, in order to be able to calculate an error value with an acceptable statistical relevance (ρ*0.025). The compounds inhibition activity has been evaluated against β-lactamase enzymes Cephalothin-hydrolysis at final concentration equal to 100μM, for a total kinetic time equal to 300seconds at the specific wavelength for employed β-lactam antibiotic substrate (Cephalothin: 265nm), in order to evaluate the ß-lactam ring opening kinetic-profile in the time. For each β-lactamases family a well-known inhibitor-compound has been included in the compounds-panel to test: Captopril, for Metal-β-lactamases and, 3APB (3-aminophenylboronic-acid), for Serine-β-lactamases. Moreover, considering compounds were dissolved in DMSO, in the screening assay, DMSO-effect on the β-lactamases kinetic activity has been also evaluated. From an experimental point of view, it is important to add the different substrates in a specific order into the 96-well plate:Kynetic-Buffer with a specific composition depending on the specific β-lactamase enzyme considered in the assay; enzyme, in such amount to have an enzymatic activity value equal to 0.07-0.08 range values (ΔOD/min); inhibitor-compoundand antibiotic substrate, added in order to reach the enzyme saturation conditions (normally, 10 times than Km value). Absorbance-Time linear-regression relationshipraw data have been elaborated and by comparison between slope value of control enzyme kinetic activity without inhibitor and slope value of enzyme kinetic activity in presence of the different compounds to assay, it was possible to determine inhibition percentage values of library-compounds against the various tested β-lactamase proteins.

β-LACTAMASES FUNCTIONALIZED LAYER DETACHMENT FOR ALD-PROBE REGENERATION
(reported in Deliverable 2.1)

NOTES It's important to underline that despite the expression system and the bacterial host are the same of the VIM-1 beta-lactamase, KPC-2 protein is intracellular and so, it is essential to sonicate the cellular pellet to recover the enzyme activity while the VIM-1 is extracellular and it is found in the broth culture.

Task 4.4 Construction of first line testing strains.

The most clinically significant B-lactamase-encoding genes were cloned in known plasmids and introduced into Escherichia coli K-12. These engineered strains serve as both first line testing strains and B-lactamase hyperproducers. Also, a number of pathogens, expressing clinically relevant B-lactamases, were collected.

4.4.1 β-lactamase genes cloning

β-lactamase genes cloned in pGEX-6P-1
The bla genes were PCR amplified and cloned into the BamHI and EcoRI restriction sites of the pGEX-6P-1 vector. Gene sequences encoding for the signal peptide were eliminated to avoid export problems in the bacterial cell. Cloning of the gene was verified by restriction and sequencing. This cloning allowed the creation of a fusion protein between glutathione S-transferase (GST) and the Bla enzymes lacking the signal peptide. The GST tag can be cleaved off and the β-lactamases purified with the GST gene fusion system (Amersham Pharmacia Biotech, Europe GmbH), in accordance with the manufacturer's instructions.
Expression plasmids were introduced by transformation into E. coli BL21, a strain defective in OmpT protease production. It is the host of choice for expression studies with GST-tagged proteins, because it minimizes proteolysis of the tagged protein (seen as multiple bands on polyacrylamide gels).
The expressed protein is a fusion GST (Glutathione S-transferase)-B-lactamase (Figure 4.5). Once purified by affinity chromatography, the GST-tagged protein must be removed with PreScission Protease to obtain the B-lactamase. Note that, once purified and cleaved, the B-lactamase will have 5 additional aminoacids at the N terminus (GlyProLeuGlySer-).

Figure 4.5.

Cloning of VIM-1 from Pseudomonas aeruginosa
Sequence ofVIM-1 protein (signal peptide in gray):

MLKVISSLLVYMTASVMAVASPLAHSGEPSGEYPTVNEIPVGEVRLYQIADGVWSHIATQ
SFDGAVYPSNGLIVRDGDELLLIDTAWGAKNTAALLAEIEKQIGLPVTRAVSTHFHDDRV
GGVDVLRAAGVATYASPSTRRLAEAEGNEIPTHSLEGLSSSGDAVRFGPVELFYPGAAHS
TDNLVVYVPSANVLYGGCAVHELSSTSAGNVADADLAEWPTSVERIQKHYPEAEVVIPGH GLPGGLDLLQHTANVVKAHKNRSVAE

Molecular weight: 28.03 kDa
VIM-1 without signal peptide:
GEPSGEYPTVNEIPVGEVRLYQIADGVWSHIATQSFDGAVYPSNGLIVRDGDELLLIDTAWGAKNTAALLAEIEKQIGLPVTRAVSTHFHDDRVGGVDVLRAAGVATYASPSTRRLAEAEGNEIPTHSLEGLSSSGDAVRFGPVELFYPGA
Primers used for Cloning blaVIM-1 (without signal peptide) into pGEX-6P-1 (BamHI-EcoRI) for purification
V1-F-BHI TCGGATCCGGGGAGCCGAGTGGTGAG
V1/13-R-ERI TCGAATTCCTACTCGGCGACTGAGCG
Cloning of VIM-13 from Pseudomonas aeruginosa
Sequence of VIM-13 protein (signal peptide in gray):

MLKVISSLLFYMTASLMAVASPLAHSGESRGEYPTVSEIPVGEVRLYQIDDGVWSHIATHTFDGVVYPSNGLIVRDGDELLLIDTAWGTKNTVALLAEIEKQIGLPVTRSVSTHFHDDRVGGVDALRAAGVATYASPSTRRLAEAEGNEVP

Molecular weight: 28.220 kDa

VIM-13 without signal peptide:
GESRGEYPTVSEIPVGEVRLYQIDDGVWSHIATHTFDGVVYPSNGLIVRDGDELLLIDTAWGTKNTVALLAEIEKQIGLPVTRSVSTHFHDDRVGGVDALRAAGVATYASPSTRRLAEAEGNEVPTHSLEGLSSSGDAVRFGPVELFYPG

Primers used for Cloning blaVIM-13 (without signal peptide) into pGEX-6P-1 (BamHI-EcoRI) for purification
V13-F-BHI TCGGATCCGGGGAGTCGAGAGGTGAG

V1/13-R-ERI TCGAATTCCTACTCGGCGACTGAGCG

Cloning of KPC-2.
Sequence of KPC-2 protein:
MSLYRRLVLLSCLSWPLAGFSATALTNLVAEPFAKLEQDFGGSIGVYAMDTGSGATVSYRAEERFPLCSSFKGFLAAAVLARSQQQAGLLDTPIRYGKNALVPWSPISEKYLTTGMTVAELSAAAVQYSDNAAANLLLKELGGPAGLTAFI
Primers used to clone kpc-2 gene in pGEX-6P-1 (without signal peptide)
KPC-2-GST-F: 5´- BamHI-TCGGATCCCTGACCAACCTCGTCGCGGAA
KPC-2-GST-R: EcoRI-TCGAATTCTTACTGCCCGTTGACGCCCAAT
Size of PCR fragment: 826 bp

Cloning of CTX-M-15 BETA-LACTAMASE
Sequence of CTX-M-15 protein (signal peptide in gray):
MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTMSLAELSAAALQYSDNVAMNKLIAHVGGPAS
Molecular weight: 31.144 kDa.
CTX-M-15 without signal peptide
TADVQQKLAELERQSGGRLGVALINTADNSQILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTMSLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Primers used to clone kpc-2 gene in pGEX-6P-1 (without signal peptide)
KPC-2-GST-F: 5´- BamHI-TCGGATCCCTGACCAACCTCGTCGCGGAA
KPC-2-GST-R: EcoRI-TCGAATTCTTACTGCCCGTTGACGCCCAAT
Size of PCR fragment: 826 bp

Cloning of NDM-1
Sequence of NDM-1 protein (signal peptide in gray):
MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQHTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAAGIATYANALSNQ
Molecular weight: 28.499 kDa.

NDM-1 without signal peptide:
GEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQHTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATA
Primers used for cloning ndm-1 gene in pGEX-6P-1 (without signal peptide)
NDM-1-GST-F: 5´- BamHI-TCGGATCCGGTGAAATCCGCCCGACGATTG
NDM-1-GST-R: 5´- EcoRI-TCGAATTCTCAGCGCAGCTTGTCGGCCATG
Size of PCR fragment: 745 bp

B-lactamase genes cloned in pCA24N
NDM-1
The gene encoding NDM-1 was PCR amplified from a well characterized clinical strain of Escherichia coli, cloned in the expression vector pCA24N, conferring resistance to Chloramphenicol (Figure 4.6), and introduced into Escherichia coli K12 strain DH5□.
Cloning of the gene was verified by restriction and sequencing. This allowed us to assure the presence of the gene, the appropriate direction in the vector and the absence of any mutation in the structural gene.
Induction of B-lactamase expression.
The inducibility of B-lactamase expression was tested by growing the strains, harbouring the vector with the NDM-1 B-lactamase, in LB broth to an optical density of 0.5 to 0.8. 1mM IPTG was added to the cultures and incubated for 3-6 additional hours with strong shaking. E. coli cells were harvested, washed, sonicated and the supernatant was recovered after centrifugation. B-lactamase overproduction was evaluated by detection of proteins by electrophoresis and the activity against nitrocefin.
Purification should be done by standard procedures for B-lactamase purification.

Figure 4.6.

4.4.2. Pathogens expressing clinically relevant B-lactamases.
The following pathogens were collected and verified phenotypic and genotypically (by PCR amplification and sequencing of the B-lactamase encoding genes; see Tables 4.1 and 4.2).

Table 4.1.
Table 4.2. .

These first-line strains were used to test in vivo 30 different putative ligands, which could act as inhibitors of B-lactamase in living bacteria. These assays are complementary of those stated in WP-3. The results obtained are showed in the following tables 4.3, 4.4 and 4.5.
Table 4.3.
Table 4.4.
Table 4.5.

Task 4.5 Testing the device provided by WP2 against complex matrices.

AmpC β-lactamase in broth attachment tests
• AmpC β-lactamase attachment test on 3-APBA biofunctionalized probe
In the following section we report the results obtained in the β-lactamase binding tests carried out by using the general procedure described in D2.1,section 3.1onto the 3-APBA and BZD biofunctionalized sensor surface.
The dynamic of biological experiments (i.e. sensorgram) is continuously monitored through an automated spectral acquisition. In the obtained sensorgram is reported the central wavelength variation of LPG attenuation band versus time.
Thus, for each test will be reported the sensorgram, the calibration curves and the Limit of Detection (LOD) of the pure and in broth AmpC protein.
The first test has been carried out using AmpC concentrations of 0.05 and 0.4μM in broth (Figure 4.7).

Figure 4.7.

The fitting data of the test led to the calibration curve (Figure 4.8) calculated as a function of the cumulative AmpC concentration (for mathematical details see D2.1, section 3.3). In particular, we report the calibration curve vs. cumulative concentration of the pure (red curve) and in broth (blue curve, ΔλPMMA= 6.3 nm) AmpC protein.

Figure 4.8.

The two calculated sensitivities and LODs are:
Eq.1.
where the SAMPC-3APBA and SAMPCinbroth-3APBA are the sensitivities to the pure and in broth AmpC binding onto the sensor surface biofunctionalizde with BZD ligand, respectively and LODAMPC-3APBA and LODAMPCinbroth-3APBA are the related LODs.

• AmpC in broth attachment test on BZD biofunctionalized probe
The test have been carried out using AmpC concentrations of 0.1, 0.3 and 0.8μMin broth (Figure 4.9).

Figure 4.9.

Following the same procedure reported in the previous section in Figure 4.10 we report the calibration curves for the pure (blue curve) and in broth (red curve, , ΔλPMMA= 5.5 nm) AmpC for the probe functionalized with BZD ligand.

Figure 4.10.

In the following, the sensitivities to the AmpC (pure and in broth) bindingfor the probe functionalized with 3-APBA and the LODs:

Eq.2.
where the SAMPC-BZD and SAMPCinbroth-BZD are the sensitivities to the pure and in broth AmpC binding onto the sensor surface biofunctionalizde with BZD ligand, respectively and LODAMPC-3APBA and LODAMPCinbroth-3APBA are the related LODs.

NDM-1 β-lactamase in broth attachment tests
• NDM-1 in broth attachment test on 3-APBA biofunctionalized probe
The test has been carried out using NDM-1concentration of 1.2µM in broth (Figure 4.11, Table 4.6).

Figure 4.11.

Table 4.6.

In the following, the sensitivities to the NDM-1(pure and in broth) bindingfor the probe functionalized with 3-APBA and the LODs:

Eq.3.
where the SNDM-3APBA and SNDMinbroth-3APBA are the sensitivities to the pure and in broth NDM binding onto the sensor surface biofunctionalizde with 3-APBA ligand, respectivelyand LODNDM1-3APBA and LODNDM1inbroth-3APBA are the related LODs.

• NDM-1 in broth attachment test on BZDbiofunctionalized probe
The test has been carried out using NDM-1concentration of 1.2µM in broth (Figure 4.12), Table 4.7.

Figure 4.12.

Table 4.7.

In the following, the sensitivities to the NDM-1(pure and in broth) bindingfor the probe functionalized with BZD and the LODs:

Eq.4.

where the SNDM-BZD and SNDMinbroth-BZD are the sensitivities to the pure and in broth NDM binding onto the sensor surface biofunctionalizde with BZD ligand, respectivelyand LODNDM1-BZDand LODNDM1inbroth-BZDare the related LODs.

Risk
Difficulties in beta-lactamase purification were encountered and overcame by changing the original strains and the purification protocols.

Products
-5 ligands with different specificity profiles have been identified. The panel is well characterized in vitro and constitutes a kit for the functionalization of the ALD for detecting beta-lactamases in different samples.
-about 50 compounds able to target the selected beta-lactamase with different inhibition profile to be further optimized for improving the inhibition activity and the optical properties and sources of new kits;
-1 compound series suitable for patent protection
- ligand property profile concept for the probe –ligand selection provided

SME interest
-Ligands set characterized with suitable specificity profile; the compounds set represent a useful kit for the probe derivatization and for the detection of different beta-lactamases in biological sample.
-Ligands with IP protection.

WP5
Automatic laboratory detector assembling
WP leader: Andrea Cusano
For the proper use of the optical fiber biosensor developed inWP2, it is needed to have in physical contact the sensing head of the sensor (i.e. the last few centimetres of the LPG probe) and the biological sample to be tested, usually present in laboratory couvettes. Thus, a key aspect relies on the realization of a suitable sensing head holder which has to mechanically hold the final part of the fiber and allow it to perform a series of defined and controlled movements.
In order to do this, Work Package 5 is devoted to the design and development of an automated robot (an Automated Laboratory Detector – ALD) able to:
- manipulate up to 8 fiber optic biosensors (the sensing head) simultaneously;
- put them into the couvettes where the samples under test are placed;
- move the couvettes in order to change the sample to be tested;
- put the sensing head in a recovery bath between a test and a new one;

To his aim, WP5 is organized in three main tasks:
- Task 5.1 is devoted to the design and realization of an automated robotic arm as well as to the design an realization of a suitable carousel of couvettes, including a sensing head holder able to host multiple fiber optic biosensors simultaneously. The sensing head is the terminal part (a few centimeters) of a cut single-mode optical fibre which has to be immersed into the biological samples to be tested. Hence, the sensor holder has to mechanically hold the final part of the fibers but has to allow the sensing part of the FO to be in contact with the solution. In addition, the automated robot has to be able to move the sensing head and the connection fibre in order to make repetitive and controlled tests on series of biological samples.

- Task 5.2 is devoted to the development of a dedicated software for command and control of the Robotic work. The robotic arm and the carousel, which are moved by step-by-step motor servo mechanisms, will be connected and controlled by a PC. This task is devoted to the development of a suitable software able to send commands and take under control the phases of the biological tests. In addiction it is devoted to the design and development of a suitable friendly User Control Interface to permit at not specialized operators to use easily the system. In this WP will be also proposed the interface with a program dedicated to data analysis of infection recording that will be provided by WP6 for the data collected analysis and for prediction of infection development.

- Task 5.3 is devoted to the validation of the ALD following the international regulation as suggested by competent organism for medical device commercialization (followed and supported by Projeccion) (the device only).

Task 5.1 Design and realization of an automated robotic arm. Design and realization of a suitable carousel of couvettes
In the first part of WP5, CeRICT researchers have focused their attention to the design of the ALD, mainly consisting on an automatic robot able to perform repetitive and controlled tests on a series of biological samples by using multiple LPG biosensors simultaneously. The design phase started from a general view of the ALD, a schematic representation of which is shown in Figure 5.1. Here, the ALD has been conceived as composed of:
1) a sensing head holder, allowing the control of up to eight fiber optic biosensors simultaneously;
2) a couvette carousel, having Functionalization, Samples and Rinse areas, divided according to the specific needs, in order supply the ALD with the additional functionality of being able to automatically realize the chemical functionalization, the ligand immobilization along with the biological tests starting from a general purpose probe (i.e. from the LPG transducer, with the a-PS and the PMMA coatings);
3) a proper optoelectronic unit for the interrogation of the LPG–based reflection probes;
4) a PC with dedicated software for command and control of the Robotic work.

Figure 5.1.

The design of the ALD has been realized through the use of Solid Works, a 3D mechanical CAD (computer–aided design) program. Below, a snapshot of the 3D model of the ALD acquired from Solid Works displays an external view of the system, where the outer metallic enclosure has been made transparent to show the inside. The designed system is composed of two main parts: the test chamber, containing a sensor mount, able to hold up to eight LPG biosensor, a couvette carousel holding a 96-well plate, and two linear actuators, responsible for the movements of the well-plate along the vertical and the horizontal directions, and the rear region, hosting the optoelectronic interrogation unit and the electronic circuits for the control of the entire system, the connection with the host PC and the power supply.

Figure 5.2.

Once defined the design criteria, the ALD system has been assembled, and all its components have been included in a suitable enclosure, as shown below. It is worth mentioning that, during the design and development phases, slight changes on the system configurations have been taken into consideration, mainly to improve the reliability and practicality of the ALD system. Performed variations mainly related to the relative movement between the 96-well plate and LPG sensors and to the fixing mechanism for the LPG biosensors on the sensor mount, as reported in deliverable D5.1.

Figure 5.3.

The test chamber, where the biological tests take place, is shown in the following Figure, where the enclosure has been opened to show the inside. It is possible to distinguish the two-dimensional translation stages (horizontal, X and vertical, Z), the sensor holder with eight LPG transducers Mmounted, and the couvettes carousel with a 96-well plate.

Figure 5.4.

In addition, in order to help the user to manually adjust each fiber sensor height, a CMOS camera has been included in the test chamber.
The rear region, shown in the following Figure, hosts the LPG interrogation unit, the electronic circuits for the control and supply of the ALD and the connection USB cable for the host PC.

Figure 5.5.

In line of principle, any commercial spectrometer could be used for the interrogation of the LPG probes. In this case, a "Wave Capture Spectrometer", was chosen. It is an FBG interrogation analyzer, properly customized for the interrogation of LPG sensors, which enables the interrogation of up to eight probes simultaneously, and is characterized by a wavelength range of 80 nm (1530–1610 nm), an absolute wavelength accuracy of ±35 pm, a wavelength repeatability of 5 pm, a response time of ~ 5 kHz. A scheme of the optoelectronic interrogation unit, the translation stages and power supply is shown below.

Figure 5.6.

The components highlighted in this diagram (Figure 5.6) are briefly listed below:
- a FBGA (Fiber Bragg Gratings Analyzer);
- a SLED (Superluminescent Light Emitting Diodes);
- a VOA (Variable Optical Attenuator);
- an Optical Isolator (ISO);
- a 2x2 Optical Coupler;
- a 1x2 Optical Switch;
- a 1x8 Optical Switch;
- a XZ axis stage upon which to mount a 96-well plate;
- a probe alignment camera.

The developed ALD, comprising both the mechanical stages and the optoelectronic interrogation unit and its related software, has been subjected to a phase of test and debugging. Once carried out the test of the single parts, the whole system operation was tested for making simultaneous biological tests with up to eight LPG probes. The complete kinetics of the binding of a ligand on the surface of each LPG probe and a typical β-lactamase detection tests have been monitored.

Task 5.2 Development of a dedicated software for command and control of the Robotic work
During task 5.2, a dedicated software for command and control of the robotic work, including the optoelectronic spectrometer software, has been developed by CeRICT researchers and customized for the LPG sensors interrogation requested in this application. The realized software allows to acquire, display and save the reflectance spectrum of the LPG biosensors, and the related central and barycentral wavelengths (see Figure 5.7).

Figure 5.7.
Also, the central and barycentral wavelengths can be displayed vs time. This functionality is fundamental since it allows to follow the kinetics of the chemical binding occurring on the surface of the LPG probes during biological tests. In order to perform completely automated biological tests, the software allows to execute automated scripts, including the commands for the robotic work and acquisition of the LPG reflectance spectra.

Figure 5.8.
In addition, Molecular Discovery has implemented a prototype for the epidemiological software. The aim of this software (Figure 5.9 and 5.10) is to read the output of the device, so that for the sample tested the identified resistant betalactamase isoform is reported to the user (clinician) along with some indication of signal (relative concentration).

Figure 5.9.
Figure 5.10.
Task 5.3 Validation of the ALD following the international regulation as suggested by competent organism for medical device commercialization (followed and supported by Projeccion) (the device only)
Task 5.3 has been devoted to the validation of the ALD following the international regulation as suggested by competent organism for medical device commercialization. This step was followed and coordinated by Projeccion. In particular, the ALD design has been performed following the international regulation for medical device (focused on the prototype of a multianalyte automatic laboratory detector for revealing antibiotic resistant infections in vitro) and its realization has been performed following the design and applied regulation. In particular, it is worth pointing out that, from the regulation viewpoint, the ALD system can be seen as composed by two subsystems, namely, ALD-manipulator and the ALD-probe.
- the ALD-manipulator comprises the interrogation unit, the system enclosure, the optoelectronic components and the automation to manipulate the fiber optic bioprobes.
- the ALD-probe relies on the functionalised fiber optic biosensor to be integrated in the ALD-manipulator.
This distinction is consequently reflected also on the device compliance with the regulations in force, as described in deliverable D5.3.
More specifically, the ALD-manipulator has been designed and manufactured respecting the following properties (refer to the paragraph 4.1 in deliverable D5.3):
A.1. The device has been designed and manufactured in such a way that, when used under the conditions and for the purposes intended, it will not compromise, directly or indirectly, the clinical condition or the safety of the patients, the safety or health of users or, where applicable, other persons, or the safety of property.
A.3. The device has been designed and manufactured in such a way that it is suitable for the purposes referred to in Article 1(2)(b), as specified by the manufacturer, taking account of the generally acknowledged state of the art.
A.5. The device has been designed, manufactured and packed in such a way that its characteristics and performances during its intended use will not be adversely affected under storage and transport conditions (temperature, humidity, etc.) taking account of the instructions and information provided by the manufacturer.
B.1.1. The device has been designed and manufactured in such a way as to achieve the characteristics and performances referred to in section A on the 'General requirements'.
B.2.1. The device and its manufacturing processes have been designed in such a way as to eliminate or reduce as far as possible the risk of infection to the user or other persons. The design allows easy handling and reduces as far as possible contamination of, and leakage from, the device during use and, in the case of specimen receptacles, the risk of contamination of the specimen. The manufacturing processes are appropriate for these purposes.
B.3.5. The device has been designed and manufactured in such a way as to facilitate the management of safe waste disposal.
B.3.6. The measuring, monitoring or display scale (including colour change and other visual indicators) have been designed and manufactured in line with ergonomic principles, taking account of the intended purpose of the device.
B.6.1. Devices incorporating electronic programmable systems, including software, have been designed to ensure the repeatability, reliability and performance of these systems according to the intended use.
B.6.2. The device has been designed and manufactured in such a way as to minimise the risks of creating electromagnetic perturbation which could impair the operation of other devices or equipment in the usual environment.
B.6.3. The device has been designed and manufactured in such a way as to avoid, as far as possible, the risk of accidental electric shocks during normal use and in single fault condition, provided the device is installed and maintained correctly.

Conclusions
All the objectives of WP5 have been fully achieved and obtained results constitute an enormous progress beyond the state of the art in the field opening a plenty of potential market options.
In particular, the following clearly significant results have been obtained:
- a 3D design of an Automatic Laboratory Detector, able to to perform repetitive and controlled tests on a series of biological samples by using multiple LPG biosensors simultaneously, has been carried out by using a 3D mechanical CAD (computer–aided design) program (Solid Works);
- based on the design criteria defined in the first part of the WP5, the ALD system has been realized by the identification and assembly of all its components;
- a dedicated software has been developed for control for command and control of the ALD robotic work, able to send commands and take under control all the phases of the biological tests, including the LPG probes interrogation.
- a deep and detailed validation and debugging phase has been carried out to attest the functionality and reliability of all the components of the device as well as of the whole system;
- the ALD validation following the international regulation as suggested by competent organism for medical device commercialization has been carried out;

Overall, no critical aspects have been encountered, all the activities have been completed and reported in the deliverables D5.1 ('Project and Prototype of an Automated Robot for Testing'), D5.2 ('Dedicated software for command and control of the Robotic work and design of software for infection') and D5.3 ('Validation of the ALD following the international regulation as suggested by competent organism for medical device commercialization') which have been delivered on time.

Products
Project and prototype of an Automated Laboratory Detector for automatic testing of LPG biosensor, including a dedicated software for command and control of the robotic work and for the LPG probes interrogation. In addition, Molecular Discovery developed a program dedicated to the analysis of the data collected in WP6 for the prediction of infections. The aim of this software is to read the output of the device, so that for the sample tested the identified resistant beta-lactamase isoform is reported to the user (clinician) along with some indication of signal (relative concentration).
The software will also allow the clinician to explore the frequency of resistance types by location, for example to illustrate if a certain type of bacterial resistance is common to a certain location (epidemiology of infection). The software will also report antibiotics that are known should be avoided for different resistant isoforms.

SMEs interest
The realized ALD system, with its capability to suitably manipulate multiple optical fiber biosensors for beta-lactamases detection tests, and, more in general, for general purpose biological experiments, is in line with the expectations of the SMEs involved in the project. This is particularly true for Optosmart, whose core business relies also on the development of fiber optic sensors for chemical and biochemical applications.

Risk
The potential risks for this WP (as reported in the DOW) could be associated to difficulties with the ALD construction.

How to overcome
During the design and development phases, slight changes on the system configurations were taken into consideration mainly to improve the reliability and practicality of the ALD system. Performed variations mainly related to the relative movement between the 96-well plate and LPG sensors and to the fixing mechanism for the LPG biosensors on the sensor mount, as reported in deliverable D5.1.
CeRICT researchers are currently completing a debugging of the whole ALD system and, at the moment, no critical aspects have been encountered.
WP6
Clinical ALD validation
WP leader: Jesús Blazquez
1.Introduction: Beta-lactamases (BLases), bacterial enzymes able to hydrolyze Beta-lactam antibiotics, are produced by bacteria harbouring specific bla genes. The production of BLases may compromise the outcome of anti-infective treatments, specifically those involving penicillins, cephalosporins and carbapenems. Since most of these treatments are empiric, early detection of BLase presence in human fluids is crucial for the success of the treatment, particularly when the time-window for actuation is very narrow. This is the case, for instance, of patients within intensive care units (ICUs) or with bacteraemia. This project aimed to develop specific ligands to the clinically most relevant BLases. To be detected, BLases must access to the ALD sensor in the appropriate active conditions, concentration and purity. The WP6 aimed to evaluate the accuracy and precision of the ALD with carefully designated BLase-producing bacteria and to find the best methodology and conditions to i) obtain enough number of bacterial pathogens from human fluids, particularly those cases with low bacterial charge (blood, for instance) to be sufficient for detection of BLases by the ALD, ii) liberate the enzymes in the amount, purity and activity sufficient to be detected by the ALD, iii) validate the ALD with clinical samples and compare results with classical microbiological methodology and iv) provide an estimation of the time needed to give a final diagnostic and compare it with the classical methodology.

2.- Work performed: The objectives of WP-6 have been addressed as follows.

2.1.- Task 6.1.- Validation of the prototype with different artificially inoculated biological samples and the characterization of the most appropriate method to concentrate pathogens present in clinical samples and liberate their b-lactamases without lost of their binding activity. The most productive methods will be used for subsequent analyses (Deliverable 1 and 2; delivery by month 24). The optimal detection of BLases by the ALD depends on the amount of BLases in the medium and on the binding capacity of these BLases to their specific ligands (i.e. activity). Thus, it is very important to develop methods to increase the production of enzymes but maintaining their activity. An effective increase can only be reached through the increase in the number of BLase producing bacteria in the clinical sample and increasing the yield of the BLase recovery.
Therefore, we studied and defined the best methods to liberate the active BLases in the best conditions to be detected by the ALD and concentrate bacterial pathogens from a biological specimen, which usually contains very low amount of bacteria (blood samples, for instance).

2.1.1.- Method for lysis of bacterial cells.
Several protocols of bacterial lysis were evaluated. The main objective was to find a protocol able to obtain: i) maximum yield in active B-lactamase proteins; ii) without interferences for the final detection with the ALD; iii) easy use for clinical technicians and iv) low cost. Activity of B-lactamases was determined by quantitative determination of nitrocefin hydrolysis capacity.
Three protocols were tested with pure cultures of AmpC- and NDM-1- producing strains. Results from the three protocols were compared by BLase activity and western blot quantification of BLase production.

2.1.1.1- Cell disruptor with glass beads:
The bacterial lysis and protein yield obtained with a Cell disruptor (Fast Prep, MP Biomedicals) (Figure 6.1) were low, particularly for K. pneumoniae, P. aeruginosa and A. baumannii.

Figure 6.1.

2.1.1.2.- Lysis with non-ionic detergents:
A home-made lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% NP-40 with addition or not of Lysozyme 0.5 mg/ml) and a commercial reagent (Thermo Scientific B-PER Bacterial Protein Extraction Reagent) were tested. Similarly to the cell disruption with glass beads, the bacterial lysis and protein yield was low for P. aeruginosa and A. baumannii. In addition, the final lysate contained quantities of detergent, which affect B-lactamase activity and could affect the ability of detection of the ADL.

2.1.1.3.- Lysis by sonication:
Bacteria were resuspended in 2 ml water and sonicated in a Bioruptor (Diagenode) (Figure 6.2 and Table 6.1) in 3 cycles of 10 min (30 seg ON and 30 seg OFF). This protocol rendered the highest lysis and protein recovery and activity, giving final extracts without putative inhibitors of the ALD.

Figure 6.2..

Table 6.1.

2.1.2.- Method for concentrating bacterial pathogens from biological specimens.
Sterile biological samples (urine, sputa and blood) were artificially inoculated with different dilutions of viable pathogenic bacteria, expressing different B-lactamases (in particular AmpC and NDM-1).
To amplify the number of bacteria, especially in blood samples, artificially inoculated dilutions of pathogenic bacteria were incubated for 4, 6, 8 and 24 hours before concentration and lysis processes.
According to the expected number of bacteria in positive biological samples, we inoculated different number of pathogenic bacteria expressing AmpC or NDM-1 B-lactamases. Specifically, Escherichia coli-1 hyperprod chromosomal AmpC, Escherichia coli hyperprod chromosomal AmpC + deletion atenuator, Escherichia coli-1 NDM-1 and Klebsiella pneumoniae NDM-1 were inoculated in sterile samples of human urine, sputum and blood.
Urine and sputa were inoculated with high numbers (10e3 – 10e5/ml) of bacterial cells (as expected in real samples). However, blood samples typically contain very low numbers of bacteria (1-20 cells/ml). Thus, they were inoculated with 1-10 or 10-100 cells per sample of 10 ml.
To simulate the protocol followed in the Microbiology clinical laboratory, we inoculated 10 ml of the artificially contaminated samples into Bactec culture bottles (BD Bactec plus aerobic culture vials) and incubated in the detector machine (present in most of the big hospitals in Europe) (Figure 6.3), according to the exact protocol followed in most clinical laboratories.

Figure 6.3.
2 ml aliquots were taken after 4, 6, 8, 12 and 24 hours of incubation. Two concentration methods were previously tested (filtration and centrifugation), being centrifugation (8 min-10.000 rpm) the most effective for the high recovery of B-lactamase producing bacteria. Bacterial cells from 2 ml of each aliquot were then concentrated by centrifugation and lysed by the sonication method. B-lactamase concentration was analysed by PAGE and Western Blot (WB) with specific antibodies (Figure 6.4). The number of bacterial cells was quantified by plating on LB-agar plates.

Figure 6.4.

The results with sputa and urine, indicated that optimal incubation time needed for obtaining an ALD-detectable B-lactam concentration (i.e. in the order of nM) is between 6-8 hours. For blood samples, however, the necessary incubation time was between 12 and 24 hours (Tables 6.2 and 6.3). Thus, according to the hospital laboratory workflow, for blood infections the final diagnostic time is nowadays about 24 hours.

Table 6.2.

Table 6.3.

2.2.-Deliverable D6.1. Prototype validated for artificially inoculated samples

The chosen methods for pathogen lysis and concentration were, therefore, sonication and inoculation and growth in culture bottles.
Final validation of the prototype requires data from ALD.

2.3.- Task 2 Accuracy and precision studies.
Accuracy is how close a measured value is to the actual (true) value and Precision is how close the measured values are to each other.

Figure 6.5.

Accuracy and precision of the ALD will be analysed with samples previously characterised at IBIS-FISEVI by different methods, including comparison with purified BLases and WB.
The different replicas of the same extract and different extracts of the same sample, analysed at IBIS,are being measured with the ALD to analyse the accuracy and precision of the ALD.

2.4.-Deliverable 2: Accuracy and precision values.

The final table is waiting for ALD data.

2.5.- Task 3:Capacity to detect b-lactamases in real biological samples.
This task required the collection of different B-lactamase expressing pathogens isolated from hospitals and the construction of different engineered Escherichia coli K-12 strains able to overproduce specific B-lactamases.

2.5.1.- Collection of pathogens producers of B-lactamases.
207 pathogenic bacteria, suspected of expressing different classes of BLases of interest for the OPTOBACTERIA project, were prospectively collected from different Spanish hospitals. Pathogens expressing a priori interesting BLases (see below) were identified by classical microbiological methods and Maldi-Tof technology. This collection includes 20 TEM and SHV producers (not shown), 18 AmpC, 10 Metalo-B-lactamase, including 3 NDM-1, and 2 CTX-M producers (Table 6.5). Most of the specific BLases were genetically characterized by cloning and sequencing of the corresponding bla genes at the FISEVI-IBIS laboratory (see below). The characterization of the B-lactamases required PCR amplification with specific primers (sometimes including cloning of the bla genes in the appropriate vector), phenotypic characterization of the clone (substrate profile, isoelectric point, size, etc), sequencing and bioinformatic analysis of the sequence.
In addition,6 Escherichia coli K-12 overproducing specific B-lactamases were engineered to be used for B-lactamase purification and ALD testing.

2.5.1.1.- Collection of pathogenic bacteria producing B-lactamases of interest:
Table 6.5.-
1.-Escherichia coli AmpC pCMY-2,peritoneal fluid
2.- Escherichia coli-1 hyperprod chromosomal AmpC, urine
3.-Escherichia coli-2 hyperprod chromosomal AmpC, urine
4.-Escherichia coli hyperprod chromosomal AmpC + deletion atenuator,urine
5.- Proteus mirabilis AmpC pCMY-2, wound
6.-Enterobacter aerogenes hyperAmpC ESAC,broncoaspirate
7.- Enterobacter cloacae-1 hyperprod chromosomal AmpC, blood
8.-Enterobacter cloacae-2 hyperprod chromosomal AmpC, blood
9.-Klebsiella pneumoniae AmpC pDHA-1,blood
10.- Citrobacter koseri AmpC pCMY-2,blood
11- Klebsiella oxytoca AmpC pDHA-1,blood
12.- Salmonella sp AmpC pDHA-1, feces
13.-Klebsiella pneumoniae AmpC pACC1, blood
14.-Klebsiella pneumoniae AmpC pFOX5, blood
15.-Klebsiella pneumoniae AmpC pACT1, blood
16.- Pseudomonas aeruginosa hyperprod chromosomal AmpC, broncoaspirate
17.-Pseudomonas aeruginosa-2 hyperprod chromosomal AmpC, broncoaspirate
18.-Pseudomonas aeruginosa-3 hyperprod chromosomal AmpC, broncoaspirate
19.- Escherichia coli NDM-1, urine
20.-Escherichia coli-2 NDM-1, urine
21.-Klebsiella pneumoniae NDM-1, urine
22.- Acinetobacter baumannii OXA-23, broncoaspirate
23.-Acinetobacter baumannii OXA-24, broncoaspirate
24.-Klebsiella pneumoniae KPC-2, urine
25.-Pseudomonas aeruginosa IMP-15, wound
26.-Pseudomonas aeruginosa VIM-1, broncoaspirate
27.- Pseudomonas aeruginosa VIM-2, blood
28.-Pseudomonas aeruginosa VIM-13, broncoaspirate
29.-Escherichia coli CTX-M-14, blood
30.-Escherichia coli CTX-M-15, blood

2.5.1.2- Engineered Escherichia coli K-12 overproducing specific B-lactamases:
pGEX-6P-1-VIM-1,
pGEX-6P-1-VIM-13,

pGEX-6P-1-KPC-2,
pGEX-6P-1-CTX-M-15,
pGEX-6P-1-NDM-1,
pCA24N-NDM1.

2.5.1.3.- DNA and deduced amino acid sequences of the B-lactamases as obtained after PCR and sequencing at IBIS.

2.6.- Deliverable 6.3.- Final tests of the prototype with biological samples (comparison with classical microbiological methods .
As we have been able to determine the BL amount through WB methodology, we have produced artificial samples, containing "real amounts" of bacterial pathogens expressing different types of beta-lactamases, by inoculation of different human fluids.
As indicated above, the optimal incubation time for obtaining an ALD detectable B-lactamase concentration (i.e. on the order of nM) is:
- Between 6-8 hours for heavily charged samples such as urine and sputa.
- Between 12 and 24 hours for blood samples.
Taking into account that once processed the samples (about 1 hour of total processing) the time for detecting the presence of B-lactamases by the ALD is practically immediate, the time needed for a final result is between 7 and 25 hours. This is in contrast with the classical methods which lasts 48-72 hours (24h-incubation + 24h presumptive identification of resistance by automatic MIC) or (24h-incubation + 24h + isolation + 24 identification of resistance by disc-diffusion or automatic MIC). Figure 6.6 shows an example of the process and time required to reach a definite identification of a pathogen expressing a carbapenemase with the classical and the ALD methodology. A variation to the classical method, which includes a PCR reaction, has been added. Most of the required times for the classical methodology have been reduced to 12 hours. However, in the day-to-day routine, they are really closer to 24 than 12 hours, because they are processes reinitiated every 24 hours.

Figure 6.6.

2.7.- Tasks and Deliverables 6.4. and 6.5.- Analysis of prospective data (data collected using the ALD in 100-200 patients evaluated in the Hospital setting) and Standardization of the final prototype.

This deliverable was related to the "on site" and "on time" detection of BLases in parallel with the classical methods of the microbiology clinical laboratory. The standardization of the final prototype also requires the "on site" and "on time" processing of human samples. The completion of these deliverables may require the "on site" analysis of human samples as soon as they arrive to the microbiology laboratory (the hospital one) to be processed accordingly to the standardized methods.

2.8.- Conclusions

Most of the objectives of WP6 have been fully achieved and obtained results constitute a clear progress.
In particular, the following significant results have been obtained:
- A protocol for lysis of bacterial cells obtained from the clinical samples. This method maintain the functionality of the liberated B-lactamases (essential for the ALD detection).
- A protocol for amplifying the amount of B-lactamases, increasing the number of bacterial cells in the sample. This protocol has been developed by taking into account the methods used in the clinical laboratories of most big hospitals in the world. This approach allows the processing of the samples without changing substantially the clinical laboratory methods.
- A collection of more than 50 first-line pathogens expressing different isoforms of the most currently found B-lactamases in the clinical practice. The strains and their B-lactamases have been phenotypically and genotipically characterised by IBIS-FISEVI. This collection, which can be easily enlarged, has been very useful for the validation of the ALD and will be used for the development of new detection tests.
- A collection of engineered strains able to overexpress the most interesting B-lactamases from the clinical point of view. As stated before, this collection will also be very useful for the development of new B-lactamase detection tests.
- We have been able to propose that B-lactamase detection can be done, depending on the human fluid, between 7 and 25 hours.
- Final testing with prospective real human samples will be easily done as soon as the prototype is ready to be tested in the clinical setting.

3.- SMEs interest.

The developed methods for concentrating and purifying B-lactamases are of great interest for the SMEs involved in this project. Rapid and efficient protocols were needed to sustain the machine work, without changing the classic way of work in clinical laboratories, where everything is protocolised in order to process a very high number of samples (thousands) with low risks of mistakes.

4.- Deviations and corrective actions.

The standardization of each stage related to probe preparation and testing (from fabrication to ligand selection, functionalization, specificity assessment, probe regeneration, testing with purified proteins and complex matrices) required more efforts then expected, due to the challenging aspects related to each step of the complex procedure underlining the probe operation in real scenarios. This led to unforeseen time delays in complete the different tasks expected from the DOW.
Specifically, in WP4, time delay caused the problem related to the low statistics level of the data in passing from the model system to complex matrices (broths), therefore we demonstrated the proof of concept in detecting correctly BLs in complex matrices with first estimations of sensitivities and LODs for both ligands and proteins.
This obviously caused a delay in process the clinical samples in Spanish hospital as expected from the DOW.
Nevertheless, we have been able to detect the BLs amount through the Western Blot methodology in clinical samples.
This allowed to rapidly test the extracts and to send them in Napoli for preliminary testing and validation using the ALD.
Data are expected during this week and will be presented at the final meeting in Naples. This deliverable is, therefore, lacking the final results from the ALD to be completed.

Potential Impact:
The overall goal of WP7 was to disseminate project results and to transfer the project knowledge, technologies, lessons learned and best practices to interested communities and thus to ensure their worldwide impact and long-term sustainability.
The dissemination activities were tracked in the form of several versions of reports in the first stages of the project in deliverables:

o D 7.1.- Brochures
o D 7.5.- Web site for project
o D 7.6.- Project Wikipedia page and expected results
o D 7.8.- Interim plan for use and dissemination of knowledge

And they have continued in the final deliverables of the poject:

o D 7.2.- Best practices report
o D 7.3.- Seminars and workshops
o D 7.4.- Video Clip
o D 7.7.- Final project Wikipedia page and results

During the project PORYECCION has carried out the tasks below:

Task 7.1. Communication and corporate image.

The corporate image of the project through the consortium have been create, designing the logo and the rest of templates in order to show to the community an unique image of the project. Bellow it is shown the logo and other graphical materials:

Optobacteria logo

Figure 7.1.

Task 7.2. Diffusion activities.

The project web site was created in the early stage of the project with a public and private area. It has been very useful as tool to disseminate the aim and progress of the projects, as well as to communicate and publish the documentation between the partners.

Figure 7.2.

A leaflet has been produced and distributed between the partners. The leaflet explains the aim of the project. The leaflet has been very useful in seminars and workshops and also it is published in the web site of the project.

Figure 7.3.

Proyección has contributed to the workshops and seminars in the design of the posters and other graphical information and collecting the data as input for the deliverables.

Figure 7.4.

The second newsletter has been produce in this period, informing about the progress of the project and the technical meeting in Benevento.

Figure 7.5.

In the first year of the project a reference was published in wikipedia. Unfortunately wikipedia has proved inadequate channel for the dissemination of this type of project since this entry was deleted by the administrator along with many other references of European projects of the seventh framework program.

Figure 7.6.

As request of the project officer the consortium have prepared a summary report that has been published by the commission portal CORDIS.

Figure 7.7.

A video clip explaining the aim and the results of the project has been produce

Task 7.3. Analysis use of results in other sectors.

A special effort has been carried out to consider the possibilities to use the results of the project in other areas of knowledge.

From a scientific point of view the development achieved about the ligands and the simulation of them by computer software will be useful in pharmaceutical and biological field.

In the technical area, the design and development of the ALD base in fiber glass biosensor open a window to use this technology in many field of the biological, agricultural, food and environmental areas as a fast device to analysis the evolution of the ecosystems.

Task 7.4. Brief study of the good practices

The Best Practices Report describe those methods and procedures that have been discussed and identified to provide a coherent and efficient approach in the context of OPTOBACTERIA project. In this report the below areas have been taking into account:

o State of the art
o Advantages achieved in the project
o Replicability of the results
o Guidelines for the use of results
o Using the results in other sectors.

Task 7.5. Mechanisms of knowledge transfer inside the project

The SMEs are the final beneficiaries of the results of the project and in this last period of the project this terms have been discussed in order to explore the launch to the market, and IP of the results.

Task 7.6. Plan for the use and dissemination of knowledge

The final plan for the use and dissemination of knowledge has been developed and detailed in the D7.9. This plan describes the activities carried out during the project in order to disseminate the results of the project between the scientific and technical community. As well as it details the exploitable results of the project and the possibility to use in other areas of knowledge.
The list below shows the basic lines to follow up the results of the project and implement them beyond state of the art:
- The software technology developed in WP1 is a key factor for the industrial exploitation of biosensors based on LPG technology. The software allows the design of the parameters needed to reach the correct sensitivity for bio applications and to be used in bio environment.
- The LPG platform technology is the key hardware needed to afford the biosensing issue. The chemical functionalized LPG allows the bioanchoring of the biological recognition element - The specific probe to used in EBSL detection provides the basic sensor to be implemented into the complete ALD system.
- The ALD system represents the main result of the program if intended not only as the automatic function but also as the integration of all the technologies developed into the project.
ALD provides the main tool for the detection of drug resistant bacteria.
- Ligand panel for BL panel profile analysis using different technologies. The ligands consist of a number of chemical compounds that have been selected through two methods: a)known ligands tested against BL proteins never tested before;

b) virtual screening studies against the selected protein panel.
- The selection criteria for the protein panel are consistent with the ligands mode of binding. The panel profile is reported in separate documents. Guidance are given in a separate document.
- One single ligand, a boronic acid derivative, is unpublished and has a high affinity profile suitable for paper fishing. Guidance are given in a separate document.
- Linker chemistry for water soluble ligands that recognize BL in solution. Guidance are given in a separate document.
- Detachment methods. The detachment is useful for the regeneration of the optical probe and for every surface that can immobilize the BL proteins, fished out by the designed ligands. Guidance are given in a separate document.
- Scaffolds for the development of further ligands with different profile and against new beta lactamase.

The below list shows the main use of the results in other sector:
- The sotware developed can be readily integrated into the manufacturing chain of the LPG optical transducers. Such in fibre devices are technologically mature to enter in several field in security, safety and environment and the possibility of using a design tool would be an important output when Optosmart will enter the LPG production sector.
- The technology developed in the LPG platform is most exploitable technological result for Optosmart that now possess an unique platform that has been demonstrated in the case of ESBL but could be readily applied in other application fields where chemical flexibility and high sensitivity is required.
- The ALD system is the most powerful and flexible technology produced by the project. It provides the needed hardware to investigate the potentiality of a faster and on site laboratory analysis are mandatory, such as security, safety and environment.
- Ligand panel for BL panel profile analysis using different technologies. The ligands consist of a number of chemical compounds that have been selected through two methods: a) known ligands tested against BL proteins never tested before;

b) virtual screening studies against the selected protein panel.
- The selection criteria for the protein panel are consistent with the ligands mode of binding. The panel profile is reported in separate documents. Based on the accurate literature search available the said application is unique. Other than optical fiber application, other application can be foreseen as chip technology and plate reading technology for microarray technologies.
- The ligands can be used for the development of antibacterial drugs directed to BL expressing bacteria.
- One single ligand, a boronic acid derivative, is unpublished and has a high affinity profile suitable for paper fishing. Other than optical fiber application, other application can be foreseen as chip technology and plate reading technology for microarray technologies and chips.
- The ligands can be used for the development of antibacterial drugs directed to BL expressing bacteria.
- Linker chemistry for water soluble ligands that recognize BL in solution. Other than optical fiber application, other application can be foreseen as chip technology and plate reading technology for microarray technologies and chips.
- Detachment methods. The detachment is useful for the regeneration of the optical probe and for every surface that can immobilize the BL proteins, fished out by the designed ligands.
- Other than optical fiber application, other application can be foreseen as chip technology and plate reading technology for microarray technologies and chips. This method is particularly useful for fluidic technology.
- Scaffolds for the development of further ligands with different profile and against new beta lactmase.
These scaffolds can be used for the development of antibacterial drugs directed to BL expressing bacteria.

List of Websites:

www.optobacteria.eu

Contact

Venturelli, Alberto (Legal representant)
Tel.: +390592055143
E-mail

TYDOCK PHARMA S.r.l
Italy

Subjects

Industrial Manufacture

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