

NAPPA-Based Vaccines for a New Proteogenomics Approach for Public Health

Nicola Luigi Bragazzi^{1,2}, Eugenia Pechkova^{1,2} and Claudio Nicolini^{1,2}

¹Nanoworld Institute Fondazione EL.B.A. Nicolini (FEN), Largo Redaelli 7, 24020, Pradalunga, Bergamo, Italy

²Laboratories of Biophysics and Nanobiotechnology (LBN), Department of Experimental Medicine (DIMES), University of Genoa, Via Pastore 3, 16132, Genova, Italy

Correspondence to:

Nicola Luigi Bragazzi, MD, PhD
Laboratories of Biophysics and Nanobiotechnology (LBN)
Department of Experimental Medicine (DIMES)
Via Pastore 3, 16132, Genova, Italy
University of Genoa, Italy
Tel: +39 010 35338217
Fax: +39 010 35338215
E-mail: 2896313@studenti.unige.it

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Abstract

Vaccinology was developed for the first time in 1796 when Jenner empirically implemented a vaccine against smallpox using animal-to-human cowpox inoculation. Since then, it has become a very complex science due to the merging of disciplines ranging from structural and functional, cellular and molecular biology and immunology to bioinformatics and nanobiotechnology, as well as systems biology and synthetic biology and engineering. In the frame of evidence-based medicine (EBM), evidence-based vaccinology emerged as an important sub-field: vaccinology has nowadays become more and more predictive and personalized. With the discovery that many patients with cancer develop antibodies against p53 (the so-called oncoantibodies), it was evident that oncoprotein are immunogenic and can be used for immunotherapeutics purposes. In this manuscript, we report Nucleic Acid Programmable Protein Arrays (NAPPA)-based Quartz Crystal Microbalance (QCM) measurement of p53 immunogenicity and kinetics, in the perspective of developing an effective p53 therapy. NAPPA-based QCM_D can be a useful platform for proving the immunogenicity of oncoprotein-based vaccines. Recently, the field of vaccinology has extended from vaccines for infectious diseases to vaccines not only preventive but also therapeutic for chronic-degenerative diseases such as cancer. Peptide-based immunotherapeutics has been proven to be quite effective for cancer treatment and NAPPA-based QCM_D has the promise of providing clinicians with quick, rapid and cheap measurement of oncoprotein kinetics and bindings with immune cells. Moreover, it can be a precious tool for implementing personalized and predictive vaccinology.

Keywords

Cancer, Nucleic Acid Programmable Protein Arrays (NAPPA), p53-based vaccine, Personalized vaccinology, Predictive vaccinology, Quartz Crystal Microbalance (QCM), Vaccinology

Introduction

Vaccinology was developed for the first time in 1796 when Jenner empirically implemented a vaccine against smallpox using animal-to-human cowpox inoculation [1]. Since then, it has become a very complex science due to the merging of disciplines ranging from structural and functional, cellular and molecular biology and immunology to bioinformatics and nanobiotechnology, as well as systems biology and synthetic biology and engineering [2, 3].

In the frame of evidence-based medicine (EBM), evidence-based vaccinology emerged as an important sub-field [4] vaccinology has nowadays become more and more predictive and personalized [5-7].

In 1986 for the first time genetic engineering was applied to vaccine development and recombinant virus-like particle (VLP) vaccine produced in yeast was launched as a vaccine against hepatitis B. In the Nineties, Rino Rappuoli, Global Head of Vaccines Research at Novartis, Siena, Italy, introduced the concept of reverse vaccinology for the design of a vaccine [8-10] first against *Neisseria meningitidis* and very recently against *Streptococcus* B group. Since then due to the explosion of OMICS sciences [11, 12] and technologies, biomolecular arrays have emerged as an important tool for vaccine development and implementation: research has focused above all on the exploitation of DNA arrays for different purposes, assessing safety and immunogenicity, evaluating the stability of the virus and bacterium strain, identifying sub-types and genotyping the strain responsible of the outbreak. Only recently protein arrays were used to discover new antigenic determinants for vaccine development, such as membrane proteins and other components or virulence factors, especially those expressed at high values, which are expected to better stimulate the lymphocyte reactions.

Plant biotechnology is another field which has greatly contributed to the advancement of vaccine production, making it possible to yield a large amount of vaccine at a lower cost and faster [13-15].

Vaccines represent an important tool of global health policy and vaccinomics is a new strategy emerged from converging disciplines, like bioinformatics, nanobiotechnologies, plant biotechnology and OMICS sciences, which has enabled scientists to design and developed personalized vaccines [5, 16-23].

Among the avenues being presently explored, NAPPA-based vaccines identification appears to represent an additional promising future perspective in the frame of the new OMICS-based Public Health. Vaccinology has emerged as a complex interdisciplinary science, especially because of the contributions of the new OMICS disciplines. In addition to what was anticipated some time ago, only recently were protein arrays used to discover new antigenic determinants for vaccine development [24-30]. NAPPA-based sensors could be used for screening the affinity between the identified proteins and the immunological synapse (CD4, TCR, MHC complex). Affinity kinetics can be evaluated also using classical techniques, or new efforts to evaluate it via Atomic Force Microscopy (AFM) and Surface Plasmon Resonance (SPR).

In this manuscript, we report and discuss some preliminary results of protein expression of genes related to vaccinology, in particular to cancer vaccines and immunotherapeutics. Experiments have been carried out coupling Nucleic Acid Programmable Protein Array (NAPPA) with a recently improved nanogravimetric apparatus which exploits the quartz crystal microbalance with frequency (QCM_F) and quartz crystal microbalance with dissipation monitoring (QCM_D) technologies, both in static and in flow condition. The selected

protein is p53 and its role will be discussed further in this manuscript.

NAPPA Technology

NAPPA is an innovative technology which avoids the time-consuming task of obtaining highly purified proteins [31]. Complementary DNAs (cDNAs) of selected genes tagged with a C-terminal glutathione S-transferase (GST) are spotted on the microarray surface and expressed using a cell-free transcription/translation system (IVTT, in vitro transcription and translation). The newly expressed protein is then captured onto the array by an anti-GST antibody that has been co-immobilized with the expression clone on the microarray surface. The advantages and benefits of NAPPA technologies can be summarized as follows:

It spares time and the challenging process of obtaining highly purified proteins since expression, purification, stabilization and preservation of the protein and its subsequent spotting on the assay are replaced by a single step.

Availability of cDNAs and clones began with an old cooperation with the Virginia G. Piper Center for Personalized Diagnostics, Biodesign Institute, Arizona State University, Tempe, AZ, USA.

Protein integrity and stability, being freshly expressed in a cell-free mammalian system at the needed time, avoiding any not natural folding or post-translational modifications.

Proteins expressed on the NAPPA arrays preserve their functions and are properly folded and biologically active. NAPPA microarray has, in fact, been successfully used for the study of different kinds of protein-protein interaction. Our previous published data taken together with data from the extant literature show that proteins displayed on the array can be used for functional assays up to 24 hours after the protein expression. Moreover, NAPPA arrays printed with the cDNAs of interest can be even stored for more than six months. The expression of the proteins is performed just when the microarray is needed, and for this reason it is not necessary to worry about protein stability above the 24 hours window.

NAPPA microarrays can be useful in biomarkers discovery and for other clinical applications [32, 33], especially in the effort of moving towards Personalized Medicine [34-38]. NAPPA can be used also as a sensing system platform enabling the development of biosensor [39]. For this task we coupled NAPPA with a new generation of conductometric devices, namely QCM. QCM_D indeed appears a promising tool to study protein-protein interactions especially in the field of oncology, both cellular and molecular.

To the best of our knowledge, we coupled for the first time QCM_D with NAPPA technology for biomedical applications. The objective of the present research regards the analysis of multiple protein-protein interaction towards potentially useful clinical applications, namely in the field of vaccinology. QCM-based application for vaccine design and implementation has been described only by Rutledge and collaborators [40]. The authors developed an antigenic mimic of the Ebola glycoprotein.

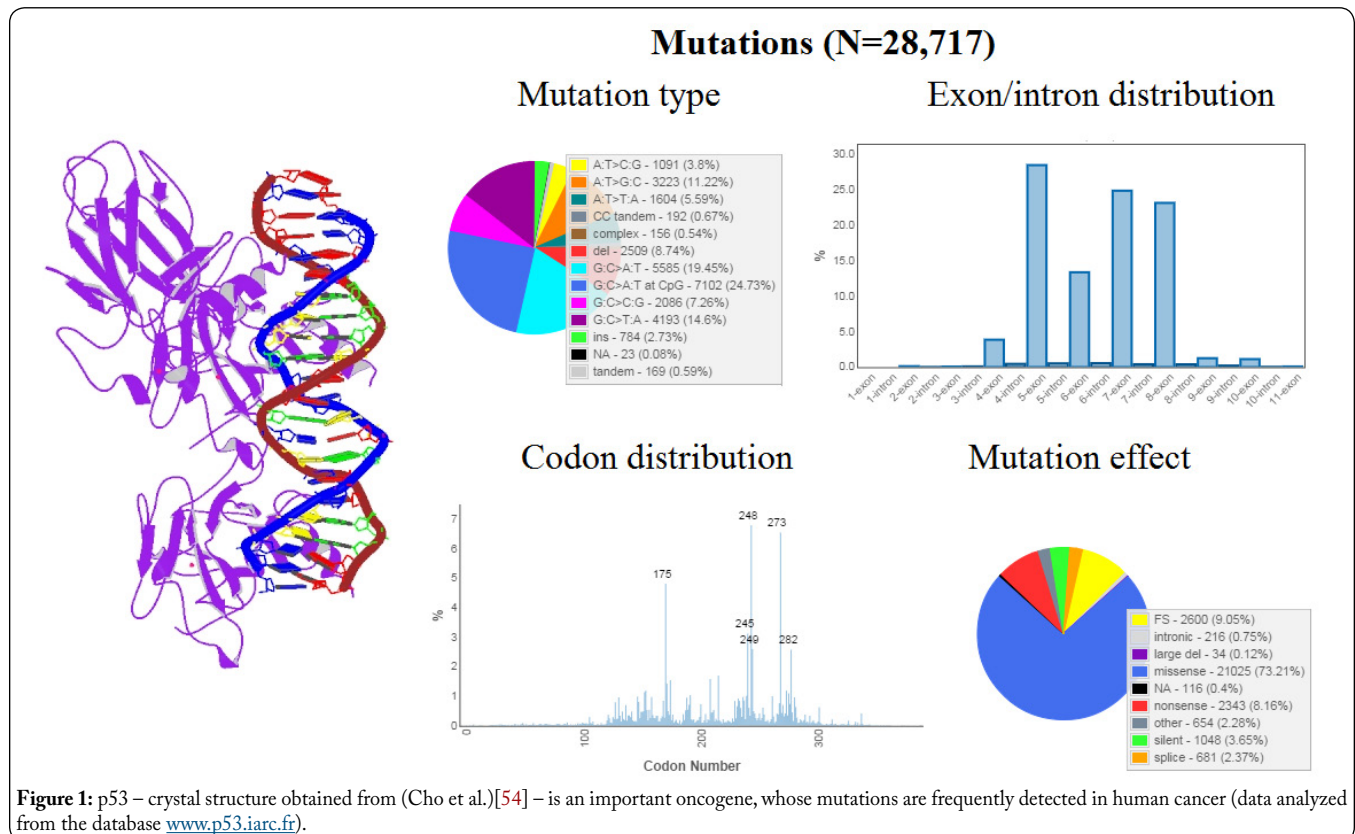
p53 as Peptide Vaccine for Tumors

With the discovery that many patients with cancer develop antibodies against p53 (the so-called oncoantibodies), it was evident that oncoprotein are immunogenic and can be used for immunotherapeutics purposes [41-49].

p53 is a 53-kiloDalton phosphoprotein oncosuppressor, encoded by a 20-kilobases gene situated on the short arm of human chromosome 17 and, due to its biological importance, termed as the “guardian of the genome” and the “policeman of oncogenes” [50, 51]. Mutated, it is involved in up to 70% of human tumors, being responsible of cell growth arrest, senescence, apoptosis in response to an array of stimuli such as DNA damages (DSBs, or double-strand-breaks), hypoxia, telomeres shortening, cell adhesion, oncogene activation and other molecular and cellular stresses [52].

Mutations in the TP53 gene characterize 50% of all reported cancer cases [53]. In the other cases, the gene is not mutated but the p53 pathway is often impaired (Figure 1). p53-based therapy is quite effective in the cure of cancer. TP53 may be delivered as a gene therapy or since the p53 protein is over-expressed in many cancers, may be exploited as a peptide-based vaccine. p53 is indeed immunogenic, since it elicits the response of immune system and in some patients autoantibodies are found (these antibodies are also called oncoautoantibodies).

and attenuated recombinant *Listeria monocytogenes* expressing human p53 (LmddA-LLO-p53) as a homologous/heterologous prime/boost in a human p53 knock in Hupki mouse model. As adjuvant, they employed to synthetic double-strand RNA (polyinosinic:polycytidylic acid) and unmethylated CpG-containing oligodeoxynucleotide to activate the innate immune system via Toll-like receptors. Some groups [56, 57] assessed the response of an injection of p53 peptides in patients with metastatic melanoma. van der Burg and coauthors [58] evaluated the effect of a recombinant canarypoxvirus (ALVAC) vaccine encoding wild-type human p53 in patients with colorectal cancer. A similar experimentation was carried out by other groups [59, 60], with similar encouraging results. Hoffmann and co-workers [61] used the HLA-A2.1-restricted wild type p53(264-272) epitope to generate cytotoxic T-lymphocytes from circulating precursor T cells of healthy donors and patients with squamous carcinoma of the head and neck. Autologous peptide-pulsed dendritic cells-based approach proved to be quite effective. Antonia and collaborators [62] experimented a dendritic cells-transduced approach with the full-length wild-type p53 gene delivered via an adenoviral vector in patients with small cell lung cancer. Similar results were obtained by another group [63]. Shi and colleagues [64] experimented a p53-based vaccine for lung adenocarcinoma. Andrade and coauthors [65] used the HLA-A2-restricted wild type polymorphic p53(65-73)



So far p53-based vaccines have been exploited for the following tumors: head and neck squamous carcinoma, breast and ovarian cancer, colorectal tumor, lung cancer, pancreatic adenocarcinoma, sarcoma, melanoma, glioma and hepatocellular carcinoma, as well as for metastatic diseases.

Ishizaki and collaborators [55] used a modified vaccinia Ankara (MVA) vaccine expressing human p53 (MVA-p53)

against squamous carcinoma of the head and neck. Rahma and coworkers [66] used p53:264-272 peptide for treating patients with recurrent ovarian cancer, with Montanide, GM-CSF and interleukin-2 as adjuvant molecules. Schuler and collaborators [67] used adjuvant dendritic cell-based vaccination against p53 for head and neck squamous carcinoma, using a HLA-restricted p53 epitope and an additional Th tetanus toxoid peptide. Svane and collaborators [68] investigated the usefulness of a dendritic

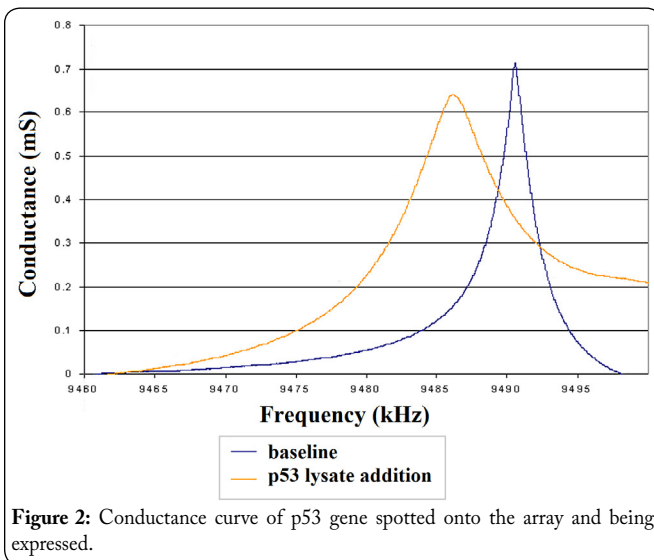


Figure 2: Conductance curve of p53 gene spotted onto the array and being expressed.

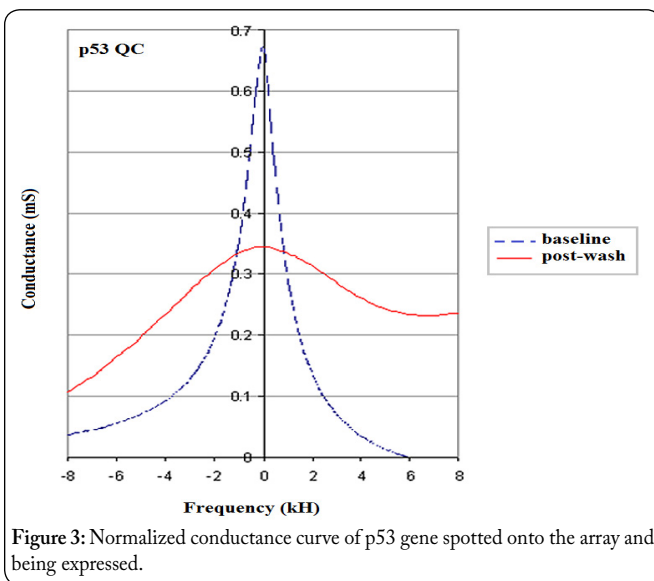


Figure 3: Normalized conductance curve of p53 gene spotted onto the array and being expressed.

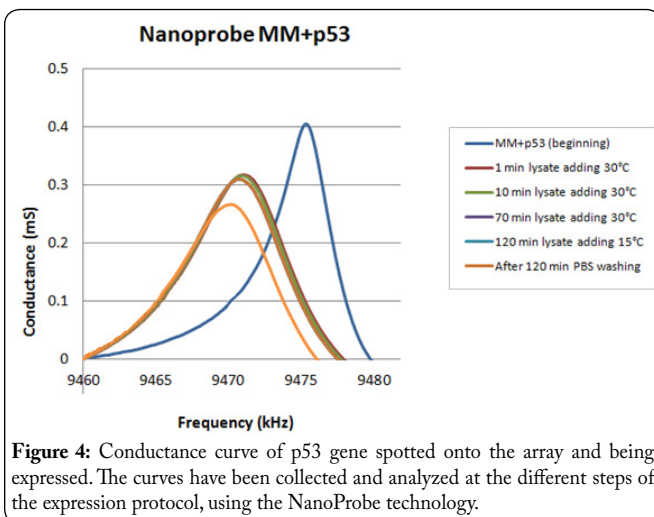


Figure 4: Conductance curve of p53 gene spotted onto the array and being expressed. The curves have been collected and analyzed at the different steps of the expression protocol, using the NanoProbe technology.

epitopes. Terashima and collaborators [71] demonstrated the effectiveness of p53-peptide therapy for pancreatic carcinoma. Lomas and collaborators [72] assessed an idiotypic vaccine, composed of a pool of eight peptides derived from the complementarity determining regions (CDRs) of human anti-p53 antibodies.

QCM_D Conductometer

Nanogravimetry [31] makes use of functionalized piezoelectric quartz crystals (QC), which vary their resonance frequency (f) when a mass (m) is adsorbed to or desorbed from their surface. This is well described by the well-known Sauerbrey's equation:

$$\Delta f/f_0 = -m/A \cdot \rho \cdot l$$

where f_0 is the fundamental frequency, A is the surface area covered by the adsorbed molecule and ρ and l are the quartz density and thickness, respectively.

Quartz resonators response strictly depends on the biophysical properties of the analyte, such as the viscoelastic coefficient. The dissipation factor (D) of the crystal's oscillation is correlated with the softness of the studied material and its measurement can be computed by taking into account the bandwidth of the conductance curve $2 \cdot \Gamma$, according to the following equation:

$$D = 2 \cdot \Gamma / f$$

where f is the peak frequency value.

In our analysis we introduced also a "normalized D factor", D_N , that we defined as the ratio between the half-width half-maximum (Γ) and the half value of the maximum value of the conductance (G_{max}) of the measured conductance curves [31]:

$$D_N = 2 \cdot \Gamma / G_{max}$$

D_N is more strictly related to the curve shape, reflecting the conductance variation [31, 32]. The QCM_D instrument was developed by Elbatech (Elbatech srl, Marciana – LI, Italy). The quartz was connected to an RF gain-phase detector (Analog Devices, Inc., Norwood, MA, USA) and was driven by a precision DDS (Analog Devices, Inc., Norwood, MA, USA) around its resonance frequency, thus acquiring a conductance versus frequency curve ("conductance curve") which shows a typical Gaussian behaviour. The conductance curve peak was at the actual resonance frequency while the shape of the curve indicated how the viscoelastic effects of the surrounding layers affected the oscillation. The QCM_D software, QCMagic-Q5.3.256 (Elbatech srl, Marciana – LI, Italy) allows to acquire the conductance curve or the frequency and dissipation factor variation versus time. In order to have a stable control of the temperature, the experiments were conducted in a temperature chamber. Microarrays were produced on standard nanogravimetry quartz used as highly sensitive transducers. The QC expressing proteins consisted of 9.5 MHz, AT-cut quartz crystal of 14 mm blank diameter and 7.5 mm electrode diameter, produced by ICM (Oklahoma City, USA). The electrode material was 100 Å Cr and 1000 Å Au and the quartz was embedded into glass-like structures for easy handling [31, 37, 39]. The NAPPA-QC arrays were printed with 100 spots per QC. Quartzes gold surfaces were

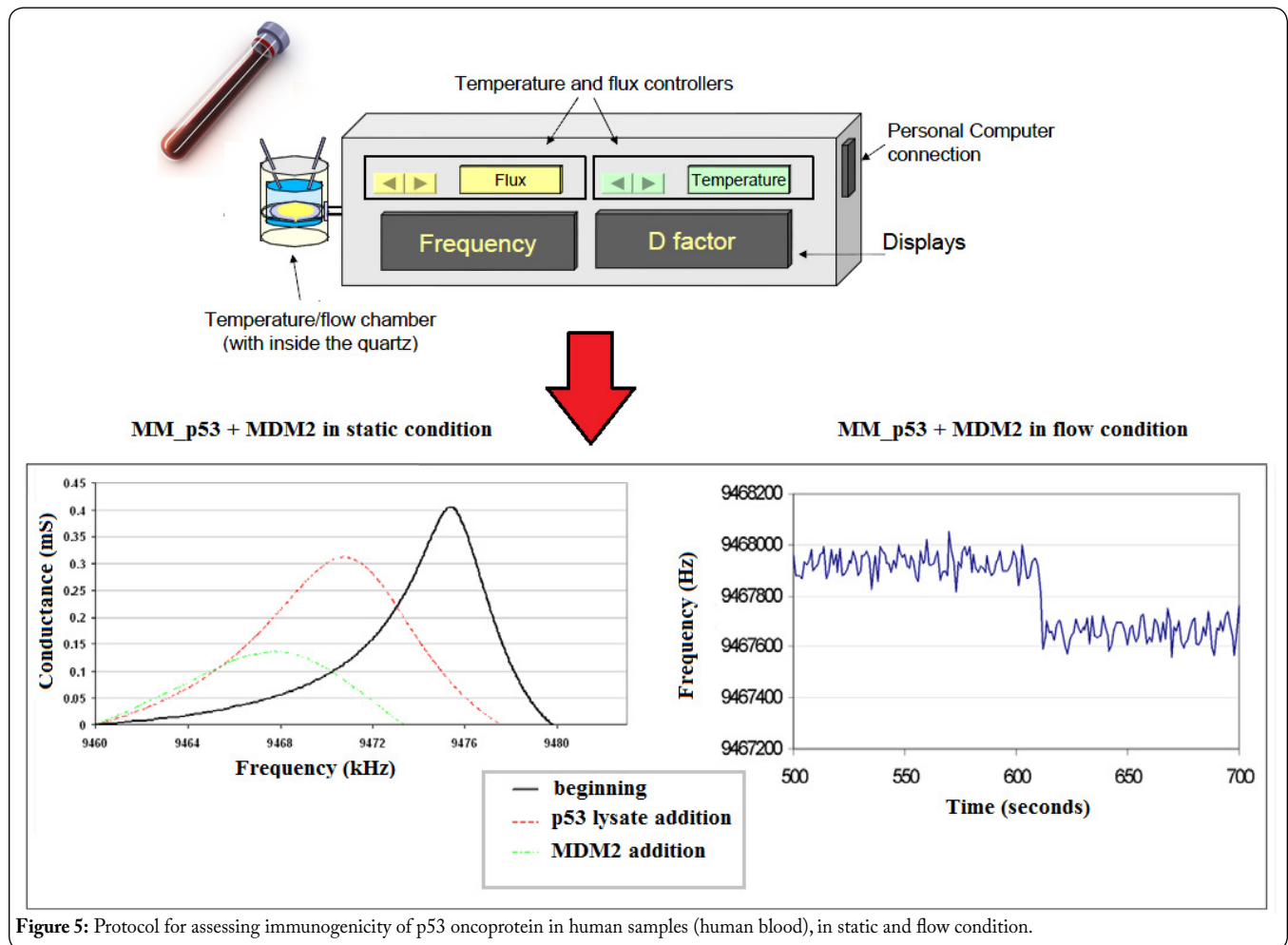


Figure 5: Protocol for assessing immunogenicity of p53 oncoprotein in human samples (human blood), in static and flow condition.

coated with cysteamine to allow the immobilization of the NAPPA printing mix. Briefly, quartzes were washed three times with ethanol, dried with Argon and incubated over night at 4°C with 2 mM cysteamine. Quartzes were then washed three times with ethanol to remove any unbound cysteamine and dried with Argon. Plasmids DNA coding for GST tagged proteins were transformed into *E. coli* and DNA were purified using the NucleoPrepII anion exchange resin (Macherey Nagel). NAPPA printing mix was prepared with 1.4 µg/ul DNA, 3.75 µg/ul BSA (Sigma-Aldrich), 5 mM BS3 (Pierce, Rockford, IL, USA) and 66.5 µg polyclonal capture GST antibody (GE Healthcare). Negative controls, named master mix (hereinafter abbreviated as “MM”), were obtained replacing DNA for water in the printing mix. Samples were incubated at room temperature for 1 hour with agitation and then printed on the cysteamine-coated gold quartz using the Qarray II from Genetix. In order to enhance the sensitivity, each quartz was printed with 100 identical features of 300 microns diameter each, spaced by 350 microns center-to-center. The human cDNAs immobilized on the NAPPA-QC was TP53. Gene expression was performed immediately before the assay, following the protocol described in [31]. Briefly, *IVTT* was performed using HeLa lysate mix (1-Step Human Coupled IVTT Kit, Thermo Fisher Scientific Inc.), prepared according to the manufacturers’ instructions. The quartz, connected to the nanogravimeter inside the incubator, was incubated for 10 min at 30°C with 40 µl of HeLa lysate mix for proteins synthesis and then, the temperature was decreased to 15°C for a period of 5 min to facilitate the

proteins binding on the capture antibody (anti-GST). After the protein expression and capture, the quartz was removed from the instrument and washed at room temperature, in 500 mM NaCl PBS for 3 times. The protocol described above was followed identically for both negative control QC (the one with only MM, i.e, all the NAPPA chemistry except the cDNA) and protein displaying QC. After protein expression, capture, and washing the QCs were used for the interaction studies QC displaying the expressed protein was spotted in PBS at increasing concentrations at 22°C. Reproducibility of the experiments was assessed computing the coefficient of variation (C_v , or σ^*), using the following equation:

$$\sigma^* = \sigma/\mu,$$

where σ is the standard deviation, and μ is the mean.

QCM_D measures were calibrated for frequency and for D factor shifts. The calibration curves equation (obtained with Ordinary Least Squares methods, OLS) are:

$$\Delta f = -7.16 - 231.18 m; \text{ with } r^2 = 0.9986,$$

and:

$$D = 0.831 + 0.286 \eta; \text{ with } r^2 = 0.9990.$$

We analyzed the conductance curves acquired in NAPPA-QCs in different steps of the expressing and capturing process: after the addition of human IVTT lysate at 30°C (“*IVTT addition*”), i.e. prior protein expression; after 10 min from the addition of human IVTT lysate, i.e. after protein expression (“*IVTT addition 10 min*”); after the final washing process with PBS (“*Post-wash*”). In Figures 2-4 are reported

the conductance curves of p53 spotted on quartz blanks being expressed. In **Figure 5**, protocol for assessing immunogenicity of p53 oncoprotein in human samples (human blood), in static and flow condition, is shown.

Conclusions

NAPPA-based QCM_D can be a useful platform for proving the immunogenicity of oncoprotein-based vaccines. Recently, the field of vaccinology has extended from vaccines for infectious diseases to vaccines not only preventive but also therapeutic for chronic-degenerative diseases such as cancer. Peptide-based immunotherapeutics has been proven to be quite effective for cancer treatment and NAPPA-based QCM_D has the promise of providing clinicians with quick, rapid and cheap measurement of oncoprotein kinetics and bindings with immune cells. Moreover, it can be a precious tool for implementing personalized and predictive vaccinology.

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Dr. Nicola Luigi Bragazzi is actually resident in Public Health, third year of specialty in Hygiene and Preventive Medicine at the School of Public Health, Department of Health Sciences (DISSAL), University of Genoa and wishes to thank the Medical School for inspiration and support during his training residence.

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