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Towards liquid biopsy: Rapid Determination of the Humoral Immune Response in Cancer Patients using HaloTag Fusion protein-Modified Electrochemical Bioplatforms

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ABSTRACT: Autoantibodies raised against tumor-associated antigens have shown high promise as clinical biomarkers for reliable diagnosis, prognosis and therapy monitoring of cancer. An electrochemical disposable biosensor for the specific and sensitive determination of p53-specific autoantibodies has been developed for the first time in this work. This biosensor involves the use of magnetic microcarriers (MBs) modified with covalently immobilized HaloTag fusion p53 protein as solid supports for the selective capture of specific autoantibodies. After magnetic capture of the modified MBs onto screen-printed carbon working electrodes (SPCEs), the amperometric signal using the system hydroquinone (HQ)/H_2O_2 was related to the levels of p53-autoantibodies in the sample. The biosensor was applied for the analysis of sera from 24 patients with high-risk of developing colorectal cancer and 6 from patients already diagnosed with colorectal (4) and ovarian (2) cancer. The developed biosensor was able to determine p53 autoantibodies with a better sensitivity than a commercial standard ELISA and using a just-in-time produced protein in a simpler protocol, less sample volume and easily miniaturized and cost effective instrumentation.

Cancer is the cause of over 8 million deaths worldwide in 2013 and the second leading cause of death just behind cardiovascular disease.[1] Although, substantial progress has been made in the last years for prevention and treatment of different cancer types,[2] cancer incidence and prevalence is increasing worldwide due to the continuous growth and aging of the global population and the maintenance or the increase in smoking, obesity, and risky dietary patterns.[1,2] Therefore, the implementation of non-invasive massive population screening programs could help reducing cancer mortality due to the detection at early stages, when the disease can be successfully treated and cured in more than 90% of patients of the eight most common cancer types.

The immune system has been largely shown to produce autoantibodies against altered tumor-associated antigens (TAAs).[3-5] The production of these autoantibodies, related to TAA overexpression, mutation, altered post-translational modification, etc[6], may serve as indicators of tumor initiation, treatment response, or disease prognosis.[7] Remarkably, the accurate detection of autoantibodies could be used for early diagnosis of cancer patients, since it has been demonstrated that the humoral immune response in different cancer types appears several months or years before the clinical symptoms.[8-12]

In addition, unlike other cancer biomarkers released by cancer cells and related to tumor mass, autoantibodies are

indicative of the presence of few tumor cells due to amplification by the immune system.[10,13] Despite the advantages of autoantibody detection for cancer diagnosis, the fact that patients develop autoantibodies to multiple TAAs is a major limitation to get clinically validated assays because on one hand autoantibody signature diagnostic panels should be accurately identified and on the other hand, new immunoassays for multiplexed autoantibodies detection should be developed.

A likely candidate in such autoantibody diagnostic panels should be p53. Serum p53 autoantibodies are reported in 10-40% of all cancer patients depending on the cancer type.[11,14,15] On the other hand, electrochemical biosensors represent a great alternative to conventional immunoassays (i.e. ELISA, WB or Luminex) for the detection of cancer biomarkers because of their simplicity of use and their versatility to detect various types of molecular targets (proteins, miRNAs, or mRNAs) simultaneously and using the same methodology.[16-20]

Other major step for the development of immunoassays consists of the production of proteins with good yield, purity and stability for functional studies. A recent very attractive alternative is to substitute the use of purified expensive cancer proteins for HaloTag fusion proteins produced just-in-time for the assay using a cell-free system. HaloTag is an engineered dehalogenase developed to covalently bind to halogenated alkanes.[21] Therefore, the use of these HaloTag fusion proteins eliminates the need to express and purify proteins separately, producing in a *mammalian milieu* the proteins for the assay, abrogating potential concerns about protein folding, stability and integrity during storage. This *in vitro* expression system is highly efficient, and has succeeded at producing thousands of different proteins for the identification of disease-specific antibodies and host-pathogen interaction profiling.[21-23] Although the uses of HaloTag fusion proteins or MBs-based electrochemical bioplatforms have been described separately in several papers of different fields, these previous works missed the obvious benefits of the combination of these two attractive technologies. This is what the strategy reported in this communication rectifies, demonstrating for the first time that the use of MBs coupled to *in situ* expressed HaloTag fusion proteins allows the efficient and selective scavenging of specific autoantibodies from sera samples. The further amperometric transduction permits the implementation of a very attractive, simple and rapid electrochemical methodology for autoantibodies determination. Considering the versatility of the methodology for protein immobilization, this novel strategy can be potentially generalized to other research fields, such as the study of other protein-(bio)molecules interactions, making it readily applicable to the determination of other (bio)molecules apart from the relevant clinical application here described for the detection of autoantibodies for early cancer diagnosis.

EXPERIMENTAL SECTION

Apparatus and electrodes. Amperometric measurements were performed with a CHI812B potentiostat (CH Instruments) controlled by software CHI812B. Screen-printed carbon electrodes (SPCEs) (DRP-110, DropSens), consisting of a 4-mm diameter carbon working electrode, a carbon counter electrode and an Ag pseudo-reference electrode, were employed as transducers and specific cable connector (ref. DRP-CAC also from DropSens, S.L.) acted as interface between the SPCEs and the potentiostat. A DynaMag-2 Magnet from Invitrogen (Spain) and a MixMate microtube mixer from Eppendorf (Germany) were employed for the magnetic separation and incubation of the MBs. Capture of the modified MBs onto the working electrode surface of the SPCE was controlled by a neodymium magnet (AIMAN GZ) embedded in a homemade casing of Teflon (see Figure 1).

Reagents and solutions. Anti-human IgG antibody HRP conjugated was from Dako (Barcelona). HaloTag magnetic beads (HaloTag MBs, 50–80 μm Ø, MBs Magne® HaloTag® beads, Cat. No: G7281) were obtained from Promega (Promega Biotech Ibérica, Madrid) and used as received according to the manufacturer instructions. Tween 20, hydroquinone (HQ) and H_2O_2 (30%, w/v) were purchased from Sigma-Aldrich. A blocker casein solution (a ready-to-use, PBS solution of 1% w/v purified casein) from Thermo Scientific was also used. All other chemicals were of analytical grade and ultrapure water (Millipore Milli-Q) was used throughout. A 0.05 M phosphate buffer, pH 6.0, solution was used to perform the electrochemical measurements.

Patient sera. The Institutional Ethical Review Board of the Clínico San Carlos Hospital (Madrid) approved this study on biomarkers. Serum samples were obtained from the biobank of the Clínico San Carlos Hospital, which belongs to the San Carlos Clinical Research Institute of health (IdiSSC) and is part of the Spanish National Biobank Network of the ISCIII, co-funded with FEDER funds.

A total of 62 individuals followed up in outpatient clinics of Gastroenterology due to a high risk of colorectal cancer (based on colorectal cancer familial history not linked to a reported inherited disease and a positive fecal occult blood test) were included in the study (see details in Table S1). In all cases, one or two different tissue samples were also obtained from each patient at the Gastroenterology Unit of the Clínico San Carlos Hospital and analyzed in the Surgical Pathology Department of the same Hospital (histologically data from all patients are also included in Table S1). The p53 autoantibody seroreactivity was analyzed to identify among those patients that should be extensively monitored for the presence of neoplastic colorectal lesions. All samples were obtained from the Hospital prior to initiating any treatment between June 2015 and March 2016. Samples were collected using a standardized sample collection protocol and stored at -80 °C until use [10,24-28].

Sera samples from 6 patients already diagnosed with colorectal (4) and ovarian (2) cancer were also obtained from La Paz Hospital (Madrid) after approval of the Ethical Review Board of the institution. Written informed consent was obtained from all patients.

***In vitro* optimized Gateway plasmid construction, gene cloning, DNA preparation and *in vitro* protein expression.** Sequence-verified, full-length cDNA plasmids containing *TP*53, *TP*63 and *TP*73 in flexible pDONR221 or pENTR223 vector systems were obtained from the publicly available DNASU Plasmid Repository (https://dnasu.org/DNASU/Home.do). ORFs were transferred to pANT7_cHalo vector by LR recombinase (Invitrogen, Carlsbad, CA) for *in vitro* expression of p53, p63 and p73 fusion proteins to HaloTag at the C-terminal end. For the construction of the Gateway compatible pANT7_cHalo plas-

mid, pANT7_cGST plasmid digested with *Xho*I and *BstB*I was used to subclone the HaloTag cDNA amplified by PCR using the pFN18a plasmid as template and the sense 5'-AAACTCGAGATGGCAGAAATCGGT-3' and the antisense 5'-AAATTCGAATTAACCGGAAATCTC-3' oligonucleotides (purchased from IDT Integrated DNA technologies). After purification, the PCR product was digested with *Xho*I and *BstB*I, ligated into digested pANT7_cGST plasmid, transformed into DB3.1 cells and grow in Luria Bertani (LB) supplemented with chloramphenicol and ampicillin. A schematic representation of the steps followed to construct the pANT7_cHalo plasmid is displayed in Figure S1. Then, the plasmid was purified using the NucleoBond® Xtra Midi kit (Macherey-Nagel Inc., Bethlehem, PA) and used in LR clonase recombination reactions (Invitrogen) to get the expression vectors according to the manufacturer instructions [29]. All donor and expression plasmids were sequence verified prior to a subsequent use.

To obtain high-quality supercoiled DNA to be used for cell-free protein expression, expression plasmids were transformed into DH5α *E. coli* cells and grown in 250 mL LB supplemented with ampicillin (100 μg mL^{-1}). Expression vectors codifying for p53, p63 and p73 HaloTag-fusion proteins were purified using the NucleoBond® Xtra Midi kit (Macherey-Nagel Inc., Bethlehem, PA).

Proteins were expressed using T7 reticulocyte lysate (Promega Corporation, Madison, WI) per manufacturer's recommendations in 20 μL volume with 500 ng of indicated expression DNA plasmids.

Preparation of the HaloTag fusion protein modified MBs-based biosensor and assay procedure. For *in situ* protein expression and selectively captured on the surface of the MBs, 1.8 μL of the *in vitro* transcription/translation (IVTT) product was incubated with 1.0 μL of HaloTag MBs suspension during 2 h at room temperature and 800 rpm for *in situ* p53-HaloTag expression and its selective capture to MBs according to manufacturer instructions. After washing, tagged HaloTag fusion proteins modified MBs were blocked in blocker casein solution diluted 1:1 in PBS for 2 h, and then incubated for 1 h at room temperature with serum samples at indicated dilutions at 800 rpm. After washing, anti-human IgG antibody HRP conjugated (Dako) diluted 1:3000 in PBS supplemented with Tween 20 0.1% (v/v) and 3% (w/v) skimmed milk was incubated with the MBs. For control experiments haloTag-protein tags were detected with anti-HaloTag (Promega), and p53 and p73 with anti-p53 (BD) and anti-p73 (R&D Systems) antibodies followed by 30 min incubation with the HRP conjugated-anti-mouse IgG diluted 1:1000.

The amperometric measurements were performed using disposable screen-printed carbon electrodes (SPCEs) by magnetically capturing the modified MBs on the SPCE working electrode by keeping the SPCE in a horizontal position after placing it in the homemade magnet holding block. The ensemble magnet holding block/SPCE with the captured modified MBs was immersed into an electrochemical cell containing 10 mL of 0.05 M phosphate buffer (pH 6.0) and 1.0 mM HQ (prepared just before the electrochemical measurement). Amperometric measurements in stirred solutions were performed by applying a detection potential of -0.20 V vs Ag pseudo-reference electrode upon addition of 50 μL of a 0.1 M H_2O_2 solution. The amperometric signal was recorded during 60 (for baseline stabilization) and 120 s (to reach the steady-state current) before and after H_2O_2 solution addition, respectively. This procedure triggered the electrochemical reactions described in Figure 1 and gave rise to an analytical signal, which corresponded to the difference between the steady-state and the background currents, and was proportional to the autoantibodies level in the standard/sample analyzed. At least two replicates were measured for each standard/sample. As positive and negative controls of the assay, pools of 4 p53 positive sera and 4 p53 negative sera were used.

Alternatively, for comparison purposes same modified MBs used for amperometric detection at SPCEs were carefully collected, placed on black Maxisorp 96-well plates (Nunc) and 100 μL of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) were employed for the detection of luminescence on a FLUOstar Optima (BMG LABTECH, Ortenberg, Germany).

p53 autoantibody ELISA kit. p53 autoantibody ELISA kit was performed following manufacturer's recommendations (p53-auto antibody ELISA-Kit (Human), Dianova GmbH, Hamburg, Germany) as control of the sensor approach. 100 μL of each control and serum samples were added in duplicate. Serum samples were diluted 1:100 and incubated for 1 h at room temperature. Bound IgGs were detected by absorbance at 450 nm using a Biochrom EZ Read 400 Microplate Reader (Biochrom, Cambridge, UK). As positive and negative controls, one p53 positive and one p53 negative sera included in the ELISA kit were tested. Epstein–Barr nuclear antigen 1 (EBNA1), a multifunctional, dimeric viral protein associated with the capsid of Epstein–Barr virus, was also tested by ELISA instead of p53 as positive control of seroreactivity [25].

SDS-PAGE, western blot (WB) analysis and peptide mass fingerprinting. SDS-PAGE and WB analysis were performed as described [30] to assess protein quality. Briefly, 2 μL of reticulocyte IVTT protein extracts were run in 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C extra). After blocking, membranes were incubated overnight at 4 °C with optimized dilutions of specific monoclonal antibodies against p53, p73 and HaloTag. Immunodetection on the membranes was achieved by using HRP-conjugated secondary antibodies (Sigma). The ECL signal was developed with ECL Western Blotting Substrate (Thermo Scientific) and detected on a Fujifilm LAS-3000 Imager (Fujifilm). As positive and negative controls, GST-HaloTag protein and reticulocyte containing pANT7_cHalo –no protein expression- were included in the assay, respectively.

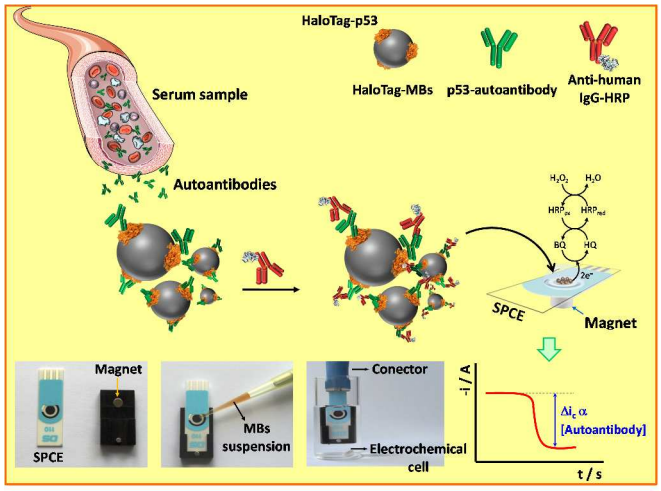
Statistical analysis. Biosensing amperometric measurements, and p53 and EBNA1 ELISA analyses in sera were performed in duplicate and positive sera were confirmed on repeated experiments. Values were plotted as mean values±SEM. p53 seroreactivity was previously defined on a commercial ELISA according to the manufacturer instructions. Analytical signals given through the manuscript corresponded to the difference between the signals measured in the presence and in the absence of HaloTag-fusion protein immobilized onto MBs or ELISA plate. In this latter case, reticulocyte containing pANT7_cHalo –no protein expression- was immobilized onto the MBs as negative control of the assay.

***In vitro* protein expression of HaloTag fusion proteins.** The developed approach involved the design and construction of a pANT7_cHalo vector bearing a Gateway™ death cassette closed to a C-terminal fusion HaloTag protein. This optimized vector for *in vitro* protein transcription/translation has a T7 promoter and an encephalomyocarditis virus internal ribosome entry site sequence upstream of the gene of interest [29,31]. We then transferred *TP*53, *TP*63, and *TP*73 genes from donor vectors to pANT7_cHalo through Gateway LR reactions and directly used the purified DNA for *in vitro* protein expression of the protein fusions to HaloTag (Figure S2).

The success of protein expression was determined by probing IVTT expression by WB with an anti-HaloTag antibody that recognizes the C-terminal tag on every antigen and anti-p53 and anti-p73 monoclonal antibodies that specifically recognize the presence of p53 and p73 fusion proteins, respectively (Figure S2B). Protein expression ranged between 5-10 ng per IVTT µL according to the amount of the HaloTag-GST protein included in the assay as control.

RESULTS AND DISCUSSION

The fundamentals of the first electrochemical biosensor involving the use of HaloTag fusion proteins-modified MBs for the specific and sensitive quantification of p53-specific autoantibodies in sera and the reactions involved in the electrochemical transduction are depicted in Scheme 1. This novel approach makes use of MBs, modified by covalent immobilization of C-terminal-tagged HaloTag fusion *in vitro* expressed proteins, as efficient magnetic microcarriers to selectively capture the autoantibodies against the immobilized protein present in the sera of patients. Subsequently, the attached autoantibodies are detected with a secondary HRP conjugated anti-human IgG. Then, the MBs bearing the immunocomplexes are magnetically captured on the SPCE and the biorecognition event is monitored by amperometric transduction at –0.20 V (vs a Ag pseudoreference electrode) of the cathodic current generated by the enzymatic reduction of H_2O_2 mediated by HQ.



Scheme 1. Scheme of the HaloTag fusion protein modified MBs-based immunosensing platform for the amperometric determination of p53-specific autoantibodies.

Remarkably, this approach may be an interesting alternative for the early detection of tumors in comparison to traditional approaches, which are still either costly in time of manufacturing (i.e. traditional methods that use purified proteins require weeks or months of protein preparation and optimization prior to the development of the immunoassay) or in price (i.e. multiplex technologies like Luminex are difficult to afford in multiple labs). In the proposed approach, the proteins used as selective capture bioreceptors are expressed in a mammalian microenvironment during the immunoassay by *in vitro* transcription/translation avoiding at the same time, the expression and purification steps regarding to bacterial, insect or mammalian cells, the concerns regarding to the stability and integrity of the protein during storage and the presence of potentially immunogenic bacterial products in the conventional immunoassays. Moreover, the use of MBs is known to be a powerful tool in a variety of bioassays. In particular, they improve the performance of disposable electrochemical biosensors in terms of sensitivity, reduced assay time and minimization of matrix effects and avoid the need for delicate electrode preparation to enable control of density and orientation of recognition probes at the surface.[32-34] In addition, the required instrumentation with MBs-based immunosensing platforms is inexpensive and might be reduced to pocket-size dimensions ideal for their integration in *point-of-care* devices.[35]

First, covalent immobilization of HaloTag p53, p63 and p73 fusion proteins expressed *in vitro* to MBs was assessed. The efficiency of the immobilization onto MBs was evaluated by using anti-HaloTag, anti-p53 and anti-p73 antibodies. Amperometric (Figure 1) and luminiscence (Figure S3 in the Supporting information) measurements were carried out for this purpose.

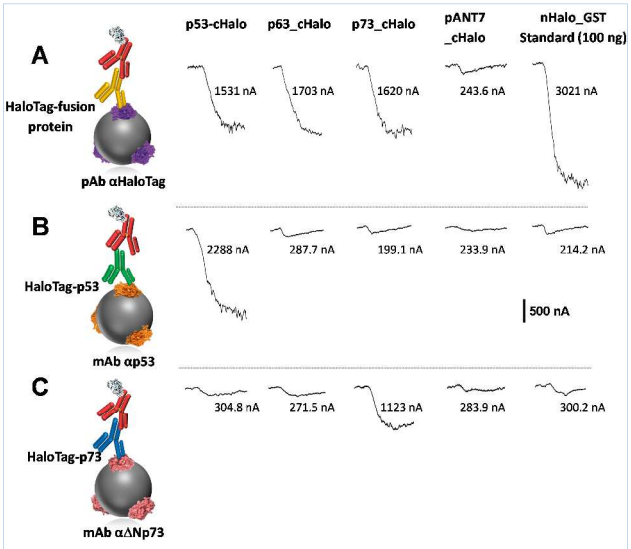


Figure 1. Specific biosensing detection of HaloTag fusion proteins. DNA expression plasmids were used to express in vitro p53, p63 and p73 proteins, which were then covalently immobilized onto HaloTag MBs and detected by amperometry using A) an anti-HaloTag pAb, B) an anti-p53 mAb and C) anti-p73 mAb followed by conjugation with specific secondary HRP-labelled antibodies. Amperometric traces recorded with the HaloTag fusion protein modified MBs-based biosensors are shown. pANT7_cHalo DNA (no protein expression) and HaloTag-GST protein were included as negative and positive controls, respectively.

Results obtained with both strategies were in full agreement. Specific responses were only obtained in the presence of anti-

HaloTag to all fusion proteins, and anti-p53 and anti-p73 antibodies when the p53 and the p73 Halotag fusion proteins were immobilized, respectively, onto the MBs. These results demonstrated the great potential of the surface chemistry involved (HaloTag fusion protein-HaloTag-MBs) suitable for the efficient immobilization of all proteins with similar yields without particular optimization parameters and the feasibility of the so prepared biosensors for the specific detection of autoantibodies. Subsequent experiments were focused on p53 autoantibodies, for their important role in early cancer detection, reported to be elevated up to 3.8 years before clinical diagnosis, and in stratification according to the cancer patient risk.[11,14]. Therefore, the use of a low-cost screening assay for the detection of p53 autoantibodies should be of worldwide public health benefit.[1,2,11,14] In addition, the detection of p53 autoantibodies is of interest on the following up cancer patients during or after treatment and to detect recurrences (since it has been reported that after cancer curation p53 autoantibodies progressively decline[13,36]).

The detection of the presence of autoantibodies to p53 was performed on a non-characterized population of 62 sera from individuals at the high-risk program to develop colorectal cancer (Table S1). ELISA test determined that 32 out of the 62 analyzed sera (mean ± SEM 0.33 ± 0.02 *vs* 0.13 ± 0.008, $p < 0.01$) were positive for the presence of p53 autoantibodies with an absorbance 5 times higher than the negative control (Figure 2 and Table S1 in the Supporting information). The seroreactivity to EBNA1 was employed as a control because 90-95% of all human individuals are reactive to that virus. Only 5 individuals in total were found to be nonreactive to EBNA1. As it can be seen in Figure 2, a similar seroreactivity to EBNA1 was found in p53 reactive and nonreactive patients (mean ± SEM 0.88 ± 0.08 *vs* 0.98 ± 0.10, p=0.414). Therefore, these data confirmed the selective seroreactivity to p53 in the analyzed population.

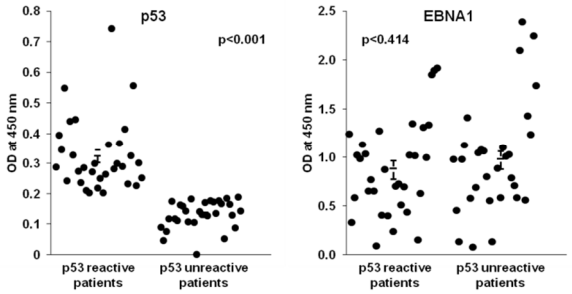


Figure 2. Analysis of the seroreactivity to p53 by ELISA. The specific autoantibody response to p53 was analyzed in comparison to EBNA1 –a protein of the capsid of the Epstein Barr Virus-.[25] Sera from the 62 individuals were analyzed at a 1:100 dilution and the specific IgGs bound to either p53 or EBNA1 were detected using a secondary HRP-labeled anti-human IgG**.**

The experimental variables involved in the amperometric detection of p53 autoantibodies were optimized by comparing the analytical responses obtained with a pool of p53 positive and p53 negative sera following the protocol described in section Preparation of the HaloTag fusion protein modified MBs-based biosensor and assay procedure. The volume of IVTT was optimized to be 1.8 µL per 1 µL of functionalized MBs. It is important to note that the one-step protocol for *in situ* production and selective capture of the HaloTag fusion protein is much simpler than the conventional procedure to get purified p53 produced in bacteria, insect or mammalian cells that usually requires weeks or months. Incubation times of HaloTag-p53-MBs with serum sample (1 h) and with the secondary antibody (30 min) were also optimized. Importantly, the whole protocol took no more than 5.5 hours. However it is important to mention that HaloTag fusion protein-modified MBs could be prepared in advance and stored at least during 7 days in filtered PBS at 4 °C (no longer times were assayed) until the determinations should be performed, reducing the assay time to only 1.5 h. Moreover, the detection potential used was previously optimized for the HRP/HQ/H_2O_2 system at SPCEs.[37]

The analytical performance of the electrochemical biosensing approach was characterized using as standard the calibrator provided in the commercial ELISA kit (diluted human serum with a defined p53 autoantibody concentration of 10 U mL^{-1}, being 1 U defined as p53 binding activity which corresponds to the binding activity of 100 µL undiluted calibrator). Under the selected experimental conditions, the calibration plot constructed with serial dilutions of this standard (Figure 3) exhibited a linear relationship (r= 0.999) between 1.1 and 5 U mL^{-1}, with a slope value of (215.2± 0.2) nA mL U^{-1}. Detection (LOD) and quantification (LQ) limits of 0.34 and 1.1 U mL^{-1}, respectively, were calculated according with the $3\times s_b/m$ and $10\times s_b/m$ criteria, respectively, where m is the slope of the linear calibration plot and s_b was estimated as the standard deviation of ten amperometric signals measured for a 2 times diluted negative control provided by the ELISA kit (a 1:100 dilution of serum containing no p53 autoantibodies). A relative standard deviation (RSD) value of 6.2% was estimated from the measurements made with 5 different sensors for the 1.25 U mL^{-1} p53-autoantibodies standard solution, demonstrating a great reproducibility of the whole MBs-based sensor fabrication including *in situ* expression of the HaloTag fusion protein and the signal transduction protocols. It is important to mention also that although the LOD is not included in the specifications of the ELISA kit, the minimum recommended dilution of the calibrator is 32 times (~0.3 U mL), very similar to the value estimated with the electrochemical bioplatform. Moreover, the sensitivity of the developed biosensor is 440 higher than the ELISA methodology (215.2 nA mL U^{-1} vs 0.4876 absorbance units mL U^{-1}). As it will be demonstrated below, this significantly higher sensitivity allows both a better discrimination between p53 reactive and non-reactive serum samples and the possibility of using 2 times larger dilutions of serum samples for the analysis.

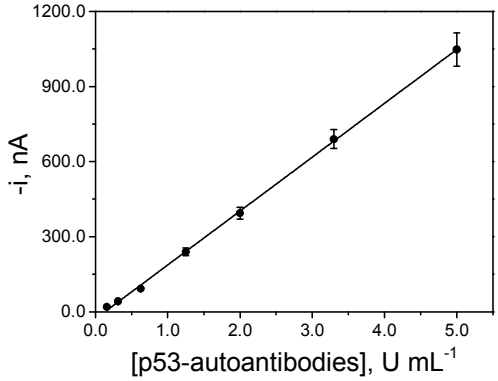


Figure 3. Calibration plot constructed for p53 autoantibodies standard with the developed electrochemical biosensor. Error bars estimated as triple of the standard deviation (n=3).

The applicability of the developed bioanalytical scaffold to the analysis of real samples was checked by analyzing serial dilutions (between 1:50 and 1:1200) of raw human sera from a pool of patients nonreactive to p53 and a pool of seroreactive individuals to p53 using two detection antibodies -a HRP-labelled secondary antibody and a biotin-labeled secondary antibody with further labeling with streptavidin-HRP (Figure S4). For all sera assayed, the non-specific amperometric signal (obtained with MBs modified with reticulocyte containing pANT7_cHalo –no protein expression-) was subtracted from each specific signal to p53. Best results for the detection of p53 autoantibodies with biotin labeled anti-Human IgG followed by streptavidin-HRP was observed using a serum 1:600 dilution (Figure S4). However, the best results in terms of positive/control signal ratio for the sera pool were achieved by using a 1:200 human serum dilution followed by a 1:600 dilution of HRP-labeled secondary antibody (Figure S4).

The diagnostic effectiveness of the biosensing approach was then evaluated by analyzing 24 human sera -12 random p53 seroreactive and 12 random non-seroreactive patients identified by ELISA- (Table S1) and the results were compared with those obtained by using the ELISA test. In general, both methodologies significantly detected higher levels of autoantibodies against p53 in p53 seroreactive patients in comparison to p53 non-seroreactive patients (Figure 4). However, the results from sera of CSC16, CSC30, CSC31, CSC36, CSC46 and CSC47 individuals were discordant between ELISA and biosensing methods involving MBs (both using amperometric and luminescence detection). In addition, no significant amperometric signal was obtained with the developed immunosensor for the non-seroreactive patients, whereas ELISA measurements for these patients gave always OD background values at 450 nm ranging between 0.05 and 0.2, which can lead to the misidentification of patients. These discordances can be attributed to the differences in concentration, type and immobilization of bioreceptors (p53 expressed in bacteria in the ELISA test and HaloTag-p53 expressed in a *mammalian milieu* in the MBs-based approaches) which for sure affect the autoantibodies capture efficiency. In addition, the remarkably lower sensitivity and the lower sera dilution factor required (100 vs 200 times) by the ELISA kit compared with the electrochemical biosensor made the conventional methodology more prone to produce false positive results. Moreover, the presence of bacterial contaminants can produce an IgG response by ELISA non-related to p53.

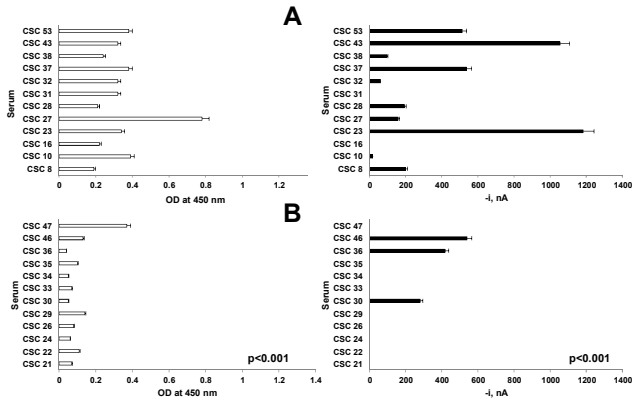


Figure 4. Comparison of the analysis of p53 autoantibody levels in 24 serum samples from p53 reactive A) and p53 non-reactive B) patients as identified by ELISA with the commercial ELISA kit (based on p53 produced in bacteria coated plates) (left) and the p53-HaloTag fusion protein modified MBs-based biosensor (using p53 produced in a *mammalian milieu* –rabbit reticulocyte-) (right). Error bars represented the SEM. *p* value calculated with the data from p53 seroreactive patients' *vs* p53 non-seroreactive patients' as identified by both approaches.

It is important to remark that better discrimination between p53 seroreactive patients and p53 non-seroreactive individuals was achieved with the electrochemical biosensor in comparison with the ELISA test. Mean values (estimated from data of Figure 4) for p53 seroreactive and non-seroreactive patients were 404.6 ± 101.4 nA and 0 ± 0 nA, respectively (p value < 0.001) while the mean ODs with the ELISA test were 0.34 ± 0.045 and 0.11 ± 0.026, respectively (p value < 0.001). Therefore, all p53 seroreactive patients and those discordant between ELISA and the biosensor will be exhaustively managed on the Hospital since they are considered at very high risk to develop colorectal cancer.

Quantification of p53 autoantibodies in these complex biological samples could be accomplished simply by interpolation of the amperometric signals measured for the 200 times diluted sera samples into the calibration graph constructed with the developed biosensor using as standard the calibrator provided in the ELISA methodology (Figure 3). The obtained results (expressed in U per mL) in all sera analyzed are summarized in Table S2. Mean values (estimated from data of Table S2) for p53 seroreactive and non-seroreactive patients were (445.08 ± 105.34) and non-detectable (27.21 ± 1.22) U mL^{-1} p53 autoantibodies, respectively (p value < 0.001), in good agreement with the cut-off value of 120 U mL^{-1} established in the specifications by the commercial ELISA kit to discriminate between positive and negative samples for p53 autoantibodies.

Additional data were obtained in the analysis of sera samples collected from patients already diagnosed with cancer (4 colorectal and 2 ovarian). Results are summarized in Table S3 and demonstrated again the reliability of the developed electrochemical biosensor also to perform selective determination of the p53 autoantibodies in sera samples from different cohorts of patients.

CONCLUSIONS

To our knowledge, this work reports the first electrochemical biosensor to monitor the humoral immune response of cancer patients through the determination of p53 autoantibodies using HaloTag technology. The methodology, involving the use of MBs modified with HaloTag, *in vitro* expressed fusion proteins and disposable electrodes, exhibits an excellent performance to provide accurate determination of p53 autoantibodies, which might serve to monitor patient specific levels of p53 antibodies during the course of the disease or therapeutic manipulations. Compared with the conventional ELISA, the developed biosensor provided more sensitive determination (440 times) and better discrimination between p53 seroreactive and non-seroreactive patients and allowed the analysis to be performed with lower cost and in shorter time. Moreover, the developed approach substitutes the use of purified expensive cancer proteins for their *just-in-time* production for the assay using a cell-free system, abrogating potential concerns about stability and integrity of the proteins during storage. All these interesting capabilities make the developed approach a promising tool, compared both to the ELISA and also to the luminiscence approach, to be readily implemented in low-cost, simple use, short time analysis, and high-throughput and multiplexed devices for diagnostic, patient follow-up and monitoring of cancer patients, alone or in combination with other biomarkers. Furthermore, given the suitability of the developed HaloTag surface chemistry-based electrochemical approach for the immobilization of multiple proteins with similar yields without particular optimization parameters, the presented approach could also find wide-spread interest in the evaluation and study of other protein-(bio)molecule interactions such as fundamental studies involving protein-protein or drugs-proteins interactions.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information including Figures S1-S4 and Tables S1-S3 is available free of charge on the ACS Publications website.

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Author Contributions

MG, AGA, CP, MJFA, RMTR, VRVM, SC and RB performed research. AGA, JMP, MV, GD, SC and RB contributed with reagents and essential samples to the work. The manuscript was written through contributions of all authors. MG, RBMTR, VRVM, JMP, SC and RB wrote the manuscript. All authors have given approval to the final version of the manuscript. *These authors contributed equally.

Notes

The authors declare no competing financial interest.

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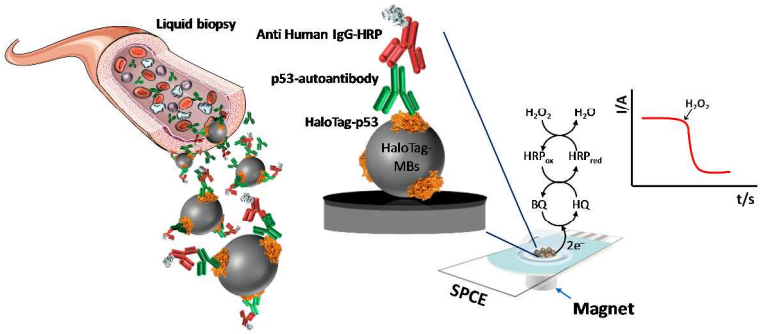

Disposable amperometric bioplatforms using a 1-step protocol for *in situ* production and selective capture of the p53-HaloTag biorecognition elements onto magnetic microcarriers (MBs) for the determination of autoantibodies to p53 in sera from cancer patients.