Functionalized TiO2 nanoparticles as labels for immunoassay

UNIVERSITÀ DEGLI STUDI DI TORINO

FULL PAPER

Functionalized TiO_2 Nanoparticles as Labels for Immunoassay

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Abstract: We designed a versatile inorganic probe based on functionalized TiO_2 nanoparticles for labeling immunoreagents and developing immunological tests. Similarly to enzymatic probes traditionally used in immunoassay, the TiO_2 probe could be covalently linked to antigens or antibodies and exploited as the signal reporter in immunoassays. The TiO_2 probe allowed revealing the rate of antigen-antibody complex formation by promoting the oxidation of a suitable chromogenic substrate with absorption in the visible. We demonstrated the suitability of the TiO_2 probe as a label for immunoassay by coupling it to human serum albumin and developing a direct competitive assay to measure micro- and macro-albuminuria for diabetes diagnosis. The developed TiO_2-based assay showed high sensitivity (detection limit 1.4 mg l^{-1}), wide dynamic range (6-1270 mg l^{-1}) and acceptable precision (within- and between-assay coefficient of variation %<20%) and accuracy (75-95%) for measuring albumin in human urine. The method is fully compatible with materials and equipment of standard enzyme immunoassay (except for the need of UV irradiation), while the inorganic probe is more robust towards chemical and physical conditions and shows better long term stability compared to enzymes.

Introduction

Immunochemical methods of analysis are widely used in many applications, including clinical, veterinary, environmental, forensic investigation, and food safety assessment, due to their sensitivity and selectivity, combined with rapidity, cost-effectiveness, and simplicity.

In most cases, immunoanalytical techniques require a probe that generates a signal upon immunocomplex formation. The optimal probe for immunoassays should: (i) generates a detectable, intense and stable signal; (ii) allows for easy conjugation with biological reactants, without introducing structural variations that interfere with the formation of the antibody-antigen complex; and, (iii) minimize non-specific interaction with other biological molecules and materials involved in the assay.

The first immunoassay probes were radioisotopes; however, they have been almost completely replaced with other probes, such as fluorophores, nanoparticles, and primarily, enzymes, which catalyse the conversion of a substrate into a coloured, fluorescent, chemiluminescent, or electroactive species. Enzymes owe their popularity as probes for immunoassays to the huge amplification of the signal obtained through their catalytic activity, and to the versatility of the detection afforded by the variety of available substrates [1]. Still, enzymes are limited by their stability as a function of several parameters, such as temperature, pH, ionic strength, and the presence of inhibitors.[2] Thus, to widen the applicability of immunoassays and, more in general, to increase their ruggedness, more robust probes need to be developed.

A promising opening in this direction was made in 2007, when Gao and co-workers first reported that inorganic nanoparticles (NPs) show a catalytic activity similar to that found in natural peroxidase.[3] After this report, other metal-based materials have been proposed as enzyme-mimics[4,5] and, recently, peroxidase-like characteristics have been described also for carbon-based materials.[6] Despite these exciting news, the potential use of these materials as probes for bio-sensing is largely unexplored. The described applications are limited to glucose determination, more as a further demonstration of peroxidase-mimicking properties of NPs than suggesting new assays based on these NPs.[5,6] Recently, the oxidizing activity of noble metals NPs towards chromogenic substrates has been investigated and exploited for bio-sensing applications. Liu et al.[7] reported that graphene-supported gold nanoparticles were able to oxidize 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of hydrogen peroxide, as the enzyme peroxidase, and that this catalytic activity depended on surface passivation. In particular, single-stranded DNA (ssDNA) adsorbed onto the surface probe and inhibited gold NPs activity, while double-stranded DNA did not adsorbed. Therefore, authors envisaged applications of the biocomposite between ssDNA and graphene-supported gold NPs as DNA probe, based on the re-activation after treatment with the complementary DNA.

The peroxidase-like activity towards the same substrate has been described also for platinum nanoparticles (Pt NPs), which have been exploited for developing immunochemical-based tests, both in the classic microplate and in the lateral flow assay formats.[8] The adopted strategy for the development of the two assays involved exploiting Pt NPs to label a specific antibody, which was used as the detection antibody in the traditional two-site immunometric assay format. Similarly to natural enzymes, Pt NPs oxidized the chromogenic substrate, thus providing a measurable signal (as VIS absorption) that is directly related to the amount of

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detection antibody present in the system. Although clearly innovative, this approach suffers some limitations, mainly due to the fact that Pt NPs were used to label antibodies exploiting uncontrolled passive adsorption of the biomolecules. This synthetic strategy could not extend to other kinds of biomolecules that not interact with noble metal surfaces, and, in particular, could not apply for labeling haptens, as required by developing immunoassays for small molecules. In addition, the amount of antibody to be used does not depend on the requirements of sensitivity of the assay, but on the needs of stabilizing Pt NPs and prevent their aggregation. Furthermore, as surface modifications impact on the catalytic efficiency of the probe, often in an unpredictable way, the reproducibility of the approach should be evaluated case-by-case.

Titanium dioxide (TiO_2) nanoparticles are widely known as an efficient photo-catalyst, which oxidizes organic substrates through the generation of reactive oxygen species. There are few examples of application of TiO_2 NPs as enzyme mimics. Some authors investigated the conversion of chemiluminescent substrates by TiO_2 for bio-sensing applications, though controversial results have been reported on the resulting catalytic efficiency.[9,10] Li and co-workers exploited carbon nanotubes decorated with TiO_2 NPs as probes for a two-site immunometric assay:[11] detection antibodies were covalently linked to the nanotubes, which simply joined them to TiO_2 NPs, and these last catalysed the oxidation of a chemiluminescent substrate. The authors attributed the generation of measurable signals to the amplification achieved by multiplying the number of TiO_2 NPs bound to the nanotubes. Along the same lines, Fu et al. designed a chemiluminescent assay in which TiO_2 NPs served just as a support for holding together several molecules of the antibodies and the enzyme.[12]

In order to overcome the limitations of using NPs as mere supports and contemporary constraints on the choice of bio-reagents due to their attitude to adsorb on the NPs material, in this work, we designed a versatile probe which exploits the peroxidase-mimicking catalytic properties of TiO_2 NPs, and, furthermore, shows chemical functionalities (i.e.: amino-groups) that allow its covalently linking to diverse biomolecules and small organic molecules, such as haptens. Our approach is based on grafting the surface of TiO_2 NPs to introduce chemical functionalities allowing their direct conjugation to antibodies, protein antigens, haptens and, moreover, allowed further surface modifications aimed at introducing other chemical functionalities. Therefore, the proposed strategy makes the probe almost universally applicable for all formats of immunoassay.

The principal advantage of using inorganic NPs instead of enzymes as labels for immunological methods is their increased resistance to physical and chemical conditions (like temperature and pH) for storage purpose. However, the use of such nanoparticles also enlarges the spectrum of possible synthetic strategies to link them to immuno-reagents, particularly to haptens, due to their increased tolerance towards the same physical and chemical conditions and towards the presence of organic solvents.

In order to demonstrate the applicability of the TiO_2-based probe as the label for immunoassays, we tested it in a heterogeneous direct competitive assay for measuring human serum albumin in urine. Urinary excretion of albumin in the absence of overt nephropathy is known as microalbuminuria and can be used to predict diabetic nephropathy.[13] The early detection of kidney damage allows starting treatments when the damage is minimal and reversible and the monitoring of urinary albumin represents a principal component of treatment for both Type I and Type II diabetes mellitus.[14] Indeed, diabetic nephropathy is a major cause of death in individuals with insulin-dependent diabetes and, thus, is a major factor in the decision to initiate haemodialysis.[14] As a demonstration of the validity of using the TiO_2-based probe, we optimized the immunoassay for measuring urinary albumin and validated it by measuring ten human urine samples and comparing results with those obtained by a reference method.

Results and Discussion

Preparation and characterization of the TiO_2 probe covalently linked to antigens

TiO_2 NPs were produced via hydrothermal synthesis. The obtained nanoparticles were homogeneous and have different shapes, both of truncated bipyramids and ellipsoid, as shown by TEM images (Figure S1 in the Supporting Information). Dimensions varied from approximately 10-15 nm for the shorter side to 15-20 nm for the longer. The crystalline phase was attributed to anatase, based on main Raman signals (Figure S2 in the Supporting Information).[15]

To introduce coupling functionalities for the direct conjugation to biomolecules and immuno-reagents, we modified TiO_2 NPs with the insertion of amino-groups (Figure 1).[10] In this way, TiO_2 probe could be easily conjugated to antibodies, antigens, and haptens through synthetic protocols commonly used in the labeling of such compounds. In particular, we used glutaraldehyde as cross-linker to bind the TiO_2 probe to two model antigens (BSA and HSA). Conjugation to proteins was qualitatively confirmed by staining particles with the Bradford reagent (Figure S3 in the Supporting Information) and the obtained BSA-TiO_2 and HSA-TiO_2 were supposed to serve as tracers for developing competitive direct immunoassays.

We preliminary studied the oxidizing activity of the tracers on TMB, since this compound is widely employed in colorimetric immunoassays based on the peroxidase probe. Usually, the chromogenic substrate for peroxidase-based tracers is a combination of TMB and H_2O_2; in fact, the enzyme readily combines with H_2O_2 and the resultant peroxidase-H_2O_2 complex can oxidize a wide variety of chromogenic hydrogen donors including TMB. The oxidized form of TMB is colored and permits measuring the enzymatic activity by recording absorbance in the Vis region. Typically, an acidic stop solution is added before absorbance measurement that produces a more stable product with enhanced extinction coefficient.

Therefore, we studied the catalytic activity towards TMB of TiO_2-labelled BSA under UV irradiation. Oxidation of the substrate increased with irradiation time (Figure S4 in the Supporting Information) and showed a clear dependence on H_2O_2 concentration (Figure S5 in the Supporting Information).

According to previous works,[6,7] we interpreted this finding as confirmation of the peroxidase-like activity of the modified TiO_2 nanoparticles, according with the mechanism of oxidation that involves the formation of a complex between the catalyst and H_2O_2. Under UV irradiation, TMB underwent limited photo-oxidation also in the absence of the catalyst (Figure S4 in the Supporting Information). We measured and subtracted this background signal in the following experiments.

We identified 8 mM TMB and 48 mM H_2O_2 as the optimal concentrations and TMB/H_2O_2 rate for the of TMB and in TiO_2 probe linked to the antigen to give a sufficiently large signal compared to auto photo-oxidation a reasonable timeframe of 1 hour (Figure S4 in the Supporting Information).

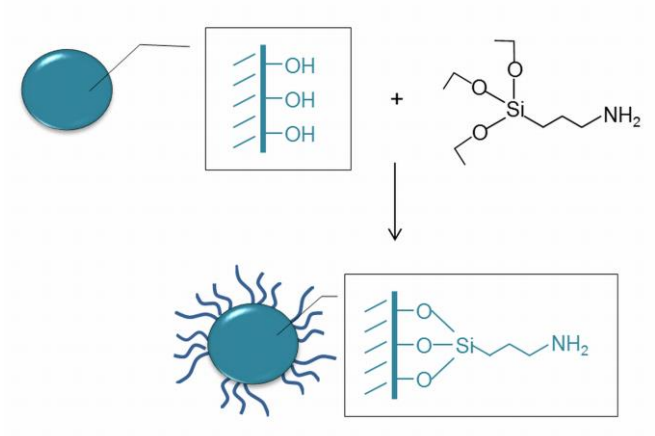


Figure 1. Functionalization of the TiO_2 NPs to insert amino-groups for the following covalent linking to immuno-reagents

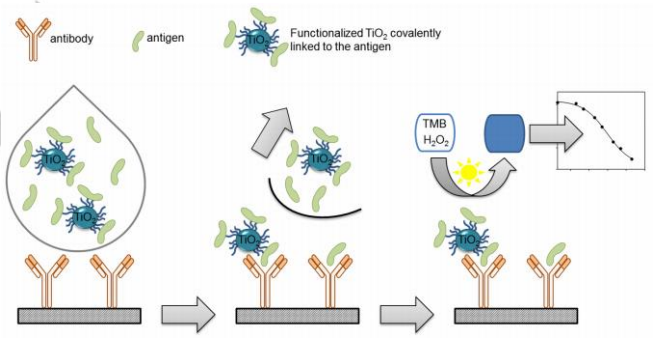
It should be noticed that, unlike natural probes, the TiO_2 probe required UV irradiation for exerting its catalytic activity. Indeed, we could not observe signals significantly differing from auto-photo-oxidation of TMB when the probes were submitted to natural and artificial ambient light. This implies the need for an additional apparatus (a UV source) for operating the assay; however, it allows us preventing unwanted oxidation of other redox compounds possibly present and easily controlling the reaction by the photo-activation.

Long-term stability and pH tolerance

We analyzed the stability of the modified TiO_2 in conditions that are known to alter the activity of the natural probes:[16] the BSA-TiO_2 preserved almost identical activity in acidic, neutral and alkaline conditions (Figure S6 A in the Supporting Information) and could be stored for 14 days at room temperature without any detrimental effect on its catalytic ability (Figure S6 B in the Supporting Information). We also checked susceptibility of the probe to high temperature (50°C for 1 hour). The TiO_2-based probes resulted unaffected by the thermal treatment (data not shown).

Binding capability of the TiO_2 probe covalently linked to BSA towards immobilized anti-BSA antibodies

Preliminary results showed the great potential of TiO_2 NPs as robust and efficient probes. To verify its actual applicability for immunological assays, we exploited the TiO_2-labeled antigen as the tracer in a heterogeneous direct competitive immunoassay, in which the free antigen (the analyte) and the labeled antigen (the tracer) compete for binding to limited amounts of immobilized antibodies in the typical assay scheme depicted in Scheme 1. In details, BSA-TiO_2 or HSA-TiO_2 as the tracer competed with the free antigen (BSA or HSA, respectively) for binding to the specific antibody anchored onto a proper support (e.g.: wells of microplates used for enzyme immunoassays). After immuno-complex formation, the unbound fractions of the free antigen and of the tracer were removed through repeated washings of the plate; then the signal produced by the bound fraction of the tracer was measured. The obtained signal is directly correlated to the amount of the bound tracer and inversely correlated to the amount of the free antigen. When the tracer is composed by the antigen linked to an enzyme, the measurement of the signal involves adding a suitable substrate, allowing the enzymatic conversion of the substrate into a product, and recording a related signal, typically absorbed or emitted light. Likewise, TiO_2 NPs are supposed to be able to promote the oxidation of the chromogenic substrate and to generate the colored product. On the other hand, the presence of TiO_2 NPs in the wells after washings is supposed to be achieved by the specific binding of the antigen moiety of the tracer to anchored antibodies.



Scheme 1. Scheme of the immunoanalytical test in the direct competitive format exploiting the TiO_2 NPs as probe for preparing the tracer (i.e.: for labeling the antigen): (i) the antigen in the sample and the tracer compete for binding to anchored antibodies; (ii) unbound tracer and antigen are removed through washings; (iii) substrate is added and well is irradiated: the TiO_2 probe converts TMB to its coloured form; (iv) absorbance of TMB is measured and the value is (inversely) related to antigen amount.

The development of the protocol in the described immunoassay format begins on studying the binding between anchored antibodies and the tracer. Hence, we investigated the kinetic of formation of complexes between the labeled antigen, BSA-TiO_2, and anti-BSA antibodies bound to the wells of a standard microtitre plate. The rate of formation of immuno-complexes

increased almost linearly in the first 2-3 hours and reached a plateau after 6 hours (Figure 2). The observed behavior is typical of antibody-tracer immuno-complex formation over time and indicated that: (i) the oxidizing activity of the functionalized TiO_2 nanoparticles allowed us to obtain a measurable signal (i.e. color related to TMB oxidation) at levels adequate for immunoassay applications, and (ii) the inorganic probe did not prevent antibody-antigen interaction.

Following the usual scheme for competitive immunoassay optimization, we carried out checkerboard titrations, in which the amounts of both the antibody and the tracer were varied to establish the best combination that would give signals measurable with acceptable precision. The experiment was conducted for the model system, i.e.: BSA-TiO_2 and anti-BSA antibody, and also for a second pair of tracer and antibodies, which included TiO_2 NPs conjugated to human serum albumin (HSA-TiO_2) and antibodies specific for HSA. Both systems qualitatively responded as expected: increasing the antibody or the tracer amount increased the observed signal. However, each pair gave a specific behavior, which could be related to the polyclonal nature of the antibodies used and to their different affinity towards antigens. The anti-BSA antibodies showed higher affinity towards their tracer, thus we could observe measurable signals with tracer amounts as low as 4 µg ml^{-1} (Figure 3 A), while for the HSA system we needed to increase tracer concentration up to 25 µg ml^{-1} to achieve acceptable signals (Figure 3 B). It should be highlighted, however, that tracer amounts are usually referred to the mass of the probe (e.g.: in our case the TiO_2 NPs) which do not represent the effective amount of the antigen available for complexation to the antibody. In agreement with the selectivity of antibodies used in this work, the cross-reactions (i.e. binding of the HSA tracer to anti-BSA antibodies and *viceversa*) were negligible for both systems.

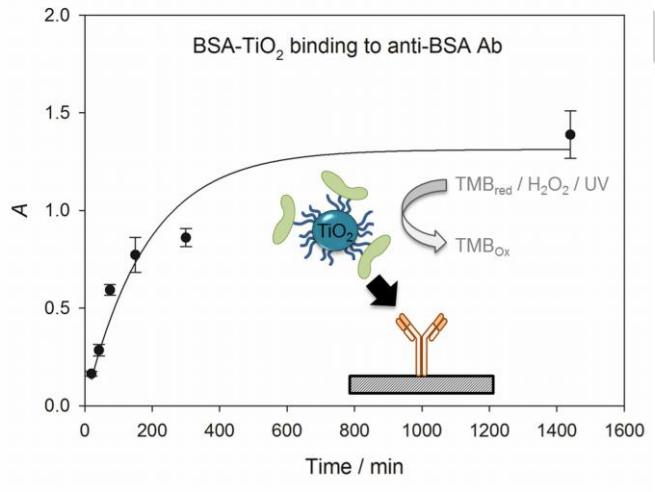


Figure 2. Rate of immunocomplex formation between the tracer (BSA-TiO_2) and anti-BSA antibodies anchored into microtitre wells. BSA-TiO_2 is incubated for varying times into wells functionalized with the anti-BSA antibodies. The unbound fraction of NPs is removed by repeated washings. To measure the bound fraction, the TMB/H_2O_2 mixture is incubated into wells under UV light for 1 hour. The absorbance of the oxidized TMB is measured at 450 nm, after adding H_2SO_4, and is proportional to the fraction of BSA-TiO_2 bound to anchored antibodies.

Overall, though, we were able to establish useful combinations of antibody dilutions and tracer concentrations for both systems that provided an acceptable signal comprised between 1 and 1.5 absorbance units. As a further confirmation of the specificity of the interaction between the TiO_2 probes and antibodies, we checked the non-specific adsorption of the probes to wells functionalized with other proteins (casein, pork skin gelatin, and ovalbumin). Similarly to traditional immunoassays, saturation with solutions containing proteins showed to be effective at reducing the non-specific absorption of the tracer to the plastic wells (data not shown) and no interactions to tested proteins was observed.

Competitive immunoassay for measuring HSA based on using TiO_2-labelled HSA as the tracer

To prove definitely the applicability of TiO_2 NPs as probes for developing effectively working immunoassays, we applied experimental conditions defined as above in a competitive direct assay for measuring HSA. Immunoassay in many formats is routinely used for the determination of the urinary content of HSA for diagnosing and typifying diabetes.[14] According to the general scheme depicted in Scheme 1, we carried out a calibration curve for HSA by variably diluting the analyte, and mixing it to a fixed amount of the HSA-TiO_2 tracer. The plot of the absorbance given by the oxidized TMB towards the log of HSA concentration has the typical shape of the inhibition curve expected for competitive assays (Figure S7 in the Supporting Information). We fit experimental data with the four parameter logistic equation: the IC_{50} value (e.g.: the analyte concentration that inhibits binding of the tracer to anchored antibodies at 50%) was 45 mg l^{-1}; the assay is comparable to existing immunoanalytical methods for HSA measurements in term of sensitivity[17] and also the overall time required for the analysis is acceptable for immunoassays.

Determination of serum albumin in human urine

Preliminary, we studied the influence of the matrix on the test and found that the matrix affected the results. In particular, human urine samples were strongly underestimated. Using the artificial urine[18] as the HSA diluent to calibrate the system mitigated the matrix effect, however unsatisfactory recovery values remained. Therefore, we applied a two-step protocol, in which samples were allowed to react with the anchored antibodies, the matrix components were removed through washing and the HSA-TiO_2 tracer was added in the second step to allow measuring unbound antibodies. The figures of merit of the test were only slightly altered and remained suitable for measuring HSA at levels of clinical interest. In particular, the calibration curve obtained by diluting HSA in artificial urine showed IC_{50}, limit of detection, and dynamic range of 68, 1.4, and 6-1270 mg l^{-1}, respectively (Figure 4). The calibration curve obtained in artificial urine overlap that obtained by diluting HSA in PB, except for the regions at low and

high HSA concentrations, where the use of PB-diluted calibrants determined a significant over and underestimation, respectively. The HSA content in human urine samples was therefore measured by using calibrators prepared in artificial urine.

A cross-validation of the TiO_2-based assay with a turbidimetric reference assay was carried out by measuring HSA concentration in ten human urine samples, representing very different levels of protein content (4-1223 mg l^{-1}). Agreeing results were obtained with the two methods (Table 1), thus demonstrating the validity of the developed assay in the entire range of albumin concentration investigated. According to the American Diabetes Association, normal albumin levels, microalbuminuria, and macro- (clinical) albuminuria levels are below 30 mg l^{-1}, comprise between 30 and 299 mg l^{-1}, and above 300 mg l^{-1}, respectively.[19] Therefore, the immunoassay using the TiO_2-based probe is applicable for the early diagnosis of diabetes, because it allows detecting microalbuminuria, and also for the follow-up of the therapy, as it is able to measure clinical albuminuria.

The precision of the method was evaluated at a preliminary level by analyzing replicates of three urine samples, at low, medium and high HSA concentrations (Table S1 in the Supporting Information). We evaluated the within-and between-day precision and the values of relative standard deviations calculated at each nominal concentration were below 20%, which is acceptable for a screening method.

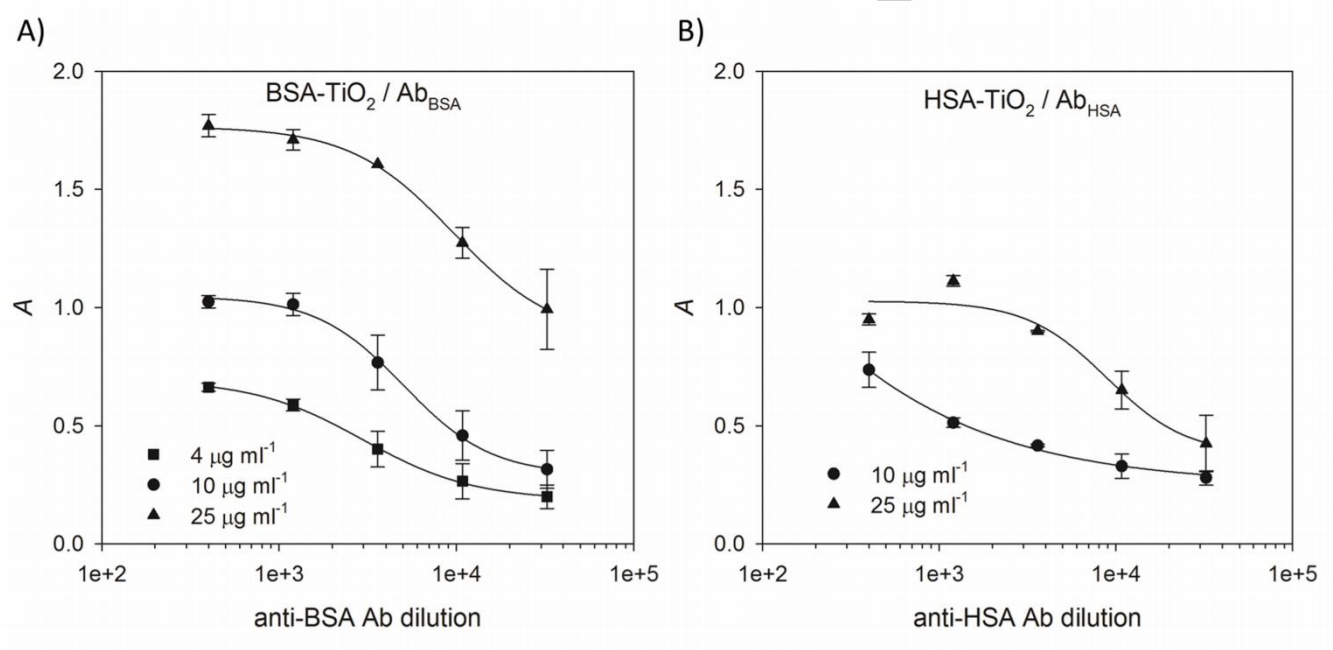


Figure 3. Binding of the tracers (HSA-TiO_2 and BSA-TiO_2) to anti-BSA (A) and anti-HSA (B) antibodies anchored to microtitre wells. The amounts of both the tracer and the antibody are varied to identify best compromise between signal and quantity of immuno-reagents. Tracers were incubated overnight in wells functionalized with respective antibodies. After removing unbound tracers, we added the TMB/H_2O_2 substrate and recorded the absorbance of oxidized TMB.

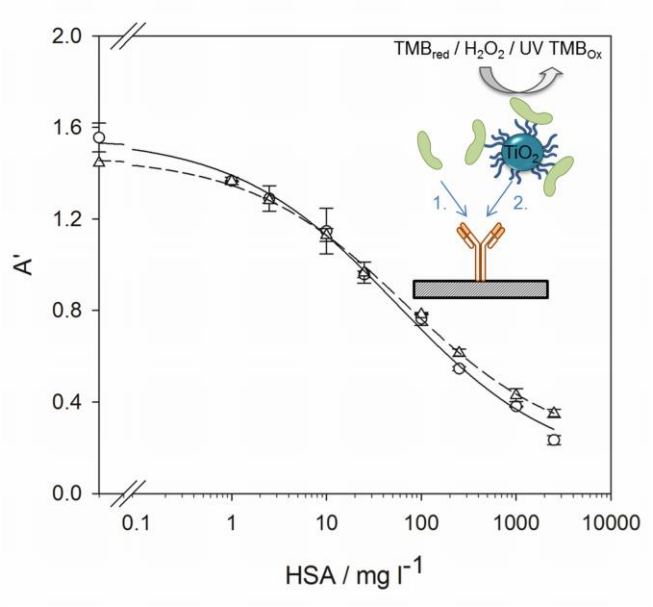


Figure 4. Typical inhibition curves for the direct competitive immunoassay for HSA, in which HSA calibrators were prepared in PB (open circles,○) and artificial urine (open triangles,△). The assay involved two steps: first, calibrators or samples were allowed to react into immunoreactive wells for 30 min, then unbound components were removed and the HSA-TiO_2 tracer added. It was allowed to react for further 30 min, followed by color development. The absorbance of oxidized TMB was recorded and plotted against HSA concentrations, after subtracting the signal due to the photo-oxidation of TMB (A').

Table 1. Cross-validation of the TiO2-based and the reference turbidimetric immunoassay

Sample	HSA [mg l^{-1}]		Accuracy [%] [b]
	Reference turbidimetric assay [a]	TiO_2-based assay ± SD (n=4)	
1	4	3.5 ± 0.5	88
2	86	65 ± 4	75
3	690	683 ± 89	99
4	578	509 ± 62	88
5	428	381 ± 87	89
6	176	165 ± 26	94
7	21	17 ± 1	80
8	1223	1084 ± 105	89
9	253	241 ± 33	95
10	133	103 ± 6	77

[a] Multigen Microalbuminuria assay (Abbott), CV% ≤ 5%. [b] Calculated as the HSA value measured by the TiO_2-based immunoassay divided by the reference HSA value x 100

Conclusions

Our work shows for the first time that functionalized TiO_2 can be effectively used as versatile labels in immunochemical assays. Besides the work of Gao et al. and Park et al.[8], who exploited the tendency of antibody molecules to passively adsorb on noble metals and used such conjugates for developing a two-site immunometric assay, we here demonstrate the superficial modification of an inorganic probe to insert functional groups available for the covalent conjugation to virtually all different immunoreagents, including antibodies, protein antigens, and haptens.

The modified TiO_2 probe maintains the catalytic activity, and is able to oxidize a chromogenic substrate involving H_2O_2 in the process. The conversion rate obtained by the TiO_2 catalyst is sufficient to achieve measurable signals with a very standard substrate and a simple colorimetric detection. We proved also that it could be linked to a model antigen and used as an indicator of the rate of formation of antibody-antigen complexes.

Our work proves that TiO_2 NPs effectively substitute the natural probe in a classic enzyme immunoassay and allow the detection of HSA at levels of clinical relevance using standard materials and standard equipment (except for the need of UV irradiation), with accuracy comparable with that obtained through a reference method. The time required to complete the analysis is also similar to typical enzyme immunoassays. Conversely, the inorganic probe is more robust towards chemical and physical conditions (pH and temperature) and shows better long term stability compared to natural catalysts.

The immunological format developed to evaluate TiO_2-based probes is very general and could extend to detect very different analytes (such as other proteins, but also small molecules) for several fields of application (i.e.: clinical chemistry, food safety assessment, veterinary, forensic, etc) Therefore, future applications would be directed to the exploitation of the TiO_2 probe for the most challenging development of immunological tests for measuring small analytes, such as steroid hormones, drugs, and chemical contaminants of foods.

Supporting Information Summary

The Supporting Information paragraph shows: TEM images of the TiO_2 nanoparticles (Figure S1); the Raman spectrum of the TiO_2 nanoparticles (Figure S2); the confirmation of protein binding to TiO_2 nanoparticles through staining with Bradford reagent (Figure S3); the oxidation of the chromogenic substrate (TMB) due to the catalytic activity of BSA-TiO_2 as a function of the irradiation time measured by UV-vis absorption (Figure S4); the catalytic activity of BSA-TiO_2 probe as a function of TMB concentration and TMB:H_2O_2 ratio (Figure S5 in the Supporting Information); the evaluation of BSA-TiO_2 stability at room temperature for 14 days and as a function of pH (Figure S6); a typical inhibition curve for measuring HSA in a direct competitive immunoassay obtained by using the HSA-TiO_2 probe as the tracer (Figure S7). Furthermore, it includes the analytical parameters for the reference turbidimetric method to quantify urinary albumin and the determination of precision of the TiO_2-based immunoassay evaluated by measuring three samples of human urine with different levels of HSA in replicates in the same day and on three different days for calculating the within- and between-assay precision (Table S1 in the Supporting Information).

Acknowledgements

Authors would like to thank Dr. M. Migliardi and Dr. L. Gallo of the Laboratory of Clinical Chemistry and Microbiology of the Umberto I Hospital (Turin, Italy), who kindly provided human urine samples and reference HSA values. We acknowledge support from a Marie Curie International Research Staff Exchange Scheme Fellowship (PHOTOMAT, proposal n° 318899) within the 7th European Community Framework Programme, and from the Canada Research Chair foundation.

Keywords: Enzyme mimic • Immunoassay • nanoparticles • titanium dioxide

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