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X4C - Innovative surface functionalization for efficient diagnostic tests

1. Company

X4C SA is a Gosselies-based (Charleroi, Belgium) company that emerged in 2016 as a spin-off from the Université libre de Bruxelles (ULB). X4C was co-founded by Alice Mattiuzzi, CEO, who holds a PhD in chemistry from ULB and Prof. Ivan Jabin, who is the co-director of the Laboratoire de Chimie Organique at ULB. Through the development of calix[4]arene-based advanced coatings, X4C aims to provide robust solutions for providing *à la carte* surface properties and functionalities to materials for high-end applications. A major priority of X4C is to grow on the market of *in vitro* diagnostic tests (IVD) by improving the overall sensitivity, specificity and cost-effectiveness of IVD diagnostic kits. To reach these goals, X4C develops and licenses tailored coating solutions to answer not only specific needs of companies that develop IVD tests, but also those that supply reagents such as functionalized nanoparticles (NPs) and surfaces as essential components of high-performance IVD tests.

2. History and main activities

X4C's technology is based on scientific discoveries made by Alice Mattiuzzi, Ivan Jabin and French colleagues (Profs Corinne Lagrost and Olivia Reinaud) in the field of surface functionalization. They showed that calix[4]arene-diazonium salts (Figure 1) can be used as molecular platforms for the modification of surfaces (of any nature, shape and dimension) through the formation of ultra-stable, post-functionalizable and covalently grafted monolayers. From 2016 to 2020, X4C focused on developing industrial partnerships that could *a priori* benefit from monolayers of calix[4] arene coatings for improving or creating new surface properties or functionalities. Companies in both non-medical sectors (electronics and opto-electronics, surface treatment etc.) and the medical sector (medical devices and IVD) have been approached and their yet unmet needs have been collected. In the non-medical sectors, improvement avenues encompassed self-cleaning, anti-biofouling, anti-frosting, anti-fogging, and water-proofing. In the medical sector, the main unmet needs have emerged from companies developing implantable medical devices, where a strong desire to improve bio-/hemocompatibility and surface lubrification of stents was expressed, as well as from companies developing IVD tests that seek to improve their sensitivity and specificity. Nowadays, X4C focuses on further reducing the "gap" between their technology implementation and the unmet needs of main

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players. To bridge that gap, X4C develops proofs of concept (PoCs) for evidencing the advantages of X4C technology compared to other existing methods for surface functionalization. This approach has enabled X4C to identify the medical sector as the most promising one, because of the necessity for high-end surface functionalization where classical surface treatments often fail or generate unsatisfactory results. X4C has carried out several technical feasibility studies to measure the suitability of its technology. These have allowed to i) better show the basic strengths of the calix[4]arene coating technology, ii) document the unmet expectations of stakeholders in the fields of *in vitro* diagnostics and implantable medical devices (stents) and iii) collect preliminary data on X4C differentiation factors compared to other coatings available on the market. Since mid-2020, X4C has decided to focus solely on medical applications and especially on the improvement of IVD diagnostic kits in view of leading to commercial deals.

3. IVD field

Today, the vast majority of *in vitro* diagnostic tests are based on the biorecognition between an analyte (e.g. an antigen) present in a matrix (physiological fluid, possibly pretreated) and a receptor (e.g. an antibody) located at the surface of a transducer that converts the information related to biorecognition into a measurable signal (e.g. an optical signal). Transducers can have different shapes and dimensions (a flat surface in the case of biosensors, a microparticle or a nanoparticle used as colorimetric reporters in the case of lateral flow immunoassays (LFIAs)). The intensity of the signal depends on the characteristics and intrinsic properties of the transducer (e.g. nature, shape, size and optical properties of (nano)particle-based colorimetric reporters such as gold nanoparticles, silver nanoparticles or latex beads) as well as on the quality of the biorecognition (e.g. antibody-antigen) in terms of specificity, reproducibility and accuracy.

Currently, companies who are active in the development of immunoassays (antibody-antigen recognition) *in vitro* diagnostic devices are seeking to improve detection sensitivity and specificity, thereby improving their bioanalytical method. To this end, they aim to achieve the ideal combination of the most appropriate transducers on the surface of which the receptor (e.g. antibody) is grafted in an optimal arrangement in terms of packing and orientation for effective recognition. The main challenges associated with such optimized arrangement include: (a) developing a robust grafting methodology to immobilize the receptor (e.g. antibody) and ensure high stability of resulting bioconjugate (stability over long periods of time and stability in the experimental conditions of the test and storage); (b) controlling the grafting density and its regularity for yielding a homogeneous distribution of the receptor (e.g. antibody) all over the surface; (c) controlling the orientation of the antibody during its immobilization to optimize the antibody-antigen recognition process; (d) limiting non-specific responses.

Polystyrene microbeads and gold nanoparticles (AuNPs) are widely used in immunoassay diagnostic devices (bead-based ELISA, immuno-turbidity, lateral flow, etc.). Most frequently, receptors are immobilized by passive adsorption, which often leads to considerable desorption or denaturation in the case of antibodies or other proteins. Other strategies, such as the covalent bioconjugation by peptide type coupling between -COOH groups located at the surface of the polystyrene microbeads or gold nanoparticles and $-NH_2$ groups of the receptor have been developed. However, relying on the currently available COOH-functionalized AuNPs and polystyrene beads, neither passive adsorption nor peptide type coupling-based covalent bioconjugation offer a means to control the number of immobilized antibodies and/or their orientation.

4. X4C core Technology

In the field of *in vitro* diagnostic devices, X4C's technology offers different key differentiating advantages for coating the surface of a broad scope of frequently used transducers (Figure 1). X4C's core technology consists of the covalent and controlled grafting of a robust and compact organic monolayer of calix[4]arenes either on conductive

(gold, silver, carbon, steel, etc.) or semi-conductive (e.g. germanium) surfaces as well as on insulators (e.g. glass) and polymeric materials (PS, PE, etc.) [1]. Besides, these surfaces can be of any shape or dimension (flat surface, micro- or nanoparticle, large or small). Calix[4]arene-tetradiazonium salts present four anchoring points and can thus potentially form up to four chemical bonds with the surface (each aryldiazonium group can generate an highly reactive aryl radical in reductive conditions). This leads to a remarkable stability of the calixarene-based coating that outperforms that of other classical organic coatings (SAMs of thiols, classical aryldiazoniums, etc.). The monolayer of calix[4]arenes is also bearing additional reactive groups (R = carboxyl, alkyne, etc.) that enables post-functionalization with specific molecules or biomolecules (antibodies, peptides, DNA, etc.). Hence, this approach is highly customizable and can be rapidly adapted to meet the customers' needs.

Since a few years, X4C has developed a strong collaboration with the groups of Gilles Bruylants and Ivan Jabin (both at ULB) aiming at developing new calixarene-coated nanomaterials and their use as enhanced colorimetric reporters for IVD applications (patent application filed). In this context, X4C has been granted an exclusive license to use a straightforward one-pot method of preparation of metal nanoparticles (gold, silver, etc.) coated with functionalizable calix[4]arenes (Figure 1). The robustness of the monolayer of calix[4]arenes is a striking feature of the resulting calix[4]arene-coated nanomaterials. Indeed, classical gold nanoparticles (AuNPs) capped by citrate anions readily degrade or aggregate with changes in pH, ionic strength, or in the presence of large concentration of fluoride. In contrast, calixarene-capped AuNPs (calix-AuNPs) remain stable upon extreme pH variation, in the presence of large concentrations of fluoride ions (between 0.15 and 0.75 M) or under increased ionic strength

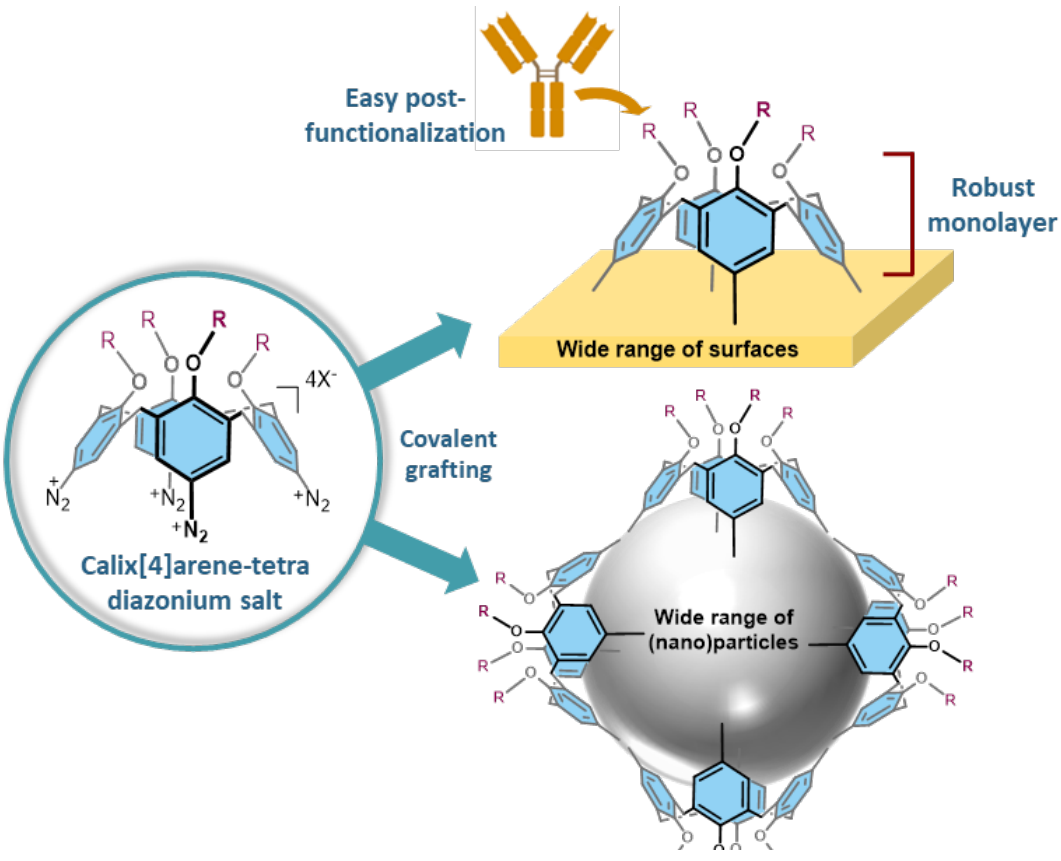

Figure 1: Core technology of X4C.

(Figure 2a) [2]. Even more remarkably, calix-AuNPs can be completely dried, yielding a gold-colored film, and then resuspended, without any loss in their optical properties (Figure 2b).

X4C technology shows its full effect especially for the preparation of particles known to be unstable so far. This is the case of silver nanoparticles (AgNPs). The latter are particularly attractive as colorimetric reporter candidates in IVD tests such as LFIAs because their intrinsic optical properties are better compared to AuNPs and they are significantly cheaper than AuNPs [3]. However, AgNPs are currently not commercially used for *in vitro* diagnostic tests because of major concerns regarding their stability. Functionalizing their surface remains thus an obstacle, limiting the bioconjugation possibilities [4]. This stability problem has been solved by using X4C's technology. Ultra-stable calixarene-capped silver nanoparticles (AgNPs-calix) have been successfully prepared. They do not aggregate even after drying (70°C) and withstand severe etching conditions [5]. Moreover, calixarene-capping prevents aggregation and degradation during and after bioconjugation (covalent and adsorption) to proteins without any loss of stability observed after at least 6 months (Figure 2c).

In addition to provide extremely robust monolayers, X4C's technology is also used to efficiently control the grafting density of specific functions. For example, a mixture of calix[4] arenes, each bearing different functional groups, e.g. oligo(ethylene glycol) chains and carboxyl groups, leads to the formation of a monolayer whose composition reflects the ratio of calix[4] arenes used in the grafting solution [6]. To our knowledge, this is a unique and simple approach to control the 2D surface structuring, grafting density and thus bioconjugation.

5. Applications in IVD field

The use of calix[4]arene tetradiazonium derivatives has already found applications in biosensing, lateral flow immunoassays (LFIAs) and immunoturbidimetry [7]. Two recent examples are described hereinafter.

Germanium-based biosensors for proteins [8]. Fourier transform infrared (FTIR) spectroscopy is a promising technique for the detection of proteins in complex media [9]. Classical FTIR-based biosensors require an organic layer functionalized with a biological receptor, which is directly grafted onto the internal reflection

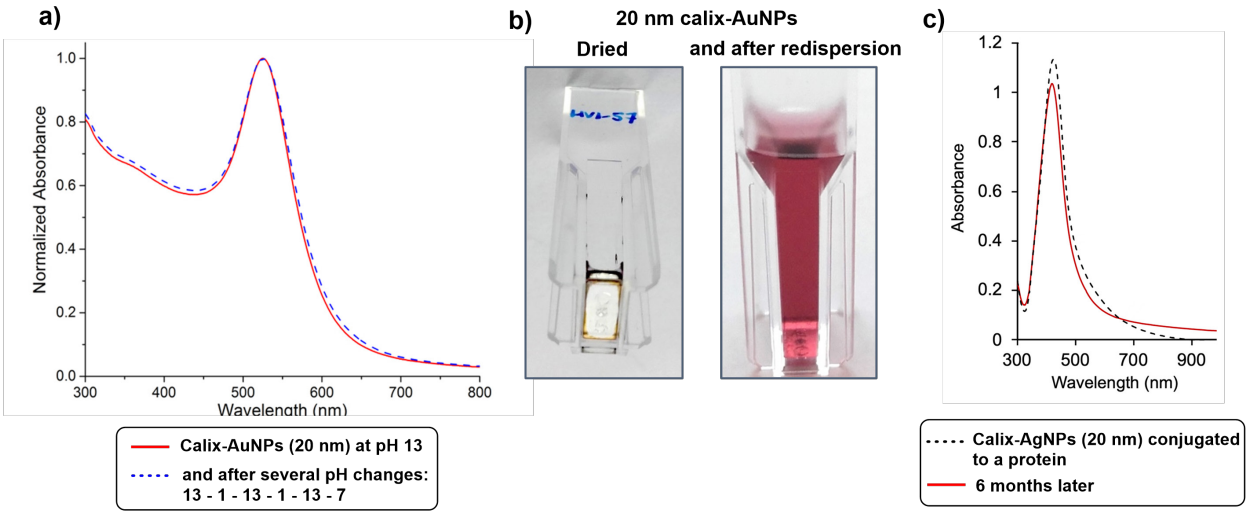

Figure 2: a) UV-Vis spectra of 20 nm calix-AuNPs at pH 13 (red solid line) and after several pH changes (blue dashed line); b) Pictures of dried 20 nm calix-AuNPs (left) and after redispersion in water (right); c) UV-Vis spectra of calix-AgNPs conjugated to a protein (black dashed line) and 6 months later (red solid line).

element [10]. However, the grafting of stable and thin organic layers on germanium surfaces remains a topical challenge [9[a], 11]. X4C's technology was able to break technological barriers through the robust anchoring of a calix[4]arene derivative decorated with three oligo(ethylene glycol) (oEG) chains terminated by a methoxy group and one oEG chain terminated by a carboxyl group (Figure 3 left). The grafted calixarene monolayer prevents non-specific adsorption of proteins through the oEG chains while the carboxyl group allows bioconjugation with biomolecules such as bovine serum albumin or biotin. The resulting calix[4]arene-biotin-based germanium biosensors were then used to selectively detect streptavidin (SA) from a complex medium by a combination of ATR-FTIR spectroscopy and fluorescence microscopy. The IR spectra show the characteristic amide-I and amide-II IR absorption bands at 1637 cm^{-1} and 1535 cm^{-1}, respectively (Figure 3a), which confirms the recognition of streptavidin (100mg/mL). Recognition was also effective with streptavidin in the presence of bovine serum albumin (BSA) (100 µg/mL). Very interestingly, no contribution from BSA at 1657 cm^{-1} can be detected in the spectra obtained from incubation experiments in the presence of this protein, which highlights the remarkable antifouling properties of the calixarene-coated germanium surfaces. Similar strategies were also developed using fluorescent FITC-BSA (100 µg/mL) and streptavidin-ATTO655 (100 µg/mL), for which the specific interaction with biotin-spotted germanium surface generated a fluorescent microarray corresponding to the immobilized biotin pattern (Figure 3b).

Use of AgNPs-calix for the serological detection of Anti-SARS-CoV-2 IgG in human samples [12]. Among all of the rapid diagnostic tests developed over the past years, LFIAs are probably the most widely used [13]. Indeed, LFIAs combine all of the POC (Point-Of-Care) features such as simple read-out signal, low cost and ease of use [14]. AuNPs are classically used as the colorimetric reporter in LFIAs because these plasmonic nanomaterials exhibit interesting optical properties [15]. Nevertheless, if compared to ELISAs current AuNPs-based LFIAs suffer from a poor sensitivity. One approach for improving the sensitivity of LFIAs is to use a colorimetric reporter exhibiting better optical properties than AuNPs, such as AgNPs [3]. Provided AgNP-based bioconjugates are stable, corresponding AgNP-based LFIAs

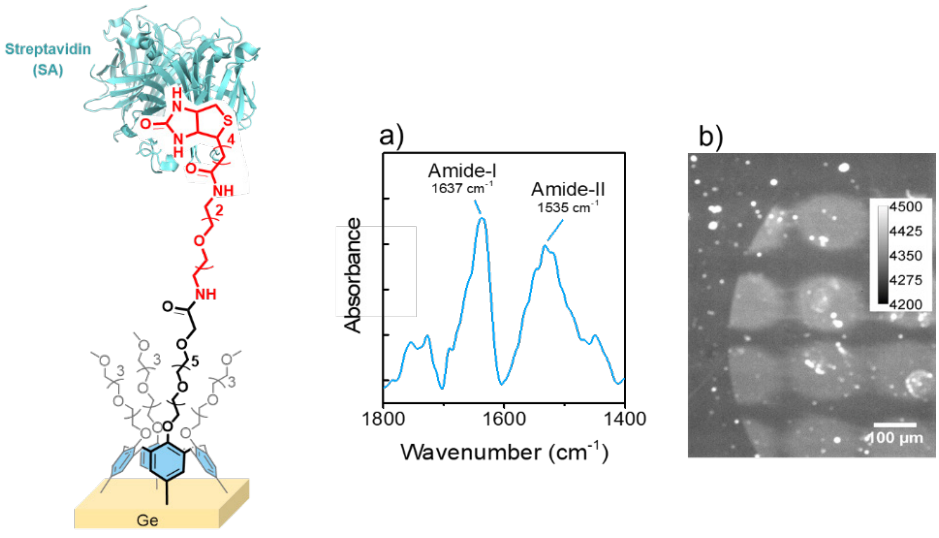

Figure 3: Left: FTIR-based selective biosensor for streptavidin (SA) composed of a calix[4]arene-biotin based germanium surface. Right: a) ATR-FTIR absorption spectra obtained after incubation in a solution of SA; b) fluorescence image acquired on Ge-calix[4]arene-biotin-spotted surface that was incubated with fluorescent streptavidin-ATTO655.

are expected to offer serological tests with lower detection limits compared to AuNP-based LFIAs. The use of AgNPs has, however been scarcely reported for the detection of proteins due to the weak chemical and colloidal stability of AgNPs in general and in complex media in particular. Here again, X4C's technology led to very promising results, paving the way to a commercial use of silver nanomaterials in the IVD field. The covalent bioconjugation of the receptor binding domain of the SARS-CoV-2 Spike Protein (Prot-S) was performed on calix[4]arene-functionalized AgNPs. The calixarene coating conferred remarkable stability to the resulting bioconjugated AgNPs in complex media such as human plasma, as no degradation was observed over several months. Simplified LFIA tests (dipstick experiments) using the resulting bioconjugated AgNPs were developed and used for the detection of Anti-SARS-CoV-2 IgG in clinical samples (Figure 4).

In comparison with LFIAs based on classical gold nanoparticles, calixarene-based AgNPs significantly improved the limit of detection (LOD) for Anti-SARS-CoV-2 IgG since the LOD was reduced by one order of magnitude and similar signals were observed with 10 times fewer particles. In real clinical samples of patient who tested positive (RT-PCR) for SARS-CoV-2 infection, the AgNP-based dipstick assays showed impressive results: 100% specificity was observed for negative samples, while a sensitivity of 73% was determined for positive samples (Figure 5).

6. Conclusion

The use of calix[4]arene-diazonium salts for the modification of surfaces enables the formation of robust, dense and post-functionalizable monolayers on a great variety of materials, in particular, those that are frequently used in the field of IVD such as (nano)particles. In addition, monolayers of controlled composition of different calix[4]arenes can be readily obtained when a mixture of them is used during the grafting process, which offers an elegant approach to fine-tune the subsequent organization of receptors at the surface of such materials. One of the most spectacular achievement is the formation of ultra-stable nanomaterials such as calixarene-coated gold or silver nanoparticles. X4C has developed several demonstrators to show corresponding key advantages. Alongside, X4C has improved its knowledge of the IVD market and has initiated several co-developments with industrial partners in this field in view of leading to commercial deals. X4C is continuously balancing its efforts between,

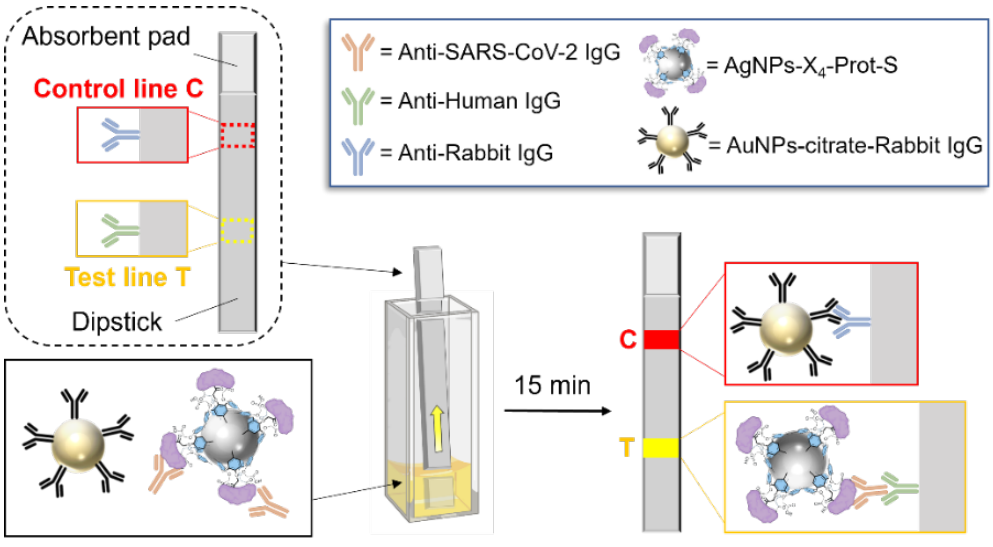

Figure 4: Dipstick assay principle for the detection of Anti-SARS-CoV-2 IgG with calixarene-coated AgNPs.

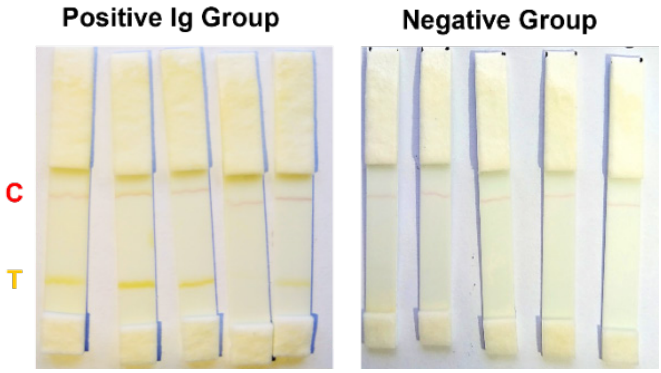

Figure 5: Pictures of dipstick assays for the serological detection of Anti-SARS-CoV-2 IgG in real human samples. Left) Example of positive sample group; Right) negative samples.

on the one hand, solving client's issues related to IVD tests performance linked to the transducer surface properties and functionalities and, on the other hand, expanding its own portfolio of calix[4] arene-coated materials including next generation particles in the field of IVD.

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