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Nanostructured electrodes based on multiwalled carbon nanotube/glyconanoparticles for the oriented immobilization of bilirubin oxidase: Application to the electrocatalytic O₂ reduction

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Here we describe the design and the characterization of novel electrode materials consisting of multi-walled carbon nanotubes coated with glyconanoparticles (GNPs) functionalized with anthraquinone sulfonate. The resulting modified electrodes were characterized by scanning electron microscopy and cyclic voltammetry. Their electrochemical behavior reveals a stable pH-dependent redox signal characteristic of anthraquinone sulfonate. Immobilization of bilirubin oxidase on these three-dimensional electrodes leads to the electroenzymatic reduction of O₂ to water with an onset potential of 0.5 V/SCE (saturated calomel electrode). A catalytic cathodic current of 174 μA (0.88 mA cm⁻²) at 0.1 V/SCE, demonstrates that glyconanoparticles modified by anthraquinone sulfonate were able to interact and orientate bilirubin oxidase by electrostatic interactions.

Keywords: nanomaterial design; MWCNTs-based electrode; glyconanoparticles, host-guest interaction, oxygen electroreduction.

1. Introduction

Among the multicopper oxidases for which direct electron transfer (DET) between redox centers of enzymes and electrodes has been observed, bilirubin oxidases have been widely used in recent years in different bioelectrochemical applications such as the design of bilirubin sensors and biofuel cells [1]. In particular, bilirubin oxidase from *Myrothecium verrucaria* (MvBOD) is well known for its high stability, high activity at neutral pH for O₂ reduction and for its commercial availability [2]. MvBOD contains four copper ions classified into Type 1 (T1) Cu site close to the protein envelope (from 5 to 8 Å) [3] and a Type 2–3 (T2/3) Cu cluster. It is well admitted that the first step of the O₂ reduction is the transfer of electrons (from substrates or electrodes) to the mononuclear site (T1), followed by the transfer to the trinuclear site (T2/T3) where the four-electron four-proton dioxygen reduction into water takes place [4]. Electron transfer between MvBOD and an electrode can be achieved by mediated electron transfer (MET) using either a redox mediator as an electron shuttle or by DET between the enzyme active site and the electrode surface [5–10]. Regarding MET, various redox mediators have already been successfully used such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium [11], metal complexes [12,13], redox polymers [14] and more recently glyconanoparticles modified with redox compounds [15,16].

However, the use of redox mediators can be confronted with their potential toxicity in solution or even with a low stability and their immobilization can prove to be difficult. Furthermore, redox mediators generally induce a minimum overvoltage necessary to achieve the maximum rate of enzyme-mediator electron transfer. In contrast, the DET reaction by definition occurs in the absence of any mediator and therefore offers a simpler setup. In addition, electron transfer can take place at a potential close to the natural standard redox potential of MvBOD. Nevertheless, to ensure a high DET rate, the ideal is obviously to shorten the distance between the T1 redox site of the enzyme and the electrode surface. Besides complex enzyme downsizing methods like deglycosylation [17], many research efforts have been conducted to design electrode materials allowing a close proximity of the T1 site of MvBOD to a conductive surface via a controlled orientation of MvBOD.

Considering that the T1 site of MvBOD presents positively charged amino acid residues [3], the favorable positioning of MvBOD at the electrode surface was mainly achieved by electrostatic interactions combined with π - π interactions with negatively charged electrodes.

Electrodes modified with carboxylate and sulfonate groups have thus been used successfully to promote efficient DET [18]. In a similar vein, MvBOD cathodes were thus developed by modifying the surfaces of the electrodes with negatively charged compounds such as syringaldazine, bilirubin and its analogs [19–23].

In order to increase the surface density of enzyme connected to the electrode, three-dimensional electrodes such as single-walled carbon nanotube forest or multi-walled carbon nanotube compression were developed [9,24–26]. With the aim of designing new biodegradable or even biocompatible 3D nanostructures allowing a high density of spatially oriented enzyme, an elegant approach consists in immobilizing glyconanoparticles functionalized by orienting agents. Glyconanoparticles (GNPs) are obtained by self-assembly of polystyrene-block- β -cyclodextrin copolymer [27] and were already used in solution as a versatile redox platform to connect enzymes [15,16]. More recently, unmodified GNPs have also been immobilized on electrodes for the design of biosensors [28] or fluorescent materials [29].

Here, we describe the functionalization of GNPs by anthraquinone sulfonate via host-guest interactions and the immobilization of these redox GNPs onto an electrode coated with multiwalled carbon nanotubes. The resulting modified electrodes were used for the spatial orientation of MvBOD at the electrode and applied to the electroenzymatic reduction of O₂.

2. Results and discussion

2.1. Characterization of GNP_{PSCD} and GNP_{PSCD}/AQS

The self-assembly of the amphiphilic polystyrene-block- β -cyclodextrin copolymer by direct nanoprecipitation in water provides spherical GNPs consisting of a polystyrene core covered with a shell composed of cyclodextrin groups [15,16,28,29]. The morphology of GNPs in suspension in water used in this work (GNP_{PSCD}) were characterized by dynamic transmission electron microscopy. Fig. 1a shows that spherical nanoparticles were obtained with a relatively narrow size distribution, the average particle diameter being 20 nm. Thanks to the hydrophobic character of the cyclodextrin cavity, GNP_{PSCD} can be easily functionalized by host-guest interaction with hydrophobic compounds [15,30]. According to this principle, the GNP_{PSCD} has been modified by the inclusion of an aromatic anthraquinone sulfonate (AQS) thus creating a negatively charged shell. Compared to unmodified GNP_{PSCD}, stable functionalized nanoparticles (GNP_{PSCD}/AQS) showed no change in morphology or size distribution (Fig. 1b).

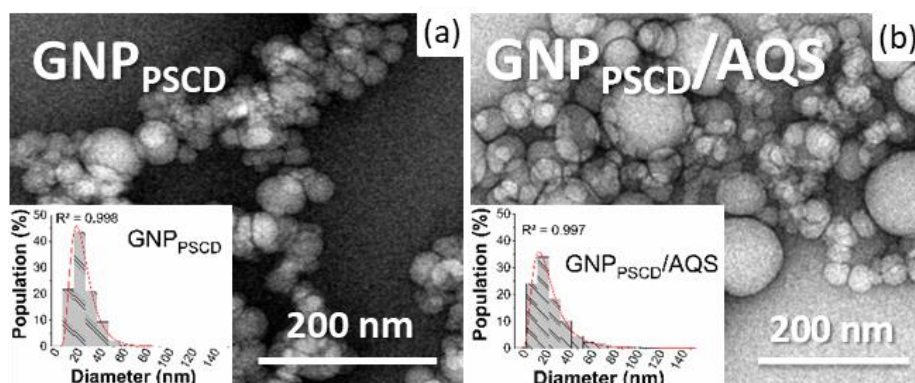


Fig. 1 TEM images showing (a) GNP_{PSCD} obtained by direct precipitation of polystyrene-block- β -cyclodextrin copolymer and (B) GNP_{PSCD} post-functionalized with AQS ($\text{GNP}_{\text{PSCD}}/\text{AQS}$). Inset: Its respective histogram showing the size distribution of the respective glyconanoparticles.

2.2. Electrochemical properties of MWCNT/ $\text{GNP}_{\text{PSCD}}/\text{AQS}$

$\text{GNP}_{\text{PSCD}}/\text{AQS}$ were deposited on carbon electrode previously modified with a multiwalled carbon nanotubes (MWCNTs) coating and the electrochemical properties of the resulting modified electrode was investigated by cyclic voltammetry in Britton-Robinson buffer at different pH values. At pH 2.5, Fig. 2a shows a well-defined reversible peak system at $E_{1/2} = -0.24$ V/SCE ($\Delta E_p = 17$ mV) that may be attributed to the two protons/two electrons reduction of anthraquinone groups. In accordance with Nernst's law, this redox couple was shifted to more negative values when the pH was increased. A linear potential/pH dependence ($R^2 = 0.998$, corresponding plot not shown) with a slope of -56 mV per pH unit was observed corroborating the ratio of one proton to one electron. This phenomenon was already observed for the electrochemistry of $\text{GNP}_{\text{PSCD}}/\text{AQS}$ glyconanoparticles in aqueous solution [30].

In addition, the current intensity of reductive peak varies linearly ($R^2 = 0.997$, corresponding plot not shown) with the scan rate, confirming the immobilization of $\text{GNP}_{\text{PSCD}}/\text{AQS}$ onto MWCNT electrodes (Fig. 2b). The apparent surface coverage of AQS ($\Gamma_{\text{max}} = 8.0 \times 10^{-9}$ mol cm^{-2}), was estimated by integration of the charge under this peak system. Considering that the theoretical maximum surface coverage of a compact AQS layer is $1.1 - 1.7 \times 10^{-10}$ mol cm^{-2} [31,32], the immobilization of $\text{GNP}_{\text{PSCD}}/\text{AQS}$ equivalent to 48-72 compact monolayers of AQS clearly shows the interest of the three-dimensional structure. The stability of the adsorbed GNPs was investigated by continuous scanning the electrode potential through the AQS redox system (50 scans) in aqueous electrolyte. A negligible decrease of the current intensity of the anthraquinone redox system (12 %) was recorded, confirming the firm adsorption of the nanoparticles and the stable inclusion of AQS into the cyclodextrin cavity (Fig. 2c).

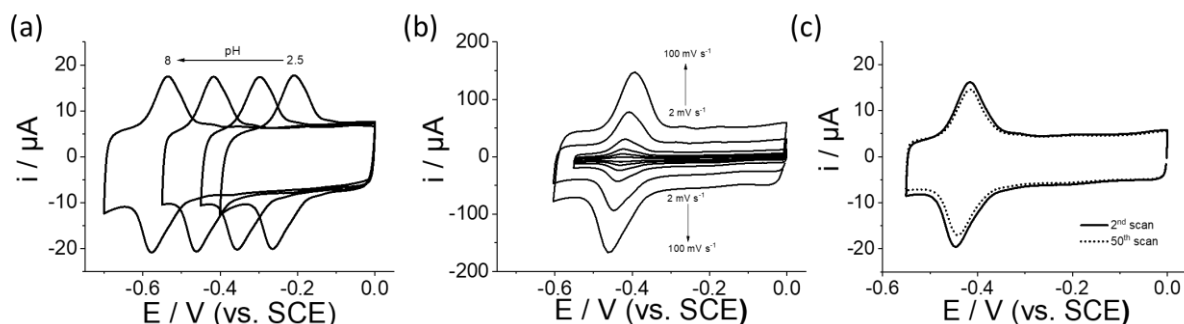


Fig. 2 Cyclic voltammetry recorded at glassy carbon electrode (3 mm diameter) covered with 20 μL of MWCNTs (5 mg mL^{-1} in NMP) and 50 μL of $\text{GNP}_{\text{PSCD}}/\text{AQS}$ (a) in Britton-Robinson buffer at different pH 2.5, 4.0, 6.0 and 8.0 at 10 mV s^{-1} under Ar; (b) at different scan rates 2, 5, 10, 20, 50, and 100 mV s^{-1} in McIlvaine buffer at pH = 6.0 and (c) voltamperogram recorded after one cycle and 50 cycles at 10 mV s^{-1} in McIlvaine buffer at pH = 6.0.

To examine the configuration of $\text{GNP}_{\text{PSCD}}/\text{AQS}$ after immobilization on the MWCNT electrode, SEM images were taken after depositing 50 μL of $\text{GNP}_{\text{PSCD}}/\text{AQS}$ on the MWCNT electrode (Fig. 3a and 3b). A scratch was made on the modified electrode in order to observe the different layers successively deposited on the surface of the vitreous carbon electrode. Thus, the surface of the vitreous carbon electrode (1), a compact layer of MWCNTs (2) and a homogeneous deposit of nanoparticles (3) can be distinctly observed. Additionally, the inset of Fig. 3b shows well-defined nanospheres confirming the conservation of the $\text{GNP}_{\text{PSCD}}/\text{AQS}$ morphology observed in solution after adsorption.

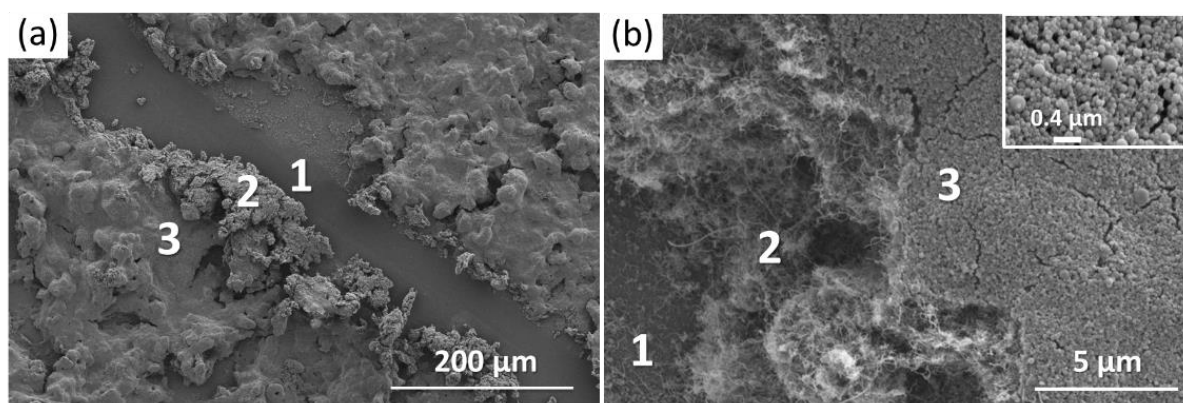


Fig. 3 (a) and (b) SEM images of $\text{GNP}_{\text{PSCD}}/\text{AQS}$ deposited on MWCNTs at different magnifications. 3 layers are visible: the carbon electrode (1), the MWCNTs (2) and the $\text{GNP}_{\text{PSCD}}/\text{AQS}$ (3).

2.3. Application to the electrical wiring of MvBOD

50 μL of MvBOD (5 mg mL^{-1}) were incubated on an MWCNT/ $\text{GNP}_{\text{PSCD}}/\text{AQS}$ electrode overnight and then the modified electrode was rinsed thoroughly for 10 minutes in a phosphate buffer solution $\text{pH} = 7$. The resulting bioelectrode was then characterized by cyclic voltammetry successively in an argon and dioxygen-saturated solution (Fig. 4a). While no faradaic current is observed under argon in the potential range from 0.1 to 0.5 V/SCE, a strong cathodic current appears from 0.5 V/SCE in the presence of dioxygen. The latter corresponds to the electro-enzymatic reduction of oxygen into water by the immobilized MvBOD via a DET process leading to a maximum current value of $-174 \mu\text{A}$ at 0.1 V/SCE.

Control experiments were carried out by incubating the enzyme under the same conditions on electrodes modified by a deposit of MWCNTs or by a deposit of MWCNTs functionalized by unmodified GNP_{PSCD} (namely without AQS). Negligible catalytic current values of $-13 \mu\text{A}$ and $-8 \mu\text{A}$ were observed for MWCNT/MvBOD and MWCNT/ $\text{GNP}_{\text{PSCD}}/\text{MvBOD}$ configurations, respectively. With the aim to evaluate more precisely the influence of AQS on the catalytic reduction of O_2 , the performance of a MWCNT electrode successively modified by AQS then MvBOD is examined. Compared to the same modified electrode without AQS, it appears that the catalytic current at 0.1 V/SCE ($-35 \mu\text{A}$) is greater reflecting the efficient orientation of the enzyme generated by the sulfonate groups of AQS. These results clearly illustrate the beneficial role of AQS for the electrical connection of MvBOD whether in the presence or absence of GNPs. Additionally, the comparison with the MWCNT/ $\text{GNP}_{\text{PSCD}}/\text{AQS}/\text{MvBOD}$ configuration shows that although the onset potential seems identical, it appears that the catalytic current is 5 times lower than that obtained with the presence of adsorbed $\text{GNP}_{\text{PSCD}}/\text{AQS}$. This unambiguously demonstrates the interest of the three-dimensional structure of $\text{GNP}_{\text{PSCD}}/\text{AQS}$ immobilized on MWCNT electrodes to obtain efficient DET from MvBOD.

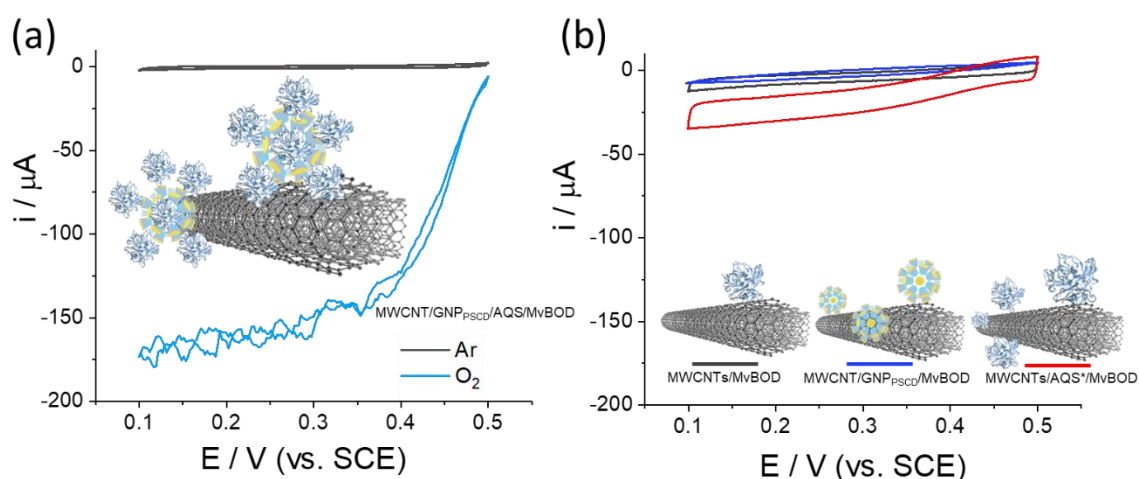


Fig. 4 Cyclic voltammetry recorded at 10 mV s^{-1} of (a) MWCNT/ $\text{GNP}_{\text{PSCD}}/\text{AQS}/\text{MvBOD}$ electrode in 0.2 mol L^{-1} phosphate buffer at $\text{pH} = 7$ under Ar and O_2 saturated solutions; (b) MWCNTs/MvBOD, MWCNTs/AQS/MvBOD and MWCNTs/ $\text{GNP}_{\text{PSCD}}/\text{MvBOD}$ electrodes in 0.2 mol L^{-1} phosphate buffer at $\text{pH} = 7$ under O_2 saturated solutions.

3. Conclusion

This work reports on the design and characterization of new original electrode material based on MWCNTs and glyconanoparticles modified by anthraquinone sulfonate as a negatively charged redox compound. It appears that the immobilization of the GNPs previously modified by AQS in solution, on the MWCNTs leads to a stable adsorption of the GNPs and does not affect the electroactivity and the spherical shape of the latter.

Importantly, these nanostructured electrodes have been successfully applied to the three-dimensional and spatially oriented immobilization of MvBOD for the efficient electroenzymatic reduction of O₂ paving the way for the design of novel biocathodes for bioelectrochemical applications.

4. Experimental section

4.1. Materials and reagents

All reagents (analytical grade) were purchased from Sigma-Aldrich. MvBOD (E.C.1.3.3.5 bilirubin oxidase from *Myrothecium verrucaria*, 10.8 U mg⁻¹ solid) from Aldrich was used as received without further treatment. Multiwalled carbon nanotubes (MWCNTs) were obtained Sigma-Aldrich and used as received.

4.2. Material and methods

4.2.1. Electrochemistry measurements

Glassy carbon electrodes (diameter 3 mm) were obtained from ALS Co. (Japan) were polished with a 2 μm diamond paste purchased from Presi (France) and rinsed successively with acetone, ethanol, water and left to sonicate for 10 min in a mixture water/ethanol. The electrochemical characterizations were carried out in a three-electrode electrochemical cell using an Autolab potentiostat with Nova software version. A saturated calomel electrode (SCE) was used as reference electrode, while a wire of Pt was used as counter electrode. Stability and electrochemical tests were performed in 0.1 mol L⁻¹ phosphate buffer, Britton-Robinson buffer or in McIlvaine buffer solutions at desired pH. Distilled water was passed through a Milli-Q water purification system (Millipore, 18 MΩ).

4.2.2. Preparation and characterization of glyconanoparticles (GNP_{PSCD})

GNP_{PSCD} were obtained as already described [30]. Specifically, an amount of 30 mg of copolymer synthesized as was already described was dissolved in 4 mL THF/H₂O solution (80: 20) w/w and stirred at 1000 rpm for 24 h. This resulting solution was added drop by drop using a syringe pump debit at 10.2 mL h⁻¹ in 160 mL of Milli-Q water under stirring at 500 rpm. The solution was then stirred for 2 h at room temperature before THF removal under reduced pressure at 40 °C. The glyconanoparticles suspension obtained with a final concentration of 0.1875 mg L⁻¹ was kept at 4 °C without further purification. For morphological glyconanoparticles characterization, TEM images were achieved.

4.2.3. Functionalization of GNP_{PSCD} with sodium anthraquinone-2-sulfonate (GNP_{PSCD}/AQS)

As already described [30], 3 mg of sodium anthraquinone-2-sulfonate (AQS) was added in 10 mL of GNP_{PSCD} suspension freshly prepared. After exposure of ultrasound for 2 h, the resulting solution was stirred for 12 h at room temperature and dialyzed to eliminate AQS excess for 72 h at room temperature (cut-off = 3.5 – 5 kDa, 72 h; 20 × 2 L of water, 300 rpm). The different GNP_{PSCD}/AQS suspensions were kept at 4 °C without further purification. These GNP_{PSCD}/AQS were characterized by TEM.

4.2.4. Preparation of the electrodes

- MWCNT/GNP_{PSCD}/AQS modified electrodes: MWCNT films were prepared from a 5.0 mg mL⁻¹ suspension in NMP kept for 60 min under sonication. From this resulting black solution, 20 μL was deposited onto glassy carbon electrode (GCE). The organic solvent was removed under vacuum leading to the formation of a MWCNTs layer (step 1). The MWCNT electrodes were then incubated with 50 μL of a GNP_{PSCD}/AQS fresh solution. The electrodes were dried at room temperature and thoroughly rinsed with deionized water before CV characterization (step 2).

- MWCNT/GNP_{PSCD}/AQS/MvBOD modified electrodes: After steps 1 and 2, MWCNT/GNP_{PSCD}/AQS modified electrodes were incubated with 50 μL of MvBOD fresh solution (5 mg mL⁻¹ in McIl pH 5.0, respectively) overnight at 4 °C (step 2). Before testing, electrodes were thoroughly rinsed with deionized water.

- MWCNT/GNP_{PSCD}/MvBOD modified electrodes: The same steps 1 and 2 were carried out in the same conditions but with unmodified GNP_{PSCD}.

- MWCNT/MvBOD modified electrodes: After step 1, electrodes were incubated with 50 μL of MvBOD fresh solution (5 mg mL⁻¹ in McIl pH 5.0, respectively) overnight at 4 °C (step 2). Before testing, electrodes were thoroughly rinsed with deionized water.

- MWCNT/AQS/MvBOD modified electrodes: After step 1, electrodes were incubated with AQS ($\Gamma = 5.25 \times 10^{-9} \text{ mol cm}^{-2}$) in order to get the equivalent AQS recovery observed to of MWCNT/GNP_{PSCD}/AQS/MvBOD modified electrodes. The electrodes were thoroughly rinsed with deionized water and then were incubated with 50 μL of MvBOD fresh solution (5 mg mL⁻¹ in McIl pH 5.0, respectively) overnight at 4 °C. Before testing, electrodes were thoroughly rinsed with deionized water

The surface coverage Γ of AQS was determined, where applicable, by integration of the charge recorded under the reduction peak of AQS by cyclic voltammetry at low scan rate by using the following equation $\Gamma = \frac{Q}{nFA}$, where Q is the integrated charge under the reduction signal of AQS, F is the Faraday constant, A is the geometric area of the electrode ($A = 0.196 \text{ cm}^2$), and n is the number of electrons transferred ($n = 2$).

4.2.5. Scanning Electronic Microscopy (SEM) and Transmission Electron Microscopy (TEM) Images

SEM images of the different electrodes were carried out by FEI/Quanta FEG 250 scanning electron microscope (SEM, Hillsboro, OR, USA) with an accelerating voltage of 5 kV. The TEM images were registered with TEM-CAM 216 (TVIPS) camera at 200 kV using a JEOL 2100 Plus microscope equipped with a RIO16 (GATAN) camera.

Author Contributions

The manuscript was written through contributions of all authors. Conceptualization, M.C., P.H.M.B., K.G., F.G., R.B. and S.C.; formal analysis, M.C., P.H.M.B., K.G., F.G. and H.L.; investigation, M.C., P.H.M.B., K.G., F.G., H.L. and S.C.; data curation, M.C., P.H.M.B., K.G. and F.G.; validation, P.H.M.B., K.G., F.G. and S.C.; writing—original draft preparation, M.C., P.H.M.B., K.G. and S.C.; writing—review and editing, P.H.M.B., K.G., F.G., R.B. and S.C.; supervision, R.B. and S.C.; funding acquisition, R.B. and S.C..

Conflicts of interest

“There are no conflicts to declare”.

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