Electrochemically active bacteria (EAB) have the capability to transfer electrons to cell exterior, a feature that is currently explored for important applications in bioremediation and biotechnology fields. However, the number of isolated and characterized EAB species is still very limited regarding their abundance in nature. Colorimetric detection has emerged recently as an attractive mean for fast identification and characterization of analytes based on the use of electrochromic materials. In this work, WO$_3$ nanoparticles were synthesized by microwave assisted hydrothermal synthesis and used to impregnate non-treated regular office paper substrates. This allowed the production of a paper-based colorimetric sensor able to detect EAB in a simple, rapid, reliable, inexpensive and eco-friendly method. The developed platform was then tested with *Geobacter sulfurreducens*, as a proof of concept. *G. sulfurreducens* cells were detected at latent phase with an RGB ratio of $1.10 \pm 0.04$, and a response time of two hours.

Electrochemically active bacteria (EAB) transfer electrons outside their cells toward insoluble electron acceptors during their respiration\(^1\). These organisms can be found in diverse environments, such as oceans, lakes and river sediments as well as domestic and industrial wastewater streams\(^2\). The most common application of EAB is the production of electrical current from the bacterial metabolism in microbial fuel cells (MFCs)\(^3,4\). Nevertheless, significant optimization of MFCs is still required. Besides electricity production, other applications of EAB include wastewater treatment, hydrogen production and *in situ* monitoring of microbial respiration\(^5,6\). Further research into the physiology and ecology of EAB is essential to design microorganisms with improved electron transfer capabilities\(^6,7\). Nowadays, the available screening methods are based on MFC principles, such as, voltage-based screening assay\(^8\), microfabricated MFC array\(^9\) and U-tube-shaped MFC\(^10\). However, these methods are relatively slow (\(\sim 5 \text{ to } 6 \text{ days}\)) and expensive. The development of rapid and simple screening methods using low cost and available materials is today a key issue to identify EAB. The use of electrochromic materials has emerged recently as an attractive mean for colorimetric identification and characterization of EAB, making use of their reducing ability. Yuan et al\(^10\) have described an optical probe method for the detection of EAB in solution using an electrochromic (EC) material, tungsten trioxide (WO$_3$). EC materials change their optical properties (between two coloration states) with the appliance of a sufficient electrochemical potential. In this work, the bioelectrochromic response is achieved by an electron-transfer (redox) process of the EAB\(^11,12\). The electrochemical reaction that results in the chromic response of WO$_3$ can be described considering the injection of an equimolar amount (\(x\)) of positive ions (M$^+$) and electrons (e$^-$) as depicted in Equation 1:

\[
\text{WO}_3(white/yellow) + xM^+ + xe^- \leftrightarrow M_x\text{WO}_3(\text{blue})
\]  

(1)

Typical cations are H$^+$, Li$^+$ and Na$^+$, with a stoichiometry that can vary between zero and one\(^11\). In this work, nanostructures of WO$_3$ were synthesized through a microwave assisted hydrothermal synthesis, which offers...
Results and discussion

Tungsten trioxide synthesis. The crystallographic structure of the synthesized WO3 nanoparticles was determined by X-ray diffraction (XRD) (Figure 1) and corroborated by Fourier transform infrared spectroscopy (FT-IR) (Fig. S1). Tungsten oxides follow a well-known ReO3-type structure built up of layers containing distorted corner-shared WO6 octahedra. The growing process of WO3 nanostructures can be described in three major steps: (i) formation of the tungstic acid (H3WO4), (ii) formation of WO3 clusters by decomposition of H3WO4 and (iii) growth of WO3 crystal nucleus. In the synthesis with sodium tungstate dihydrate (Na2WO4·2H2O) as precursor and NaCl as structure-directing agent (SDA) (Figure 1A), WO3 nanoparticles grow in a monoclinic (m-WO3) crystallographic structure (ICDD #00-043-1035) at pH 0.0 and orthorhombic (o-WO3-0.33H2O) (ICDD #01-072-0199) at pH 1.8. At pH 0.4 the WO3 nanoparticles are a mixture of the two phases, monoclinic and orthorhombic, together with the precursor (marked with *) and tungstic acid (marked with ▲). Using Na2SO4 as SDA (Figure 1B), orthorhombic and hexagonal (h-WO3) (ICDD #01-075-2187) phases were obtained at pH 0.4 and pH 1.8, respectively. At pH 0.4 the sample also shows a peak assigned to the acid tungstic (▲) and two unidentified peaks (▲) that are due to lattice distortions of the crystallographic structure, as previously reported for WO3 nanoparticles prepared by hydrothermal synthesis. Finally, using peroxypolytungstic acid (PTA) as precursor (Figure 1C), the crystallographic structure of the synthesized nanopowder is monoclinic for the lowest and highest pH values, which is in agreement with previous reports, although with different crystallographic plane intensities. For the intermediate pH value, the WO3 nanoparticles present an orthorhombic phase. The FT-IR analysis is in accordance with the crystallographic structures attributed by XRD. However, the samples prepared with PTA precursor also revealed the presence of a W = O vibration bond that are assigned to some impurities. In general, the formation of nanoparticles is favorable for pH values lower that 2.0, however at pH 0.4 tend to form bundle structures and a mixture of phases and/or impurities.

Figure 2 shows the morphological analysis performed by scanning electron microscopy (SEM). The WO3 nanoparticles synthesized with Na2WO4·2H2O show different morphologies: (i) nanocubes assigned to m-WO3, (ii) nanosheets assigned to o-WO3·0.33H2O, (iii) nanowires assigned to h-WO3 and (iv) bundle structures. Regarding the WO3 nanoparticles synthesized with PTA, the obtained structures present well-defined edges with nanosheet-like morphology at pH 0.0, a mixture of nanowires and nanocubes at pH 0.4 and single nanocubes at pH 1.8.

The sulphate ions added to the synthesis process, act as capping agents covering some facets of WO3 crystal nuclei. At pH 1.8 a faster growth rate along c-axis is observed, yielding to one-dimensional wire/rod-like structures. In the meantime, a certain amount of sodium cations is required as stabilization ions for the hexagonal and triangular tunnels in the formation of h-WO3. When chloride ions are added it is believed that a similar process occurs prompting the growth of the nanoparticles in a specific direction.

Additionally, electrochemical impedance spectroscopy (EIS) was performed in all the samples, in order to compare the conductivity of the nanostuctures, since this affects its electron transfer ability during the electrochromic process. The Bode plots represented in Fig. S2 display, in general, lower impedances values for the orthorhombic and hexagonal crystallographic structures, which is in accordance with the literature.

Office paper as a platform for EAB identification. The above described WO3 nanoparticles were used as an active layer in a regular office paper substrate in order to develop a colorimetric electrochemical device for EAB detection. This paper is optimized for printing, and therefore has a more uniform surface, lower porosity and higher hydrophobicity (water-contact angle of 106°) when compared to chromatography paper (water-contact angle of 12°), the most common type employed in paper-based devices. Office paper allows a superficial adhesion of the WO3, which facilitates the interaction of EAB with the electrochromic nanoparticles, promoting an intense and uniform coloration of the test well. SEM-EDS and XRD analysis (Figure 3A–C) revealed a high-density structure of intertwined cellulose fibers with a cylindrical and flat shape, and the presence of agglomerates, especially calcium carbonate, as confirmed by a FT-IR analysis (Fig. S3).

The sensor layout was designed to resemble a conventional microplate (Figure 4A) for parallel assays and prototyped into single-use sensors containing only a test and a control wells (Figure 3D–E). The layout was then patterned by wax printing, a method previously optimized by M. N. Costa et al., that increases the surface hydrophobicity (water-contact angle of 119°) ensuring no cross contamination between adjacent samples as well as the confinement of the WO3 nanoparticles dispersion to one particular area. A thermal analysis of the office paper (Fig. S4) was also performed in order to guarantee that the material could withstand the heating process. The patterned office paper was then impregnated with the synthesized WO3 nanoparticles by a drop casting process. A drop was spotted on each well delimited by the hydrophobic pattern, in order to create the electrochromic layer.

Colorimetric assays. A screening colorimetric assay of all the synthesized WO3 nanoparticles was performed in the developed paper-based sensor (Figure 4A) and in the conventional 96-well plate (Fig. S5). For the paper platform, an RGB analysis of the results was carried out using ImageJ software, and the ratio of the average intensities in blue and red channels was recorded (Figure 4B). An electrochromic response translated by the deep blue color of the tungsten bronze (Equation 1) was achieved with sample 6, which corresponds to the synthesized hexagonal WO3 nanowires. The h-WO3 nanoparticles has attracted much attention due to its well-known tunnel structure where openness degree is
higher when compared to the layered structure of orthorhombic or monoclinic geometries. This feature results in an easier intercalation of cations to form tungsten bronzes and concomitant enhancement of the electrochromic properties. Moreover, the one-dimensional nanowire shape originates a structure with a high surface area and increased surface atom density that can easily interact with the EAB. Additionally, the electrochromic response was also observed in the conventional assay with other crystallographic WO₃ structures, due to the higher concentration of the nanoparticles and enhanced interaction with EAB (Fig. S5).

For the fabricated paper-based sensor, the bioelectrochromic response is conditioned by the time that the cell suspension drops (V = 50 μL) take to dry (approximately 4 hours). Therefore, an equal scale down on the well’s diameter (d = 3.38 mm) and in the sample volume (V = 20 μL) was carried out, with a reduction (2.5 times smaller) from the first paper-based sensor (Figure 4A). Additionally, an optimization assay was carried out (Figure 5A) to evaluate the influence of the h-WO₃ nanoparticles concentration in the detection of EAB. Figure 5B represents the RGB analysis of the resulting color of the Geobacter sulfurreducens cells in contact with h-WO₃ nanoparticles. From this analysis it is possible to conclude that 15 g/L and 20 g/L h-WO₃ nanoparticles dispersion renders higher RGB ratios when compared to the other studied concentrations. Moreover, 15 g/L nanoparticles dispersion presents a linear response to the increasing G. sulfurreducens cells concentration. Therefore, henceforward the sensors were produced with h-WO₃ nanoparticles dispersion at 15 g/L, with the same ratio nanoparticles per area than the first sensor (0.014 g/mm²). With the described scale-down the response time decreased to half the time (approximately 2 hours).

Figure 5C represents an RGB analysis of a positive control (Geobacter sulfurreducens), a negative control (Escherichia coli) and a blank well (no sample) for background information. Geobacter sulfurreducens cells in a late-exponential phase of growth (Abs₆₀₀ nm ~ 0.5) display an RGB ratio of 1.33 ± 0.005, while the negative control, Escherichia coli under the same conditions, and blank sample display an equal ratio of 0.99 ± 0.001/0.004. This result reveals a clear statistically significant difference between a positive and negative sample (P < 0.0001), proving the specificity of the developed paper-based device (Table S2). Moreover, it was also possible to detect EAB at latent phase (Abs₆₀₀ nm ~ 0.1), with an RGB ratio of 1.10 ± 0.040, thus confirming that the sensor here described is sensitive for low concentrations of EAB.

Conclusions
Electrochemically active bacteria are ubiquitous in nature and have the ability to transfer electrons outside their cells, a feature that can be applicable in electricity production, which is of the outmost importance in an energy dependent world.

However, the number of identified species is still very limited and their electron transfer mechanisms are feebly understood, caressing of feasible techniques to allow the detection of these bacteria as well
as to facilitate the study of their physiology and electron transfer mechanisms.

This work reports a paper-based sensor with WO₃ nanoprobes to optically detect the presence of these bacteria.

In order to study and optimize the electrochromic response to EAB cells, three different WO₃ crystallographic structures (monoclinic, orthorhombic and hexagonal) were synthesized with the use of different precursors (Na₂WO₄·2H₂O and PTA) and structure-directing agents (NaCl and Na₂SO₄) as well as by varying solution pH.

The performance of the produced structures as electrochromic material for EAB detection was evaluated in a screening colorimetric assay both in the conventional method, using a 96-well microplate, and in an office paper platform, with the same dimensions and produced through wax-printing method. Both assays achieved a successful detection of EAB Geobacter sulfurreducens with a high color contrast for the h-WO₃ nanowires. This enhanced biocathodic response is in accordance with mentioned above that this structure presents an increased surface area and surface atom density proving to be the ideal material for EAB electrochromic detection.

The conventional assay performed in a solution based reaction also demonstrated a mild colorimetric response with other WO₃ structures, due to the easier contact with G. sulfurreducens cells.

Aiming to reduce the response time, sample and reagents volume necessary for the test, a scale down paper-based device was tested with a 2.5× reduction of wells diameter. The mentioned scale down allowed a reduction in the response time for 4 hours to 2 hours.

The described paper-based sensor showed a RGB ratio above 1 for a sample of Geobacter sulfurreducens cells at latent phase, thus providing a reliable, inexpensive, eco-friendly and simple approach to identify EAB. Moreover, this method can also be used in screening assays to aid in the understandings of the influence of certain proteins in the electron transfer chain, thus allowing further refining in the performance of the mentioned applications.

Experimental section. Tungsten trioxide synthesis. Several types of nanoparticles were synthesized through a microwave assisted hydrothermal method. Two different precursors were used (0.8 g): sodium tungstate dehydrated (Na₂WO₄·2H₂O), (Fluka, 99%) and peroxopolytungstic acid (PTA), prepared by oxidation of tungsten (W) with hydrogen peroxide (H₂O₂), previously described.49,50.
Two different salts were used as structure directing agents (SDA): NaCl (0.3 g) (Panreac, 99.5%) and Na₂SO₄ (0.72 g) (Panreac, 99%) with the Na₂WO₄·2H₂O precursor. For each set of precursor, 3 M hydrochloric acid (Sigma-Aldrich, 37% by weight) was added to the solutions thus forming the acid tungstic (H₂WO₄) intermediate, and the final pH values were set as 0.0, 0.4 and 1.8. In the samples produced from PTA precursor the influence of SDA was not studied. 

The solutions were then transferred into a 35 mL vessel, sealed, and heated in a microwave reactor (Discover SP, CEM Corporation, Matthews, NC, USA) at a constant power of 100 W for 1 h. All nine nanopowders were collected by centrifugation (4000 rpm, 30 min), and dried (80°C, 12 h). Solids were then grounded in a mortar to obtain fine powders and stored for further characterization.

**Paper sensor fabrication.** A4 (210 × 297 mm) sheets of an office paper (300%, Portucel Soporcel, Setúbal, Portugal) were fed to the manual feed tray of a commercial solid ink printer (Xerox ColorQube 8570, Xerox Corporation, Norwalk, CT, USA) designed to print a wax-based ink. All the prototypes used in this work were designed in Adobe Illustrator (Adobe Systems Software, Ireland). The prototypes were projected to resemble 96 and 384-well plates with d = 0.7 and 0.38 cm, respectively.

The printed patterns were placed on a hot plate (Heidolph MR HeiTec, Schwabach, Germany) at 140°C for 2 min, allowing the wax to melt and spread vertically through the whole thickness of the paper, creating the desired hydrophobic pattern. Each well was functionalized with 40 μL and 10 μL of WO₃ nanoparticles dispersion for the sensor with d = 0.7 cm and d = 0.38 cm, respectively. The papers were allowed to dry at room temperature overnight. The final paper sensors were stored at room temperature in a dark box with no light interferences.
2H₂O, 0.1 g/L MgSO₄·7H₂O, 1.8 g/L NaHCO₃, 0.5 g/L Na₂CO₃, H₂O, 0.19 mg/L Na₂SeO₄, DL vitamins, 100X NB salts and NB mineral elixir, with 10 mM sodium acetate as the electron donor and 40 mM sodium fumarate as the electron acceptor. To remove oxygen, the NBAF medium was placed in 10 mL pressure tubes and gassed with an 852 mix of N₂:CO₂. After the NBAF medium deaeration and sterilization, 2% of yeast extract, 1% of 100 mM pH 7.0 cysteine and 1% of Geobacter sulfurreducens cells from a frozen stock were added to the culture medium under an anaerobic atmosphere. Cultures were grown at 30°C. The bacterial growth was followed through absorbance readings at 600 nm (Fig. S6).

**EAB-WO₃ colorimetric assay.** An anaerobic chamber (LABstar Glove Box Workstation, MBRAUN, Garching, Germany, O₂ < 0.1 ppm) was used to perform all the colorimetric assays. Geobacter sulfurreducens, grown until late-exponential phase, and Escherichia coli, in the same conditions, were collected by centrifugation at 6000 rpm for 5 min and then suspended in a buffer (30 mM Na₂HPO₄·2H₂O, 30 mM KCl and 30 mM CH₃COONa, pH 6.53). Cell cultures (50 μL and 20 μL for the sensor with d = 0.7 cm and d = 0.38 cm, respectively) were added to the respectively well and the drops were allowed to dry at room temperature.

**Data acquisition and analysis.** The colorimetric paper-based results were recorded with a digital scanner (All-in-One Printer 1050A HP, Hewlett-Packard Development Company, L.P., Palo Alto, CA, USA) with a 300 dpi resolution. Color development analysis was performed with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) through an RGB control. A one-way ANOVA analysis, with Tukey’s multiple comparison test, using GraphPad (San Diego, CA) was used to validate the results. The one-way analysis of variance was used to test for differences between two groups of data (positive and negative results). Additional statistical analysis of the differences was carried out using Tukey’s multiple comparison procedures.

**Characterization.** The crystallographic structure of the WO₃ nanoparticles and the office paper were determined by XRD (X’Pert Pro, PANalytical, Almelo, Netherlands) with CuKα target and wavelength of 1.5406 Å. The nanoparticles’ morphology and paper was characterized by SEM-EDS (Carl Zeiss AURIGA Crossbeam SEM-FIB, Oberkochen, Germany) and their chemical structure by FT-IR.
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