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# Anodic Stripping Voltammetric Determination of Copper Ions in Cell Culture Media: From Transwell® to Organ-on-Chip Systems

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**Abstract:** The integration of sensing devices into cell culture systems is a topic of great interest in the study of pathologies and complex biological mechanisms in real-time. In particular, the fit-for-purpose microfluidic devices called organ-on-chip (OoC), which host living engineered organs that mimic in vivo conditions, benefit greatly from the integration of sensors, enabling the monitoring of specific chemical-physical parameters that can be correlated with biological processes. In this context, copper is an essential trace element whose total concentration may be associated with specific pathologies, and it is therefore important to develop reliable analytical techniques in cell systems. Copper can be determined by using the anodic stripping voltammetry (ASV) technique, but its applicability in cell culture media presents several challenges. Therefore, in this work, the performance of ASV in cell culture media was evaluated, and an acidification protocol was tested to improve the voltammetric signal intensity. A Transwell® culture model with Caco-2 cells was used to test the applicability of the developed acidification protocol by performing an off-line measurement. Finally, a microfluidic device was designed in order to perform the acidification of the cell culture medium in an automated manner and then integrated with a silicon microelectrode to perform in situ measurements. The resulting sensor-integrated microfluidic chip could be used to monitor the concentration of copper or other ions concentration in an organ-on-chip model; these functionalities represent a great opportunity for the non-destructive strategic experiments required on biological systems under conditions close to those in vivo.

**Keywords:** copper detection; anodic stripping voltammetry; cell culture media; acidification protocol; embedded sensors microfluidics



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## 1. Introduction

In vitro models represent a very important research tool in the medical and biological fields, as they provide insight into the behaviour of cells and can be used to study pathologies and complex biological mechanisms [1–3]. In particular, organ-on-chip (OoC) represents the latest evolution of in vitro systems where cells are cultured in microfluidic chips with sensing capabilities in order to mimic the functionality and (patho-)physiological response of the organs and to monitor specific chemical-physical parameters [1–5]. Micronutrients, such as the transition metal ions zinc, copper and iron are essential for life as they are cofactors for various proteins and enzymes; on the other hand, an excess of metals leads to the formation of free radicals that induce toxicity in the body [6]. Therefore, all organisms have developed complex mechanisms to finely regulate the levels of ions to ensure intra- and extracellular homeostasis [7]. Micro-physiological platforms that are designed

to provide physiologically relevant results, could greatly benefit from rapid and in situ technologies to measure micronutrient concentrations while mimicking biological systems.

Copper is an essential trace mineral fundamental for our survival and it is found in all body tissues [8]. Copper plays a crucial role in the production of red blood cells, and the maintenance of nerve cells and the immune system; it is also important for collagen formation and energy production. An imbalance in copper levels may be associated with certain pathologies such as Menkes, Wilson's and Alzheimer's diseases [9]; moreover, low copper levels may also lead to cardiovascular diseases [10]. Copper is assimilated from the diet and its management and regulation in the intestine are essential to preserving copper homeostasis in the whole body [11].

Dietary copper, largely in the form of $Cu^{2+}$, is reduced (likely in the form of $Cu^{+}$) and then it can be absorbed by intestinal epithelium [12]. Recent evidence suggests that intestinal mucin glycoproteins which have two copper-binding sites (one for $Cu^{2+}$ and one for $Cu^{+}$) are involved in protecting cells from excessive copper toxicity, by blocking unnecessary redox cycling and controlling the low levels of copper uptake required for physiological processes [13]. Despite the crucial importance of the regulation of intestinal copper absorption, the mechanisms involved are currently poorly elucidated. Hence, real-time monitoring of $Cu^{2+}$ concentrations in cell culture media can be an important tool to study the processes of copper transport and uptake.

Typical analytical techniques employed for the determination of copper are atomic absorption spectrometry, inductively coupled plasma atomic emission spectrometry and inductively coupled plasma mass spectrometry [14]. Such analytical techniques are very sensitive, but they involve the use of cumbersome and expensive instrumentation. Moreover, these techniques require an important preparatory phase by using strongly acidic substances, high temperatures and/or high pressures. As an alternative, optical methods are highly sensitive, selective, cheap and easy to apply. In these systems, the interaction between the recognition element (optical probe) and the target species can generate variations in the optical characteristics [15–17]. Sometimes, the interaction between the optical probes and the target analyte can induce the formation of toxic substances, and this must be deeply evaluated when the detection is performed in a cell environment [18]. Moreover, when optical methods are employed in complex media like cell culture media, the presence of other organic species could interfere with the formation of the optically active species [16]. For these reasons, the use of optical methods is not always the best solution.

In this context, electrochemical methods are cheap, easy and fast and thus they are suitable for in situ and in-line analysis. In particular, anodic stripping voltammetry (ASV) is a very versatile technique for the detection of heavy metals including copper [19]. This analytical technique is based on a two-step detection. The first step consists of the deposition/reduction of copper ions to the working electrode surface by applying a negative potential (according to the Pourbaix diagram) [20]. In the second step, a potential scan is performed starting from the potential at which the deposition was carried out up to a potential sufficient for the ion to be oxidized again and then "stripped" from the working electrode surface. The stripping process corresponds to the appearance of a peak in the voltammogram, whose current intensity is a function of the ion concentration (quantitative analysis), while the peak potential is specific to the analyte (qualitative analysis).

Electrochemical techniques in cell culture media have been already used for the monitoring of different small molecules such as glucose, lactate, amino acids, drugs, etc. [21–24], while only a few examples have been reported for the detection of trace metals through voltammetric techniques [25,26]. The presence of large amounts of organic substances in cell culture media represents a challenge to obtain reliable and intense signals with the ASV technique.

With the idea to integrate such analytical technique in an in vitro system for real-time ion monitoring, we evaluated the performance of ASV in different cell culture media and a medium acidification protocol was designed and tested to enhance the voltammetric signal

and lower the detection limits. Its potential to improve the detection sensitivity was already demonstrated by our group with a machine learning approach [27].

First, the potential of such an analytical and pre-treatment approach was applied to monitor copper concentration in a Caco-2 cell Transwell® culture model by withdrawing the cell culture media from the upper and the lower compartments. Then, a microfluidic circuit was designed by implementing a commercial chip with microfluidic controllers/sensors to regulate the fluxes of both the cell culture medium and the acidifying solution, and so to perform the acidification protocol in an automatic way. Moreover, a microelectrode was integrated into the microfluidic circuit to perform ASV measurements in situ.

The reported results represent an important step toward the integration of chemical sensors in complex organ-on-chip systems. In fact, to the best of our knowledge, there is a lack of OoC devices that integrate the ASV analytical technique with automated sample treatment; this aspect proves to be strategic to evaluate the performance of such complex systems.

## 2. Experimental Section

### 2.1. Materials

Anhydrous copper (II) sulphate, Dulbecco's phosphate buffer saline (PBS), Dulbecco's Modified Eagle's Medium—high glucose (DMEM), fetal bovine serum, penicillin-streptomycin, L-glutamine solution, MEM non-essential amino acid solution, acetic acid, sodium acetate, sulfuric acid and nitric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Minimum Essential Medium Eagle (MEM) was supplied by Corning (Corning, NY, USA), while Kaighn's Modification of Ham's F12 (F12K) was purchased from ATCC (ATCC, Teddington, UK). A Copper Assay Kit for the colourimetric test was purchased from Sigma-Aldrich.

### 2.2. Solution Preparation

The $Cu^{2+}$ calibration solutions were prepared starting from a solution of anhydrous copper (II) sulphate 0.01 M in 0.1 M nitric acid to ensure complete salt dissolution. Subsequently, a dilution process was carried out firstly in PBS and after in a cell culture medium until the desired concentrations were obtained. It was verified that the pH of the final $Cu^{2+}$ solutions (1–20 µM, suitable physiological homeostasis range) was preserved (pH 7.4) despite the acidity of the stock solution (0.01 M in nitric acid). For the measurements at pH 4, a known amount of acetate buffer at pH 3.6 (0.1 M) was added to the solutions of $Cu^{2+}$ in cell culture media (volume ratio cell culture media:acetate buffer 1:1). The $Cu^{2+}$ concentration in cell culture media was adjusted in order to obtain calibration solutions ranging from 1 µM to 20 µM after the dilution process with acetate buffer.

### 2.3. Single Working Electrode and Three-Electrode-Integrated Sensors: Fabrication Details

For anodic stripping voltammetry (ASV) measurements with the classic three-electrode setup, a single working gold electrode device was fabricated, and used with a platinum counter electrode and a Ag/AgCl reference electrode. The gold working electrode was realized by a standard fabrication process on a 500 µm thick (100) 4″ silicon wafer (n-type) with 500 nm of thermal oxide. Silicon was accurately cleaned with acetone and a 2-propanol ultrasonic bath for 15 min, then rinsed in deionized water and dehydrated at 120 °C for 600 s. A 1.4 µm thick AZ 5214 reversal image photoresist (Micro-Chemicals GmbH, Ulm, Germany) was dispensed on the silicon substrate and soft-baked at 110 °C for 50 s, then exposed with a 365 nm MA6 mask aligner tool and developed in AZ326. After the photolithography completion, a Cr/Au thin film (5 nm/200 nm) was deposited by e-beam evaporation, followed by lift-off to pattern the working electrode. A full wafer passivation was deposited at 350 °C, with 400 nm thick ultra-low residual stress (+50 MPa) PECVD silicon nitride. A final photolithography process allowed to selectively remove the silicon nitride layer from the working electrode by an inductively coupled plasma process with a SF6/O2 mixture. Using a 3D stylus profiler scan of the circular gold working electrode,

3 mm diameter, before and after the plasma process, an average final RMS roughness of about 5 nm was determined.

The three-electrode-integrated sensor (microelectrode) to be coupled with the microfluidic platform was achieved with a fabrication process similar to that used for the single working electrode device, on the same silicon wafer substrate. A schematic of the fabrication process is shown in Figure 1a. The first photolithography step was identically replicated for the definition of the working electrode and pads, then followed by Cr/Au thin film (5 nm/200 nm) deposition by e-beam evaporation. However, a second photolithography step with a similar recipe was added to fabricate the reference and counter electrodes made by a Ti/Pt thin film (10 nm/300 nm) deposited by e-beam evaporation. To follow, full wafer passivation with 400 nm thick ultra-low residual stress (+50 MPa) PECVD silicon nitride and dry etching process, as conducted for the single working electrode device, were carried out to complete the fabrication of the three-electrode-integrated sensor.

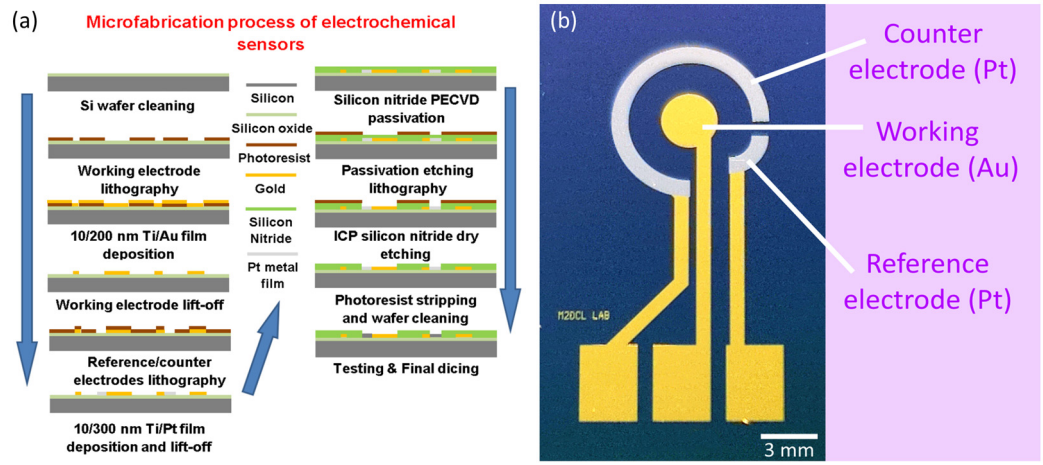


**Figure 1.** The electrochemical sensor integrated into the microfluidic chip, (**a**) schematic of the microfabrication process; (**b**) final device ready for experimental testing.

Figure 1b shows the image of the final three-electrode-integrated sensor, ready for calibration and experimental testing.

### *2.4. Anodic Stripping Voltammetry Measurements in Different Cell Culture Media*

For the detection of copper ions, different cell culture media including MEM, DMEM, F12K and MEM with the addition of non-essential amino acids, glutamine, penicillin-streptomycin and fetal bovine serum were supplemented with copper solutions and electrochemically measured at physiological pH (pH 7.4) and after acidification at pH 4.

Before ASV measurements, the single working gold electrode was cleaned by performing 10 cyclic voltammetry cycles in $H_2SO_4$ (50 mM) from −0.3 V to 1.5 V vs. Ag/AgCl. Electrochemical measurements were performed using a potentiostat/galvanostat (Ivium Vertex One, Ivium Technologies B.V., Eindhoven, The Netherlands). Different accumulation potentials and times were tested and the voltammograms reported in this work are related to −0.4 V vs. Ag/AgCl for 30 s, and the stripping was performed using square wave voltammetry in the range −0.4 V–0.7 V (pulse amplitude 30 mV, frequency 25 Hz and E step 4 mV). After each measurement, a potential of 0.7 V was applied to the working electrode for 50 s in acetate buffer as a cleaning step. After each calibration, a test measurement was carried out to ensure that the electrode response remained constant over time. The reported measurements were carried out in triplicate and the averages of the signals are shown graphically.

### *2.5. Copper Detection in Transwell: Cell Culture, Copper Exposure and MTT Viability Assay*

Anodic stripping voltammetry measurements were also performed to monitor copper ions concentration in an in vitro system of the Caco-2 cell Transwell® culture model.

Caco-2 cells (ATCC® HTB-37™) were cultured in T-75 $cm^2$ flasks in Minimum Essential Eagle Medium (MEM; Corning) supplemented with 20% fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine, 1% penicillin-streptomycin solution, at 37 °C in a humidified atmosphere of 5% $CO_2$. At 80–90% confluence, the cells were trypsinised, and then they were seeded at $5 \times 10^5$ cells $cm^{-2}$ in 6-well Transwell® plates.

After Caco-2 monolayer formation (21 days after seeding), copper solutions at 10 μM and 20 μM final concentrations were added to the cell culture media of the separated upper compartments of the Transwell® system. Electrochemical measurements, as described in the previous section, were performed by sampling both the upper and lower media at the beginning of the experiment ($T_0$) and after 72 h under physiological and acidic conditions.

To assess the copper effect on cell viability, cell metabolic activity was investigated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. An aliquot of 1 mL of MTT solution (1 mg $mL^{-1}$) was added into each well and incubated for 3 h at 37 °C. Then, the produced dark insoluble formazan was dissolved in DMSO (2 mL). The absorbance signal at 560 nm, recorded using a UV-Vis spectrophotometer (Tecan Spark®, Tecan Trading AG, Zurich, Switzerland), was correlated to the percentage of vital cells by comparing data from cells exposed to copper ions with the control cells.

### *2.6. Microfluidic Chip with Three-Electrode-Integrated Sensor and ASV Measurements in Chip*

With the aim to apply the proposed copper detection protocol in an organ-on-chip system, the electrochemical sensor was integrated into a commercial microfluidic Topas polymer 2-chamber cross-flow platform (Microfluidic ChipShop, Jena, Germany) suitably modified. The latter was adapted, for a preliminary demonstration of the potential of the proposed approach, to allow the simultaneous medium acidification and ASV detection of copper ions in the chip and real-time. The microfluidic chip includes two chambers connected with a microfluidic system based on an Elveflow® pressure and flow control instrument (OB1, MK3). A first chamber (cell culture chamber) is dedicated to the cell culture, as a porous membrane separates its cavity into two horizontally aligned compartments, apical and basolateral, in which the medium can flow separately if required. A controlled flow of fresh culture medium, possibly supplemented with copper ions, is injected into the cell chamber through an inlet tube. Then, the medium flows from the cell chamber toward a second chamber, the sensing chamber, passing through a T-shaped microchannel where it can be acidified. In this way, it is possible to perform the acidification of the cell culture medium and the related detection process without affecting the main cell culture chamber. The sensing chamber was obtained by performing a laser cut of its cover lid to allow the insertion of the three-electrode-integrated sensor bonded onto the cover lid. The bonding was performed with an acrylic, biomedical-grade pressure-sensitive adhesive, 80 μm thick (ARCARE 90445Q, Adhesive Research, Glen Rock, NJ, USA), obtaining good bonding strength and gas/medium tightness. Different mini Luer ports were used/interconnected to make the microfluidic circuit functional to cell viability, medium treatment and acidification, ASV detection and final wasting. The sensor-integrated chip was connected to the microfluidic Elveflow® system, in order to allow the flow of the medium for the cells' growth, and the controlled buffer injection for the medium acidification through the channel leading to the sensing chamber.

The signal from the sensor was acquired by a flat cable connector (Würth Elektronik GmbH & Co. KG, Niedernhall, Germany). The controlled flow rate at the inlets was set by the adopted Elveflow OB1 microfluidic controller with two Elveflow MFS2 thermal mass flow sensors.

ASV measurements in the chip by the integrated sensor were conducted by performing an accumulation step at −1.0 V vs. Pt for 100 s, and the stripping by square wave voltammetry in the range −1.0 V–0.3 V (pulse amplitude 30 mV, frequency 25 Hz and E step

4 mV). After each measurement, a potential of 0.3 V was applied to the working electrode for 150 s as a cleaning step. The reported measurements were carried out in triplicate and the averages of the signals are shown graphically.

### *2.7. Efficient Medium Acidification in the Chip: FEA Simulations*

FEA simulations allowed us to identify the best injection conditions to be used for the acidification of the medium by using the commercial Topas polymer 2-chamber cross-flow platform. To this aim, a 2D model of the acidification and sensing chamber was developed in Comsol Multiphysics for analysing computational fluid dynamics coupled with the transport of diluted species and evaluating the mixing quality obtainable in the microfluidic platform. The 2D model accurately reproduces the geometry of the considered microfluidic circuit [28]. In particular, the mixing efficiency and achievable minimum mixing times were evaluated as a function of different flow rates applied to the medium and acetate buffer inlets. The 2D geometry comprises the T-shaped microchannel junction, by which the culture medium (collected from the cell chamber) and the acetate buffer were injected into a common microchannel and mixed with each other before reaching the sensing chamber (area of 88.8 $mm^2$), where the copper ions detection in the acidified medium was performed. By FEA simulations, the flow field was determined by solving the steady-state Navier-Stokes equations for the conservation of momentum and the continuity equation for the conservation of mass, for an incompressible Newtonian fluid:

$$\nabla \cdot (-p\vec{I} + \vec{K}) + \vec{F} = 0 \tag{1}$$

$$\rho \nabla \cdot \vec{u} = 0 \tag{2}$$

where

$$\vec{K} = \mu(\nabla \vec{u} + (\nabla \vec{u})^T) \tag{3}$$

$\rho$ is the fluid density (in $kg/m^3$), $\mu$ is the fluid dynamic viscosity (in Pa·s), $p$ is the pressure (in Pa) and $\vec{u}$ is the velocity vector (in m/s). $\vec{F}$ is the volume force vector (in $N/m^3$). No-slip boundary condition ($\vec{u} = 0$) was applied on all the side walls of the microfluidic circuit, while zero pressure condition was set at the outlet of the sensing chamber.

The calculated flow field was then used to solve the convection–diffusion equation and determine the concentration of acetate buffer in the culture medium present in the sensing chamber:

$$\frac{\delta c_i}{\delta t} + \nabla \cdot \vec{J_i} + \vec{u} \cdot \nabla c_i = R_i \tag{4}$$

$$\vec{j_i} = -D_i \nabla c_i \tag{5}$$

$c_i$ represents the concentration of the species $i$ (in $mol/m^3$), $D_i$ is the diffusion coefficient (in $m^2/s$), $R_i$ is a reaction rate expression for the species $i$ (in $mol/m^3/s$), $\vec{u}$ is the mass-averaged velocity vector (in m/s), and $\vec{J_i}$ is the diffusive flux vector (in $mol/m^2/s$). The third term on the left side of the above equation describes the convective transport due to the velocity field $\vec{u}$ obtained as a solution of the Navier-Stokes equations.

The flow and mass transport models were solved to determine the concentration distribution across the modelled domain. The medium acidification degree achievable in the sensing chamber was evaluated in terms of mixing efficiency (ME), calculated as [29]:

$$ME = 1 - \frac{1}{\bar{C}} \sqrt{\frac{\sum_{i=1}^{n} (C_i - \bar{C})^2}{n}} \tag{6}$$

where

$$\bar{C} = \frac{1}{n} \sum_{i=1}^{n} C_i \tag{7}$$

$n$ is the number of sampling points in the section, $C_i$ is the concentration value at each node, $\bar{C}$ is the average concentration across the section. ME = 0 indicates no mixing, whereas optimum mixing is obtained when ME = 1.

## 3. Results and Discussion

### *3.1. ASV Measurements of Copper in Different Cell Culture Media*

Cell culture media contain a mixture of compounds and nutrients designed to support cellular growth. They consist of a balanced salt solution which is necessary to maintain optimum osmotic pressure within the cells, stabilize the optimum pH and provide the essential metal ions. ASV measurements with three separated electrodes were performed in an electrochemical cell with three different kinds of cell culture media (MEM, DMEM and F12K), commonly used for human in vitro intestinal model, at a physiological pH (pH 7.4) in a range of $Cu^{2+}$ concentrations between 1 and 20 µM (Figure 2). The same measurements were performed with a complete medium ($MEM^+$, pH 7.4), which is MEM complemented with non-essential amino acids, glutamine, penicillin-streptomycin and fetal bovine serum. Several factors related to the composition of the medium can affect the ASV measurements. In particular, ionic strength is a very important characteristic that influences the electrochemical kinetics of the reaction involved in the ASV process. However, the aim of this work is to perform ASV measurements in cell culture media and the composition of the culture media in terms of the amount of ions and associated ionic strength cannot be modified to meet the requirements for cell growth. Therefore, in this work, standard media with fixed ionic strength and composition were used without considering other compositional parameters.

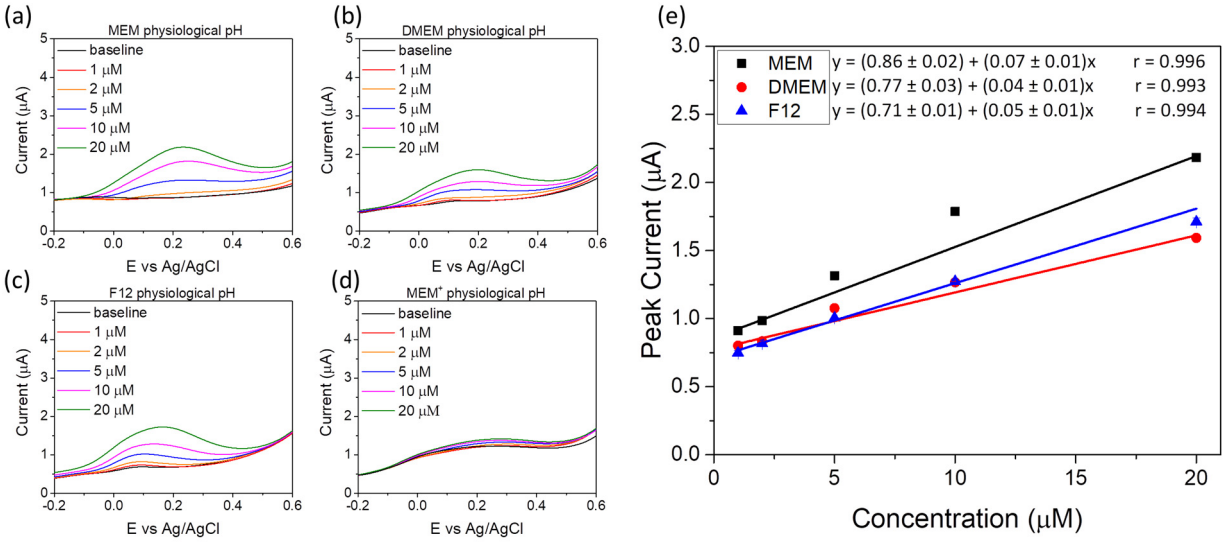


**Figure 2.** Anodic stripping voltammetry with a three-electrode setup for copper determination in (**a**) MEM at pH 7.4; (**b**) DMEM at pH 7.4; (**c**) F12K at pH 7.4; (**d**) $MEM^+$ at pH 7.4. Calibration plot of anodic stripping voltammetry in (**e**) MEM, DMEM and F12K at pH 7.4. Accumulation potential: −0.4 V vs. Ag/AgCl; accumulation time: 30 s; stripping scan: −0.4 V–0.7 V (square wave voltammetry: pulse amplitude 30 mV, frequency 25 Hz and E step 4 mV).

A preliminary study was carried out to define the accumulation parameters (potential and time) in the three-electrode configuration and the results are shown in Figure S1. Firstly, the accumulation potential was chosen by performing ASV measurements in MEM (pH 7.4) with 20 µM of $Cu^{2+}$ and the highest peak current value was obtained by using −0.4 V. Subsequently, different accumulation times were tested (at −0.4 V of accumulation potential) and 30 s was chosen as a compromise between a good intensity of the voltammetric signal and the time of the measurement.

For measurements with MEM, DMEM and F12, a linear correlation between copper concentration and peak current is visible, although some experimental points do not follow the correlation line perfectly, because the physiological pH conditions and the medium composition do not represent the best conditions for carrying out this type of measurement, as will be explained below. In particular, the most sensitive medium is MEM with a peak current of about 2.2 μA at 20 μM and a higher slope with respect to DMEM and F12. In the case of $MEM^+$, the supplemented components could be able to completely bind the copper ions, resulting in no visible peaks under physiological conditions. The details about the effect of copper binding on ASV signal intensity will be discussed below.

The reasons for the performance reported in these measurement conditions are to be found in the pH and composition of the measurement medium. Regarding the pH, according to the Pourbaix diagram of copper in aqueous systems, the $Cu^{2+}/Cu^0$ equilibrium is observed in acidic conditions up to pH 7. At higher pH values, passivation effects due to the formation of oxides can influence the equilibrium. Moreover, considering the analytical principle of ASV, free copper ions in a solution can be quantified by such a technique, while the formation of copper complexes with organic substances present in the measurement solution can reduce the quantity of free and measurable ions, thus influencing the intensity of the ASV signals [19]; such complexes are usually more stable in neutral-basic pH conditions. For all these reasons, copper analysis by ASV is usually performed at pH 4–5 in simple buffers, hence the pH and the specific employed electrolyte can strongly affect the measurement and should be evaluated.

In our specific case, the cell culture media have a neutral pH and contain organic substances such as amino acids, vitamins, sugars, etc. that could interact with copper ions by forming metal complexes, as largely reported elsewhere [26,30–34]. The formation of complexes depends on several factors, such as organic molecules concentration, metal concentration and thermodynamic driving forces [30], and is therefore difficult to predict and quantify this phenomenon in complex systems such as cell culture media. Considering such a scenario, by performing the measurements in the cell culture media under physiological conditions, the quantity of free copper ions can be estimated, which could be very low considering the large amount of potential complexing agents. The identification of suitable protocols for a reliable cell culture media treatment before the metal ions detection phase can improve the quality of the ASV measurement and promote the adoption of these sensors in in vitro and OoC platforms with greater confidence from the scientific community.

Based on this consideration, an acidification protocol was tested with the aim to improve the voltammetric signal intensity. The optimized protocol consists of a 1:1 volume ratio between cell culture media and pH 3.6 acetate buffer and it enabled a pH of 4 to be reached. The lower pH value can be useful to induce the decomplexation of copper ions, while the volume addition of acetate buffer is useful to dilute the organic species in the final solution. Indeed, it is widely reported that copper complexes, and in general metal complexes, are stable under neutral and basic conditions and that acidic conditions can induce the decomplexation of copper and the increase in free copper ions [30,35]. A similar acidification protocol was tested for measurements of copper concentration in seawater samples in order to distinguish between electroactive copper (free Cu ions) and acid-dissolved copper (labile Cu complex) [36]. In any case, the concentration of organic species in seawater samples is about two orders of magnitude lower than that present in cell culture media, so this aspect should be deeply evaluated in biological systems [37].

The results related to the ASV experiments performed after the medium acidification are reported in Figure 3. Higher current values and higher slope values are visible with respect to measurements performed at physiological pH, thus demonstrating the higher sensitivity of the system in acidic/diluted conditions. A linear correlation was obtained for all the cell culture media.

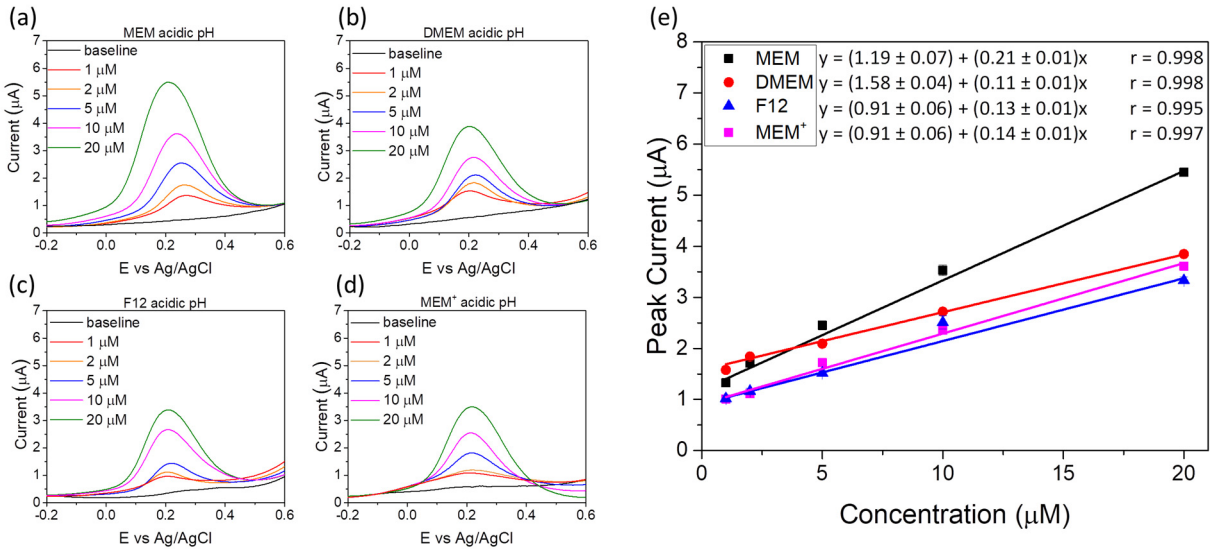


**Figure 3.** Anodic stripping voltammetry with a three-electrode setup for copper determination in (**a**) MEM at pH 4; (**b**) DMEM at pH 4; (**c**) F12K at pH 4; (**d**) $MEM^+$ at pH 4. Calibration plot of anodic stripping voltammetry in (**e**) MEM, DMEM, F12K and $MEM^+$ at pH 4. Accumulation potential: −0.4 V vs. Ag/AgCl; accumulation time: 30 s; stripping scan: −0.4 V–0.7 V (square wave voltammetry: pulse amplitude 30 mV, frequency 25 Hz and E step 4 mV).

As with all electrochemical systems, the electrodes used for measurement require periodic checks and recalibration procedures. However, the material adopted in the developed sensor did not present any specific problems with the adsorption of organic substances and/or ions, maintaining a stable response over several weeks.

As a reference method, the same copper detection measurements were performed by using a commercial colourimetric kit and the results are reported in Figure S2. The measurements carried out with the colourimetric kit do not seem to be comparable at low concentrations (1–5 μM) in the two pH conditions, whereas at high concentrations (10–20 μM) the absorbance values recorded in the two conditions tend to coincide. Therefore, the kit does not seem to discriminate between the pH of the two measured solutions and therefore whether the copper is in the form of a free or complexed ion. Furthermore, the colourimetric kit does not seem to be very sensitive, with an absorbance variation of about 0.1 between the two extreme concentrations.

It can therefore be concluded that the colorimetric kit could be used to estimate the total amount of copper in the culture medium, beyond the pH conditions. However, this type of measurement requires mixing the solution to be analyzed with different reagents and then measuring the absorbance using a spectrophotometer, which, unlike the method developed in this work, is not easy to implement in chips.

### *3.2. Copper Detection in Caco-2 Cell Transwell® Culture Model*

In order to test the applicability of this analytical method in an in vitro system, experiments were performed with a Caco-2 cell Transwell® culture model.

Human colon cancer Caco-2 cells are widely used to mimic the intestinal barrier for in vitro studies of the biological transport and toxicity of nutrients and drugs [38–40]. Over a 21-day period, Caco-2 cells form a polarized monolayer with tight junctions between adjacent cells, separating apical and the basolateral membrane compartments to ensure the epithelial barrier function.

The Caco-2 monolayer was exposed to two concentrations of copper ions (10 μM and 20 μM) by loading $MEM^+$ (supplemented with copper) into the upper compartment of the Transwell®. The copper concentration was monitored by withdrawing the cell culture media from the upper and lower compartment of the Transwell® at the beginning

of the experiment ($T_0$) and after 72 h. Measurements were performed under physiological and acidic conditions. As a control, to test the cell viability in the presence of the two concentrations of copper ions, an MTT assay was performed and results were reported in Figure S3.

Under physiological conditions, the voltammetric signal was barely visible (as already reported in Figure 2d), whereas it can be well distinguished under acidic conditions (Figure 4). At $T_0$ the peak current for 10 µM concentration was about 2 µA, while that for 20 µM was about 2.5 µA (Figure 4c,d). Obviously, the observed currents correspond to a concentration that is halved when compared to the original one, as the acidification/dilution process was carried out before the measurement. Indeed, the peak current of 2 µA matches with the peak of 5 µM concentration in the calibration line in Figure 3e, while 2.5 µA corresponds with the 10 µM concentration.

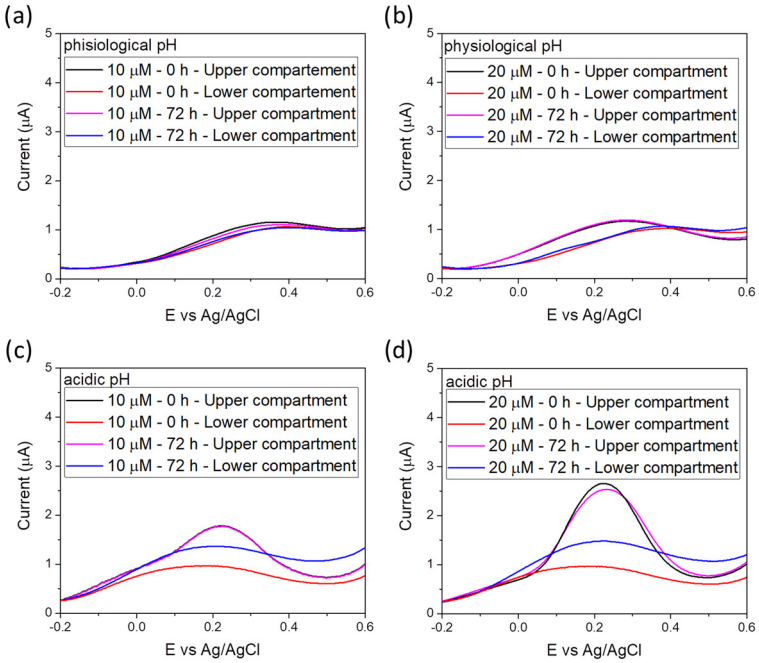


**Figure 4.** Anodic stripping voltammetry with a three-electrode setup for copper determination in Caco-2 cell Transwell® culture model: monitoring at 0 h and 72 h, (**a**) 10 µM $Cu^{2+}$—physiological pH; (**b**) 20 µM $Cu^{2+}$—physiological pH; (**c**) 10 µM $Cu^{2+}$—acidic pH; (**d**) 20 µM $Cu^{2+}$—acidic pH. Accumulation potential: −0.4 V vs. Ag/AgCl; accumulation time: 30 s; stripping scan: −0.4 V–0.7 V (Square Wave Voltammetry: pulse amplitude 30 mV, frequency 25 Hz and E step 4 mV).

Regarding measurements after 72 h, no variations were visible in the upper compartment, while in the lower compartment, only a slight increase in the base current was visible. These results suggested that the intestinal epithelial monolayer does not mediate the reduction of $Cu^{2+}$ to $Cu^{+}$, thus preventing the passage or absorption of the copper ions. Furthermore, the MTT assay demonstrated that the addition of 10 µM and 20 µM of copper ions within 72 h from the injection did not affect the cell viability of Caco-2 cells, which displayed the same metabolic activity as the control cells. These results confirmed that at these copper concentrations, the monolayer preserved its integrity, blocking the passage of copper ions from the apical to the basal compartment of the Transwell®.

Unfortunately, the investigated Transwell® system does not allow continuous measurements to be performed, because the cell culture media must be sampled from the culture plates and manually acidified before measurements. Therefore, we developed a microfluidic chip to automatically perform the acidification and the $Cu^{2+}$ sensing process with the aim of continuously monitoring the flow of copper across an epithelium such as the intestine.

### *3.3. Efficient Medium Acidification in the Adapted Commercial Platform: FEA Predictions*

In order to carry out the acidification process in a microfluidic chip, it is essential to evaluate the best and most efficient conditions for mixing the culture medium with the acidifying solution (buffer acetate). This involves taking into account both the properties of the involved fluids and the geometry of our system. The culture media solution has density $\rho = 1008\ kg/m^3$ and viscosity $\eta = 0.733 \times 10^{-3}$ Pa·s [41], and the aqueous 0.1 M acetate buffer has $\rho = 1007\ kg/m^3$ and $\eta = 1 \times 10^{-3}$ Pa·s [42]. The culture medium was modelled as solvent solution entering Inlet 1, the acetate buffer was modelled as dilute species entering Inlet 2 with a diffusion coefficient in the culture medium of $1 \times 10^{-9}\ m^2/s$. The degree of medium acidification and minimum time required to achieve homogeneous mixing in the sensing chamber as a function of different flow rates at the inlets were evaluated by FEA. Simulations were performed by considering increasing values of flow rate at the two inlets, in the range 0.8–7.2 µL/min, in accordance with the values of flow rate that can be reliably applied using the Elveflow OB1 commercial system. In the discussion to follow, $FR_M$ is used to indicate the flow rate applied to the culture medium inlet, and $FR_B$ represents the flow rate applied to the acetate buffer inlet.

FEA simulations allowed us to evaluate the mixing performance achievable in the sensing chamber, in terms of efficiency and time, for different combinations of flow rates at the inlets. The mixing efficiency (ME) was determined using Equation (6), the mixing time was defined as the time required from the acetate buffer injection to the reaching of the steady-state ME value. Both the ME and mixing time were evaluated in the middle section of the sensing chamber. Table 1 reports the most interesting results obtained by considering four different combinations of $FR_M$ and $FR_B$, which allowed us to obtain a ME comprised between 0.81 and 0.99. The results show a decrease in the ME for increasing the total flow rate in the chip. By applying the minimum value of flow rate at both the two inlets (0.8 µL/min), the ME is maximized (0.99) within a time of 4000 s from the acetate buffer injection (case #1). In cases #2 and #3 reported in Table 1, $FR_B$ was doubled (1.6 and 3.2 µL/min, respectively, with respect to the minimum applicable flow rate), and in case #4, $FR_B$ was set to the maximum applicable value (7.2 µL/min), with the aim to identify a possible flow rates combination able to reduce the mixing time without excessively compromise both the ME and the final pH of the acidified medium. For each value of $FR_B$ set in cases #2 to #4, the value of $FR_M$ that maximizes the ME was identified by simulations.

**Table 1.** Mixing time and efficiency achievable in the sensing chamber of the adapted commercial platform, for different combinations of flow rates at the two inlets.

| Case | Flow Rates (µL/min) | Mixing Time (s) | Mixing Efficiency |
|---|---|---|---|
| #1 | $FR_M = 0.8$<br>$FR_B = 0.8$ | 4000 | 0.99 |
| #2 | $FR_M = 1.5$<br>$FR_B = 1.6$ | 2000 | 0.97 |
| #3 | $FR_M = 2.7$<br>$FR_B = 3.2$ | 1000 | 0.89 |
| #4 | $FR_M = 5.1$<br>$FR_B = 7.2$ | 540 | 0.81 |

Figure 5 shows the ME versus time, calculated at the middle section of the sensing chamber for the four cases indicated in Table 1. The results show that each investigated combination of flow rates requires a mixing time to reach the steady-state ME that is inversely proportional to the total flow rate applied to the inlets. The best efficiency is obtained by applying the slowest flow rate to both inlets.

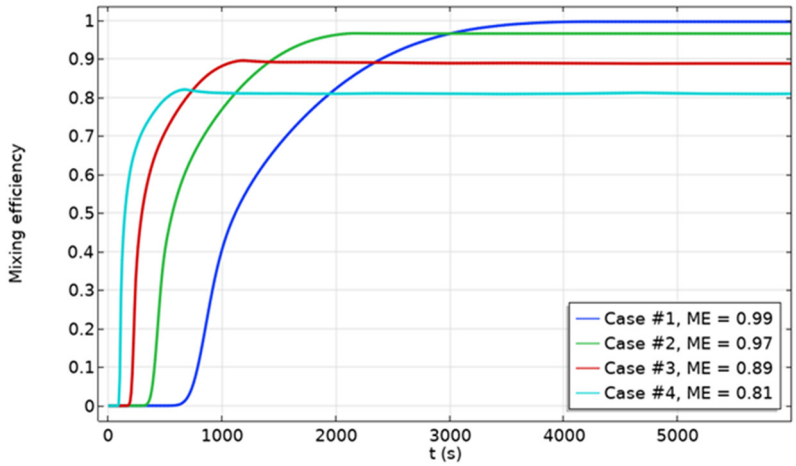


**Figure 5.** Mixing time and efficiency achievable in the sensing chamber of the adapted commercial platform, for the four cases indicated in Table 1.

Figure 6 depicts the steady-state concentration distribution achieved by FEA for the best and worse investigated combinations of flow rates (case #1 and case #4 indicated in Table 1), with a ME of 99% and 81%, respectively. In the simulations, the diluted species (acetate buffer) was assumed entering in Inlet 2 at a concentration of 1 mol/m$^3$, while the concentration was 0 mol/m$^3$ in Inlet 1. In the concentration profile depicted in Figure 6, the red colour represents the unmixed species (acetate buffer) in the culture medium (blue colour), while the mixed species is represented by the green colour (with a concentration equal to half of that entering, 0.5 mol/m$^3$). The steady-state concentration of the diluted species is clearly uniform in Figure 6a (case #1 with ME = 99%), while an evident gradient of concentration is visible in Figure 6b, due to a lower ME (81% for case #4).

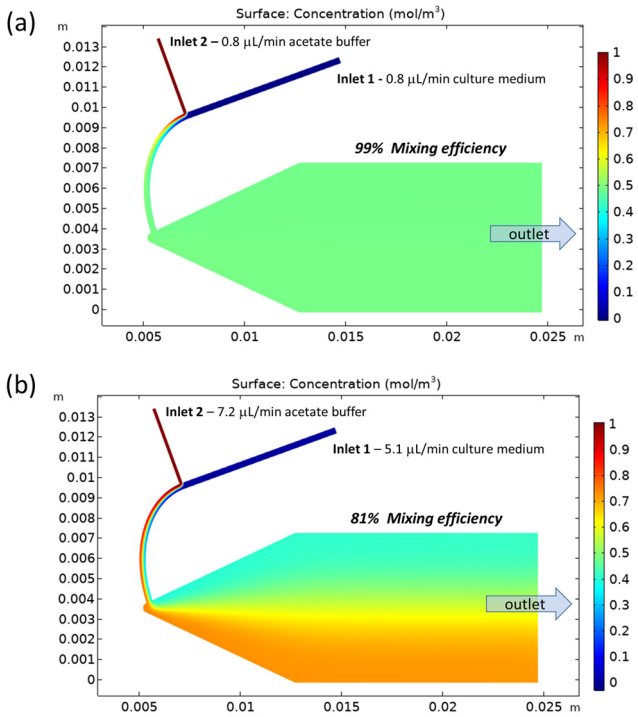


**Figure 6.** Steady-state concentration distribution achieved by FEA, for (**a**) ME = 99% and (**b**) ME = 81%.

For the experiments of ASV measurements in chip the optimum flow rates at the inlet of cell culture medium and acetate buffer were set to 1.5 and 1.6 µL/min, respectively. Compared to the best case (case #1), the selected combination of flow rates (case #2) halves

the mixing time while keeping a very high mixing efficiency (97%) and a minimal pH variation (lower than 0.1 pH units), that is experimentally negligible vs. sensor signal gain. Cases #3 and #4 were discharged because they did not allow an effective pH control of the acidified medium. In addition, the selected flow rates (case #2) guaranteed a complete renewal of the medium in the sensing chamber approximately every 30 min (67 µL cells chamber volume) and allowed different measurements approximately every 1 h. These settings enabled monitoring of the epithelium permeability to ions, drugs or molecules of interest at hourly intervals without affecting the cell culture chamber.

### *3.4. Copper Measurements in a Microfluidic Device*

To test the electrochemical copper detection in a microfluidic platform, a sensor-integrated dual chamber chip was developed (Figure 7a). In detail, the cell culture medium was supplemented with copper ions and injected into the cell culture chamber with a controlled flow (1.5 µL/min). The medium flowed from the cell culture chamber to the sensing chamber where it was mixed with acetate buffer (Figure 7a). The electrochemical measurements in the sensing chamber were always carried out with a three-electrode setup, but contrary to what was reported in Section 3.1, a microelectrode (Figure 1b) integrated into such a chamber was used instead of three separate electrodes. All of the experimental results are reported below (Figure 7b,c).

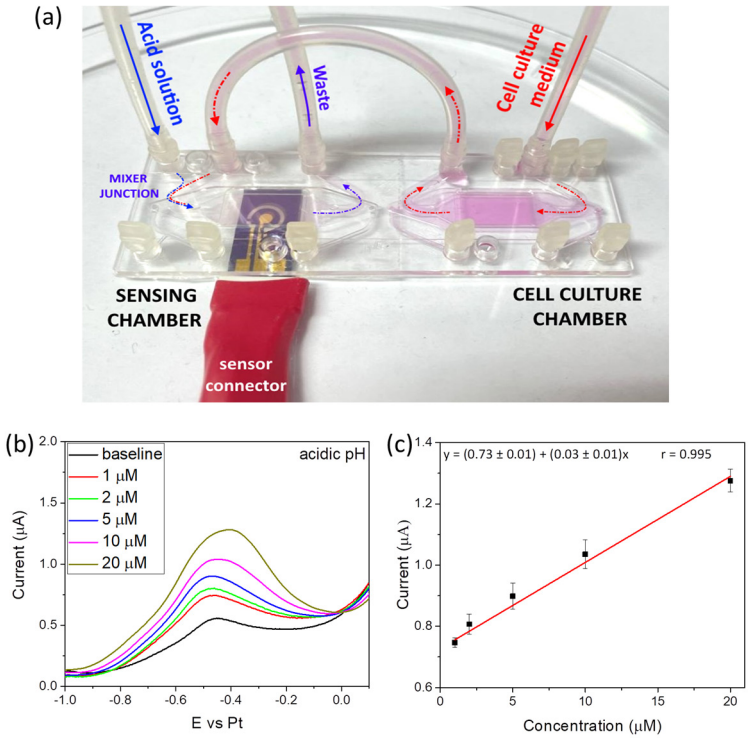


**Figure 7.** Photograph of the microfluidic chip integrated with ion sensor (**a**); anodic stripping voltammetry with three-electrode-integrated sensor (microelectrode) for copper determination performed in chip (sensing chamber) (accumulation potential: −1.0 V vs. Pt; accumulation time: 100 s; stripping scan: −1.0 V–0.3 V (square wave voltammetry: pulse amplitude 30 mV, frequency 25 Hz and E step 4 mV) (**b**); calibration plot in chip (sensing chamber) (**c**).

The microelectrode consisted of a gold working electrode, a platinum counter electrode and a platinum pseudoreference (Figure 1b). A platinum pseudoreference was used because of the durability problems associated with the use of thin film Ag/AgCl electrodes [43]. The accumulation potential was set at −1 V vs. Pt, taking into account both the shift with respect to the Ag/AgCl electrode and the minimum potential required to display the entire voltammetric peak. In addition, a longer accumulation time was used (100 s) due to

the lower surface area of the working electrode in the integrated microelectrode. In fact, while in Section 3.1, a working electrode with a 3 mm diameter was employed, due to the geometric limits of the chip, the working electrode in the microelectrode setup has a diameter of 2 mm. This difference in surface area, together with the difference in geometry of the setup, leads to differences in measurement conditions, and the accumulation time indicated here represents the best compromise between the signal intensity and a reasonable accumulation time.

By analysing the results of ASV measurements recorded by the integrated sensor, at acidic pH a voltammetric signal was visible with a linear correlation as the copper concentration increases (Figure 7c). The peak currents were lower than those reported for the three separated electrode systems due to the different geometry of the working electrode and of the entire electrochemical system.

The double chamber system developed in this work makes it possible to isolate the measurement chamber from the culture chamber, not only to perform the medium acidification without affecting the cell environment but also to carry out any cleaning of the working electrode (e.g., by cyclic voltammetry in sulphuric acid) and the periodic recalibrations required by the electrochemical analysis. The microelectrode has been tested for several weeks without any particular problems of instability of the electrochemical response at the same concentration of copper in solution.

These results demonstrated the effectiveness of the developed acidification/sensing chip and opened the way to a promising coupled use with different organ-on-chip and microfluidic devices in order to perform the acidification of the medium and ions dosing in a fully automated way.

## 4. Conclusions

Copper detection and monitoring in a cellular environment is of great interest for different biological studies, in order to understand its involvement in molecular pathways, its regulation in the body and its association with specific diseases. In particular, copper can be provided by diet and absorbed by intestinal epithelium thus preserving the copper homeostasis in the whole body.

Despite the importance of the intestinal epithelium in the regulation of copper, the involved mechanisms are not completely elucidated. Such kinds of mechanisms could be studied by integrating sensors with cellular systems and by mimicking conditions close to that in vivo.

Starting from such background, in this work the electrochemical sensing of copper ions in different cell culture media was investigated. Anodic stripping voltammetry at physiological conditions (pH 7.4) showed moderate current signals at (pato-)physiological concentrations of ions. An acidification protocol of the cell culture media was designed and tested with the aim to enhance the voltammetric peaks and a higher responsivity of the system was observed. In fact, the acidification process can induce the decomplexation of copper ions with the organic substances present in the culture medium, thus increasing the number of ions measurable with the ASV technique and allowing the total amount of copper in the medium to be estimated.

The acidification and detection protocol was tested in an in vitro system by performing measurements with a Caco-2 cell Transwell® culture model.

In particular, to indirectly estimate the amount of copper ions absorbed by the cells, the amount of copper in the culture medium in the upper and lower compartments can be monitored over time. The intestinal barrier is only able to absorb physiological concentrations of copper, preventing the absorption of excess copper which would be toxic. Therefore, by quantifying the ions in the medium, it is possible to provide information on the amount absorbed by the cells and the integrity of the intestinal barrier. The integration of such a detection protocol into organ-on-chip systems could allow the automatic monitoring of copper uptake and regulation mechanism, also by a correlative approach of such a sensing system with biochemical experiments. Therefore, a microfluidic device for

the acidification process and copper detection was implemented and investigated with an embedded integrated microsensor. FEA simulations allowed the best injection conditions to be identified in the adapted commercial microfluidic platform for obtaining the desired medium acidification within acceptable mixing times.

The results reported in this work represent a first step towards the development of organ-on-chip models that integrate more functionality through the use of sensors, microfluidics and automated biological liquids treatment/detection. Such models could be of great support to study pathologies and complex biological mechanisms.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemosensors11080466/s1, Figure S1: Parameter optimization for the accumulation step of ASV performed in the three-electrode configuration: peak current vs accumulation potential (30 s as accumulation time) for 20 μM $Cu^{2+}$ solution (a), peak current vs accumulation time (−0.4 V as accumulation potential) for 20 μM $Cu^{2+}$ solution (b). Figure S2: Colorimetric copper content quantification in MEM complete medium containing $Cu^{2+}$ with a concentration ranging from 1 μM to 20 μM at pH 7.4 (a) and at pH 4 (b). Figure S3: MTT assay to test viability of Caco-2 cells exposed to two concentration of copper ions (10 μM and 20 μM) for 72 h: histograms represent the percentage (%) calculated by comparing the data of the exposed cells with those of the control cells (ctrl, 100%). All values are reported as the means ± standard error. Statistically significant differences with respect to the control according to One-Way ANOVA; $p < 0.05$. Means comparison by Tukey's test.

**Author Contributions:** C.T.P., E.S. and L.N.F. conceived and designed the experiment. C.T.P., E.S., C.D.P., L.G., F.B., V.E. and F.C. conducted the experimental work and analyzed the data. C.T.P., E.S. and C.D.P. wrote the original draft of the manuscript. L.B., P.A.S. and L.N.F. supervised the project. All authors were involved in the reviewing and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Van Der Meer, A.D.; Van Den Berg, A. Organs-on-chips: Breaking the in vitro impasse. *Integr. Biol.* **2012**, *4*, 461–470. [CrossRef] [PubMed]
2. Wu, Q.; Liu, J.; Wang, X.; Feng, L.; Wu, J.; Zhu, X.; Wen, W.; Gong, X. Organ-on-a-chip: Recent breakthroughs and future prospects. *Biomed. Eng. Online* **2020**, *19*, 9. [CrossRef] [PubMed]
3. Bhagat, S.; Singh, S. *Cultivating Human Tissues and Organs over Lab-on-a-Chip Models: Recent Progress and Applications*, 1st ed.; Elsevier Inc.: Amsterdam, The Netherlands, 2022; Volume 187, ISBN 9780323853033.
4. Fuchs, S.; Johansson, S.; Tjell, A.; Werr, G.; Mayr, T.; Tenje, M. In-line analysis of organ-on-chip systems with sensors: Integration, fabrication, challenges, and potential. *ACS Biomater. Sci. Eng.* **2021**, *7*, 2926–2948. [CrossRef]
5. Signore, M.A.; De Pascali, C.; Giampetruzzi, L.; Siciliano, P.A.; Francioso, L. Gut-on-Chip microphysiological systems: Latest advances in the integration of sensing strategies and adoption of mature detection mechanisms. *Sens. Bio-Sens. Res.* **2021**, *33*, 100443. [CrossRef]
6. van den Berghe, P.V.E.; Klomp, L.W.J. New developments in the regulation of intestinal copper absorption. *Nutr. Rev.* **2009**, *67*, 658–672. [CrossRef] [PubMed]
7. Kozlowski, H.; Janicka-Klos, A.; Brasun, J.; Gaggelli, E.; Valensin, D.; Valensin, G. Copper, iron, and zinc ions homeostasis and their role in neurodegenerative disorders (metal uptake, transport, distribution and regulation). *Coord. Chem. Rev.* **2009**, *253*, 2665–2685. [CrossRef]

8. Bost, M.; Houdart, S.; Oberli, M.; Kalonji, E.; Huneau, J.F.; Margaritis, I. Dietary copper and human health: Current evidence and unresolved issues. *J. Trace Elem. Med. Biol.* **2016**, *35*, 107–115. [CrossRef]
9. Desai, V.; Kaler, S.G. Role of copper in human neurological disorders1. *Am. J. Clin. Nutr.* **2008**, *88*, 855S–858S. [CrossRef]
10. Liu, Y.; Miao, J. An Emerging Role of Defective Copper Metabolism in Heart Disease. *Nutrients* **2022**, *14*, 700. [CrossRef] [PubMed]
11. Ferruzza, S.; Sambuy, Y.; Ciriolo, M.R.; De Martino, A.; Santaroni, P.; Rotilio, G.; Scarino, M.L. Copper uptake and intracellular distribution in the human intestinal Caco-2 cell line. *Biomet. Int. J. Role Met. Ions Biol. Biochem. Med.* **2000**, *13*, 179–185. [CrossRef]
12. Lönnerdal, B. Intestinal regulation of copper homeostasis: A developmental perspective. *Am. J. Clin. Nutr.* **2008**, *88*, 846S–850S. [CrossRef] [PubMed]
13. Reznik, N.; Gallo, A.D.; Rush, K.W.; Javitt, G.; Fridmann-Sirkis, Y.; Ilani, T.; Nairner, N.A.; Fishilevich, S.; Gokhman, D.; Chacón, K.N.; et al. Intestinal mucin is a chaperone of multivalent copper. *Cell* **2022**, *185*, 4206–4215.e11. [CrossRef] [PubMed]
14. Planeta, K.; Kubala-Kukus, A.; Drozdz, A.; Matusiak, K.; Setkowicz, Z.; Chwiej, J. The assessment of the usability of selected instrumental techniques for the elemental analysis of biomedical samples. *Sci. Rep.* **2021**, *11*, 3704. [CrossRef] [PubMed]
15. Gerdan, Z.; Saylan, Y.; Denizli, A. Recent Advances of Optical Sensors for Copper Ion Detection. *Micromachines* **2022**, *13*, 1298. [CrossRef] [PubMed]
16. Falcone, E.; Okafor, M.; Vitale, N.; Raibaut, L.; Sour, A.; Faller, P. Extracellular Cu2+ pools and their detection: From current knowledge to next-generation probes. *Coord. Chem. Rev.* **2021**, *433*, 213727. [CrossRef]
17. Zagurskaya-Sharaevskaya, O.; Povar, I. Determination of Cu (II) ions using sodium salt of 4-phenylsemicarbazone 1,2-naphthoquinone-4-sulfonic acid in natural and industrial environments. *Ecol. Process.* **2015**, *4*, 1–5. [CrossRef]
18. Alford, R.; Simpson, H.M.; Duberman, J.; Hill, G.C.; Ogawa, M.; Regino, C.; Kobayashi, H.; Choyke, P.L. Toxicity of organic fluorophores used in molecular imaging: Literature review. *Mol. Imaging* **2009**, *8*, 341–354. [CrossRef]
19. Borrill, A.J.; Reily, N.E.; Macpherson, J.V. Addressing the practicalities of anodic stripping voltammetry for heavy metal detection: A tutorial review. *Analyst* **2019**, *144*, 6834–6849. [CrossRef]
20. Celante, V.G.; Freitas, M.B.J.G. Electrodeposition of copper from spent Li-ion batteries by electrochemical quartz crystal microbalance and impedance spectroscopy techniques. *J. Appl. Electrochem.* **2010**, *40*, 233–239. [CrossRef]
21. Rosati, G.; Scaramuzza, M.; Rotilio, V.; Monaco, L.; Pasqualotto, E.; Campolo, F.; De Toni, A.; Reggiani, C.; Naro, F.; Paccagnella, A. Culture Mediums and Buffer Effect on Screen-printed Carbon Electrodes for Continuous Voltammetric Monitoring of in vitro Cell Cultures Lactate Production. *Procedia Technol.* **2017**, *27*, 246–247. [CrossRef]
22. Sadok, I.; Tyszczuk-Rotko, K.; Mroczka, R.; Staniszewska, M. Simultaneous voltammetric analysis of tryptophan and kynurenine in culture medium from human cancer cells. *Talanta* **2020**, *209*, 120574. [CrossRef] [PubMed]
23. Orság, P.; Havran, L.; Fojt, L.; Coufal, J.; Brázda, V.; Fojta, M. Voltammetric behavior of a candidate anticancer drug roscovitine at carbon electrodes in aqueous buffers and a cell culture medium. *Monatshefte Fur Chem.* **2019**, *150*, 461–467. [CrossRef]
24. Tang, Y.; Petropoulos, K.; Kurth, F.; Gao, H.; Migliorelli, D.; Guenat, O.; Generelli, S. Screen-Printed Glucose Sensors Modified with Cellulose Nanocrystals (CNCs) for Cell Culture Monitoring. *Biosensors* **2020**, *10*, 125. [CrossRef] [PubMed]
25. Romih, T.; Hočevar, S.B.; Kononenko, V.; Drobne, D. The application of bismuth film electrode for measuring Zn(II) under less acidic conditions in the presence of cell culture medium and ZnO nanoparticles. *Sens. Actuators B Chem.* **2017**, *238*, 1277–1282. [CrossRef]
26. Baldisserri, C.; Costa, A.L. Electrochemical detection of copper ions leached from CuO nanoparticles in saline buffers and biological media using a gold wire working electrode. *J. Nanopart. Res.* **2016**, *18*, 96. [CrossRef]
27. Biscaglia, F.; Caroppo, A.; Prontera, C.T.; Sciurti, E.; Signore, M.A.; Kuznetsova, I.; Leone, A.; Siciliano, P.; Francioso, L. A Comparison between Different Machine Learning Approaches Combined with Anodic Stripping Voltammetry for Copper Ions and pH Detection in Cell Culture Media. *Chemosensors* **2023**, *11*, 61. [CrossRef]
28. Available online: https://www.microfluidic-chipshop.com/catalogue/microfluidic-chips/polymer-chips/membrane-chips/membrane-chips-fluidic-653/ (accessed on 3 May 2023).
29. Chen, Z.; Wang, Y.; Zhou, S. Numerical Analysis of Mixing Performance in an Electroosmotic Micromixer with Cosine Channel Walls. *Micromachines* **2022**, *13*, 1933. [CrossRef] [PubMed]
30. Murphy, J.M.; Powell, B.A.; Brumaghim, J.L. Stability constants of bio-relevant, redox-active metals with amino acids: The challenges of weakly binding ligands. *Coord. Chem. Rev.* **2020**, *412*, 213253. [CrossRef]
31. Hakimi, M.; Hakimi Tahereh Saberi Aliabadi, M. Coordination Chemistry of Copper α-Amino Acid Complexes. *World Appl. Program.* **2012**, *2*, 431–443.
32. Cerchiaro, G.; Sant'Ana, A.C.; Arruda Temperini, M.L.; Da Costa Ferreira, A.M. Investigations of different carbohydrate anomers in copper(II) complexes with D-glucose, D-fructose, and D-galactose by Raman and EPR spectroscopy. *Carbohydr. Res.* **2005**, *340*, 2352–2359. [CrossRef]
33. Hamed, E.; Attia, M.S.; Bassiouny, K. Synthesis, spectroscopic and thermal characterization of copper(II) and iron(III) complexes of folic acid and their absorption efficiency in the blood. *Bioinorg. Chem. Appl.* **2009**, *2009*, 979680. [CrossRef]
34. Fahrni, C.J. Synthetic fluorescent probes for monovalent copper. *Curr. Opin. Chem. Biol.* **2013**, *17*, 656–662. [CrossRef]
35. Gabryel-Skrodzka, M.; Nowak, M.; Stachowiak, K.; Zabiszak, M.; Ogawa, K.; Jastrzab, R. The Influence of pH on Complexation Process of Copper(II) Phosphoethanolamine to Pyrimidine Nucleosides. *Materials* **2021**, *14*, 4309. [CrossRef]
36. Hu, X.; Pan, D.; Han, H.; Wang, C. Anodic stripping voltammetric analysis of different species of copper in seawater using an electrochemical sensor. *J. Oceanogr.* **2019**, *75*, 149–156. [CrossRef]

37. Pain, A.J.; Martin, J.B.; Young, C.R.; Huang, L.; Valle-Levinson, A. Organic matter quantity and quality across salinity gradients in conduit- vs. diffuse flow-dominated subterranean estuaries. *Limnol. Oceanogr.* **2019**, *64*, 1386–1402. [CrossRef]
38. Tan, H.-Y.; Trier, S.; Rahbek, U.L.; Dufva, M.; Kutter, J.P.; Andresen, T.L. A multi-chamber microfluidic intestinal barrier model using Caco-2 cells for drug transport studies. *PLoS ONE* **2018**, *13*, e0197101. [CrossRef]
39. Natoli, M.; Leoni, B.D.; D'Agnano, I.; Zucco, F.; Felsani, A. Good Caco-2 cell culture practices. *Toxicol. Vitr.* **2012**, *26*, 1243–1246. [CrossRef] [PubMed]
40. Sciurti, E.; Blasi, L.; Prontera, C.T.; Barca, A.; Giampetruzzi, L.; Verri, T.; Siciliano, P.A.; Francioso, L. TEER and Ion Selective Transwell-Integrated Sensors System for Caco-2 Cell Model. *Micromachines* **2023**, *14*, 496. [CrossRef]
41. Poon, C. Measuring the density and viscosity of culture media for optimized computational fluid dynamics analysis of in vitro devices. *J. Mech. Behav. Biomed. Mater.* **2022**, *126*, 105024. [CrossRef] [PubMed]
42. Zdovc, B.; Jaklin, M.; Hribar-Lee, B.; Lukšič, M. Influence of Low Molecular Weight Salts on the Viscosity of Aqueous-Buffer Bovine Serum Albumin Solutions. *Molecules* **2022**, *27*, 999. [CrossRef]
43. Suzuki, H.; Hiratsuka, A.; Sasaki, S.; Karube, I. Problems associated with the thin-film Ag/AgCl reference electrode and a novel structure with improved durability. *Sens. Actuators B Chem.* **1998**, *46*, 104–113. [CrossRef]