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Real-time xCELLigence impedance analysis of the cytotoxicity of dental composite components on human gingival fibroblasts

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ABSTRACT

Objectives. Aim of this study was by continuous monitoring to assay the proliferative capacity of human gingival fibroblasts (HGFs), to investigate cytotoxicity of the most common monomers/comonomers in dental resin composites: bisphenol-A-glycidylmethacrylate (BisGMA), hydroxyethylenemethacrylate (HEMA), triethyleneglycoldimethacrylate (TEGDMA), and urethanedimethacrylate (UDMA) in HGFs during 24 h exposure using the xCELLigence system.

Methods. xCELLigence cell index (CI) impedance measurements were performed according to the instructions of the supplier. HGFs were resuspended in medium and subsequently adjusted to 400,000, 200,000, 100,000, and 50,000 cells/mL. After seeding 100 μL of the cell suspensions into the wells of the E-plate 96, HGFs were monitored every 15 min for a period of up to 18 h by the xCELLigence system.

Results. Half maximum effect concentrations (EC_{50}) were determined based on the dose–response curves derived by xCELLigence measurements. Following real-time analysis, significantly increased EC_{50} values of HGFs exposed for 24 h to the following substances were obtained: HEMA[a], TEGDMA[b], UDMA[c]. The EC_{50} values (mean [mmol/L] ± S.E.M.; $n = 5$) were: HEMA 11.20 ± 0.3, TEGDMA[a] 3.61 ± 0.2, UDMA[a,b] 0.20 ± 0.1, and BisGMA[a,b,c] 0.08 ± 0.1. These results are similar to the EC_{50} values previously observed with the XTT end-point assay.

Significance. Our data suggests that the xCELLigence live cell analysis system offers dynamic *live cell* monitoring and combines high data acquisition rates with ease of handling. Therefore, the xCELLigence system can be used as a rapid monitoring tool for cellular viability and be applied in toxicity testing of xenobiotics using *in vitro* cell cultures.

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1. Introduction

Amalgam has been replaced in increasing rates by dental resin composites that are tooth-colored materials most commonly used to restore dental damage in the permanent dentition [1]. Dental resin composites consist of an organic resin matrix with embedded organic particles. Besides direct filling materials, resins are also used as bonding resins, e.g., dentin adhesives and cements and as luting agents for crowns, inlays and orthodontic brackets [2]. The common components of both resin and bonding components are the monomers/comonomers: bisphenol-A-glycidylmethacrylate (Bis-GMA), hydroxyethylene methacrylate (HEMA), triethyleneglycoldimethacrylate (TEGDMA), and urethanedimethacrylate (UDMA). Previous studies have described that unpolymerized monomers/comonomers can be released from resin composites into the oral cavity [3–5] during implantation and even after polymerization [6,7]. Leaching compounds can, after dilution by the saliva, enter the intestine [8,9] where, after uptake and metabolization they can form toxic and radical intermediates [10–12].

HEMA and TEGDMA are the main comonomers released from resin-based materials [13,14]. In previous animal experiments the uptake, distribution, metabolism, and excretion of HEMA and TEGDMA were investigated [10]. *In vitro* studies revealed mutagenic, teratogenic, genotoxic and estrogenic effects of composite components [15–17]. Numerous cytotoxic responses to dental composite resins and their components have been described [18–22,10]. It has been demonstrated that UDMA and TEGDMA were more cytotoxic than HEMA to human gingival fibroblasts (HGFs) [23,24]. A significant increase in relative toxicity of the monomers/comonomers was found in the XTT-test in the following order: BisGMA > UDMA > TEGDMA > HEMA [25].

In the earlier studies several methods and techniques were used to investigate the cytotoxicity of dental resin materials, e.g., lactate dehydrogenase (LDH) assay [2], 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzol-disulfonate (WST-1) assay [26], sodium 3′[1-phenyl-aminocarbonyl]-3,4-tetrazolium bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate assay [25], 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay [17], bromodeoxiuridine (BrdU) assay [2], and fluorescence microscopy [25]. All these methods, which are designed for the analysis of cell proliferation, viability and cytotoxicity, are single end-point qualitative measures of cell fitness. The established assays are labor intensive and comprise a number of manipulation steps that potentially can induce variation of the end-points. In addition there is a great tendency for compound interference because of the optics-based detection methods for most assays, such as absorbance, luminescence or fluorescence, which are vulnerable to distortions. In this sense there is an important requirement for the competency of quantitative monitoring cell biological parameters in real-time in *in vitro* cell culture. Hence, an automated assay that combines high reproducibility with respect to *in vitro* cell proliferation and viability with easy manipulation is much appreciated.

Recently, Roche Applied Science and ACEA Biosciences conjointly launched the Real-Time Cell Analyzer Single Plate (RTCA SP®) system under the xCELLigence™ name, which follows the predecessor impedance-based Real-Time Cell Electronic Sensing (RT-CES®) system. The RT-CES system has been previously described in detail [27–29].

Real-time and continuous monitoring allows label-free assessment of cell proliferation, viability and cytotoxicity, revealing the physiological state of the cells and at the same time saves expensive reagents used in conventional cell analysis. In the xCELLigence system, the kinetic control of cellular status during entire experiment runs reveals continuous information about cell growth, morphological changes and cell death. Furthermore, the xCELLigence system allows for the calculation of time-dependent physiological EC_{50} values, which can be more informative than single EC_{50} end-points of classical toxicity testing.

In our present experiments, we conducted experiments with the new xCELLigence system that investigated the cytotoxicity of the dental composite compounds: BisGMA, HEMA, TEGDMA and UDMA on HGFs by real-time and continuous monitoring of the cell growth, proliferation and viability.

2. Materials and methods

2.1. Chemicals

The monomers/comonomers triethyleneglycoldimethacrylate (TEGDMA; CAS-No. 109-16-0), bisphenol-A-glycidylmethacrylate (BisGMA; CAS-No. 1565-94-2), hydroxyethylenemethacrylate (HEMA; CAS-No. 868-77-9), and urethanedimethacrylate (UDMA; CAS-No. 72869-86-4) were obtained from Evonik Röhm (Essen, Germany).

HEMA and TEGDMA were directly dissolved in medium. BisGMA and UDMA were dissolved in dimethyl sulfoxide (DMSO, 99% purity, Merck, Darmstadt, Germany) and diluted with medium (final DMSO concentration: 0.20%). Control experiments contained DMSO (0.20%) in medium only.

2.2. Cell culture

The human gingival fibroblast (HGF) cultures used in this study were produced by Provitro on the base of human tissues and obtained from Cell-Lining, Berlin, Germany, Cat-No.: 1210412. The HGFs (passage 9) were grown on 175 cm^2 cell culture flasks to approximately 75–85% confluence and maintained in an incubator with 5% CO_2 atmosphere at 100% humidity and 37 °C. Quantum 333 medium supplemented with L-glutamine and 1% antibiotic/antimycotic solution (10,000 Units/mL penicillin, 25 mg/mL streptomycin sulfate, 25 μg/mL amphotericin B) was used in the experiments. After reaching confluence the cells were washed with Dulbecco's phosphate buffered saline (PBS), detached from the flasks by a brief treatment with trypsin/EDTA. Quantum 333, antibiotic–antibiotic solution, PBS and trypsin/EDTA were purchased from PAA Laboratories GmbH, Cölbe, Germany.

2.3. Instrumentation

2.3.1. xCELLigence system

The xCELLigence system was used according to the instructions of the supplier (Roche Applied Science and ACEA Biosciences) [30]. The xCELLigence system consists of four main components: the RTCA analyzer, the RTCA SP station, the RTCA computer with integrated software, and disposable E-plate 96. The RTCA SP station fits inside a standard tissue-culture incubator, while an analyzer and laptop computer with software will be on the outside. The core of the xCELLigence system is the E-plate 96: this is a single use, disposable device used for performing cell-based assays on the RTCA SP instrument, which has similar application like commonly used 96-well microtiter plate. However the E-plate 96 differs from standard 96-well microtiter plates vastly with its incorporated gold cell sensor arrays in the bottom, which contributes cells inside each well to be monitored and assayed. The E-plate 96 has a low evaporation lid design [30]: the bottom diameter of each well is 5.0 mm ± 0.05 mm; with a total volume of 243 ± 5 μL, approximately 80% of the bottom areas of each well is covered by the circle-on-line-electrodes, which is designed to be used in an environment of +15 to +40 °C, relative humidity 98% maximum without condensation [30].

The electronic impedance of sensor electrodes is measured to allow monitoring and detection of physiological changes of the cells on the electrodes. The voltage applied to the electrodes during RTCA measurement is about 20 mV (RMS) [30]. The impedance measured between electrodes in an individual well depends on electrode geometry, ion concentration in the well and whether or not cells are attached to the electrodes [30]. In the absence of cells, electrode impedance is mainly determined by the ion environment both at the electrode/solution interface and in the bulk solution. In the presence of cells, cells attached to the electrode sensor surfaces will act as insulators and thereby alter the local ion environment at the electrode/solution interface, leading to an increase in impedance [30]. Thus, the more cells that are growing on the electrodes, the larger the value of electrode impedance. The RTCA associated software allows users to obtain parameters such as: average value, maximum and minimum values, standard deviation (SD), half maximum effect of concentration (EC_{50}), half maximum inhibition of concentration (IC_{50}), cell index (CI), and in addition graphics. The data expressed in CI units can be exported to Excel for any type of mathematical analysis [30].

2.3.2. Derivation of cell index (CI)

An unit-less parameter termed cell index (CI) is derived to represent cell status based on the measured relative change in electrical impedance that occurs in the presence and absence of cells in the wells [27], which is calculated based on the following formula: $CI = (Z_i - Z_0)/15$, where Z_i is the impedance at an individual point of time during the experiment and Z_0 is the impedance at the start of the experiment [30]. Impedance is measured at 3 different frequencies (10, 25 or 50 kHz) and a specific time [30]. Impedance change can occur depending on mainly two factors [28]:

(1) *The number of cells attached to the electrodes*: When there are no cells on an electrode surface, the sensor's electronic feature will not be affected and the impedance change will be 0 (Fig. 1A). Attaching of one cell onto the electrodes, this value will be 1 (Fig. 1B). When more cells attach onto the electrodes, the value will further increase (Fig. 1C). All the factors that increase the number of attached cells on the electrodes, e.g., attachment from solution or cell proliferation leads to a higher CI value. However cell death or toxicity induces cell-detachment, which will lead to a decreased CI value.
(2) *The dimensional change of the attached cells on the electrodes*: Despite the same cell numbers, dimensional changes of the attached cells on the electrodes will lead to change the CI, e.g., an increase in cell adhesion or cell spread will lead to a higher CI value (Fig. 1D) [28]. Toxicity can induce cells to spread or cluster thereby leading to a larger cell surface/sensor contact, which in turn can increase the CI value [28]. On the other hand, toxic compounds can induce cells to round up and/or to detach leading to a decrease in CI [28].

2.4. Cell growth and proliferation assay using xCELLigence system

HGF cells were grown and expanded in tissue-culture flasks. After reaching ~75% confluence, the HGFs (passage 9) were washed with PBS, afterwards detached from the flasks by a brief treatment with trypsin/EDTA. Subsequently, 50 μL of cell culture media at room temperature was added into each well of E-plate 96. After this the E-plate 96 was connected to the system and checked in the cell culture incubator for proper electrical-contacts and the background impedance was measured during 24 s. Meanwhile, the cells were resuspended in cell culture medium and adjusted to 400,000, 200,000, 100,000, and 50,000 cells/mL. 100 μL of each cell suspension was added to the 50 μL medium containing wells on E-plate 96, in order to determine the optimum cell concentration. After 30 min incubation at room temperature, E-plate 96 was placed into the cell culture incubator. Finally, adhesion, growth and proliferation of the cells was monitored every 15 min for a period of up to 18 h via the incorporated sensor electrode arrays of the E-Plate 96. The electrical impedance was measured by the RTCA-integrated software of the xCELLigence system as a dimensionless parameter termed CI.

2.5. Cytotoxicity assay using xCELLigence system

First, the optimal seeding concentration for proliferation experiments of the HGFs was determined. After seeding the respective number of cells in 100 μL medium to each well of the E-plate 96, the proliferation, attachment and spreading of the cells was monitored every 15 min by the xCELLigence system. Approximately 18 h after seeding, when the cells were in the log growth phase, the cells were exposed to 50 μL of medium containing the following substances: BisGMA (0.01, 0.3, 1, 30 mM), HEMA (0.01, 0.3, 1, 30 mM), TEGDMA (0.03, 0.1, 3, 10 mM), and UDMA (0.001, 0.01, 0.1, 1 mM). Controls received either medium only, or medium + DMSO with a final concentration of 0.20%. All experiments were run for 24 h.

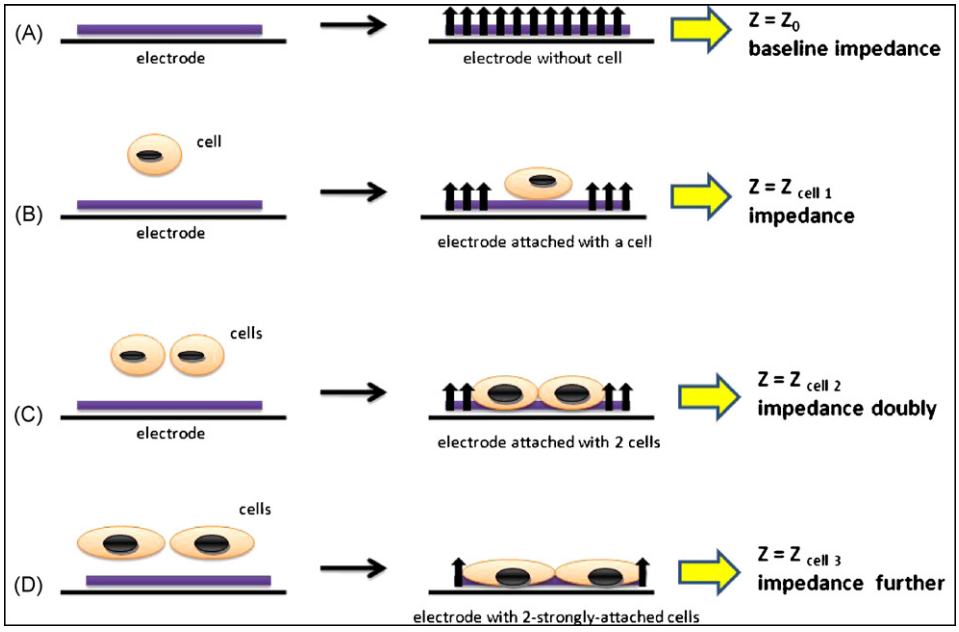

Fig. 1 – Scheme of impedance measurement.
***Baseline impedance*: There are no cells on an electrode surface (A).**
***Impedance*: A cell labels to the electrode surface and blocks partially the electrical current in the circuit, inducing an increase in the electrode impedance (B).**
***Impedance doubly*: Two cell labels to the electrode surface and reduce even further the electrical current, as compared with B inducing to doubly increased impedance (C).**
***Impedance further*: Two cell labels to the electrode surface with more extension, which induce much more impedance in comparison with C (D).**

2.6. Statistics

All calculations were obtained using the RTCA-integrated software of the xCELLigence system. The RTCA software performs a curve-fitting of selected "sigmoidal dose–response equation" to the experimental data points and calculates logarithmic half maximum effect of concentration (log [EC_{50}]) values at a given time point based on log of concentration producing 50% reduction of CI value relative to solvent control CI value (100%), expresses as log EC_{50} (M/L), which was converted into EC_{50} (mmol/L) in our results. Data are represented as mean [mmol/L] ± S.E.M. ($n = 5$).

The statistical significance ($p < 0.05$) of the differences between the experimental groups was checked using the *t*-test, corrected according to Bonferroni-Holm-modification preferred by Forst et al. [31].

3. Results

3.1. Monitoring dynamic cell proliferation and attachment in real-time using xCELLigence system

First, we determined the optimal concentration for cell proliferation and viability measurements. To this end 40,000, 20,000, 10,000, and 5000 cells/well were seeded in the E-Plate 96 and the impedance determined. While the impedance CI of 20,000, 10,000, and 5000 cells/well increased proportionally to cell number, the CI of 40,000 cells/well did not match to this correlation (Table 1 and Fig. 3).

As shown in Fig. 2 the CI of each cell concentration sharply increased after seeding up to reach its maximum at 2.5 h. Thereafter the CI of 10,000 and 5000 cells/well slowly decreased to reach a minimum at 10 h to increase again to a maximum at 18 h. The CI of 20,000 cells/well showed a minimum at 6 h and reached its second maximum at 18 h. In contrast, the CI of 40,000 cells/well made a sharp increase during the first 2 h to reach a plateau value after 7 h, where it remained thereafter. In all, we conclude that the response

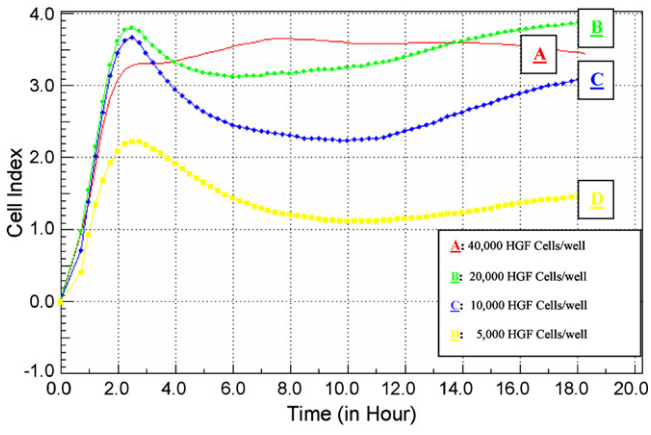

Fig. 2 – Dynamic monitoring of cell adhesion and proliferation using the xCELLigence system. HGFs at a density of 40,000, 20,000, 10,000, 5000 cells/well per well in E-Plates 96 were observed during 18 h.

Table 1 – The correlation of CI, which was measured as a dimensionless unit due to the relative change in electrical impedance, and cell numbers using the xCELLigence system.

Cell numbers (cells/well)	40,000	20,000	10,000	5000
Cell index	3.5 (0.07)	3.9 (0.1)	3.1 (0.1)	1.5 (0.1)

The cell index was calculated from repeated experiments ($n = 5$) with the xCELLigence system. Data are presented as means (mmol/L) ± S.E.M. in parentheses.

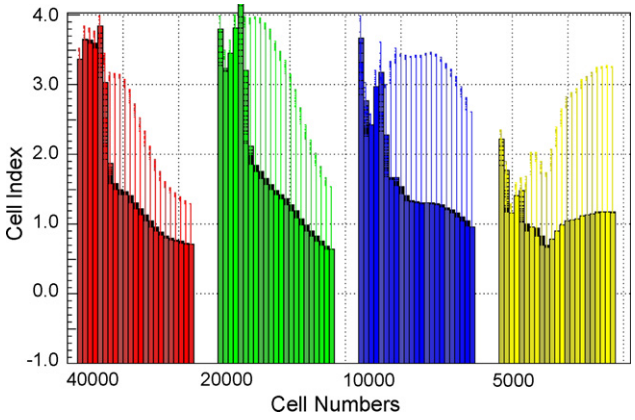

Fig. 3 – Dynamic monitoring of the correlation between cell index and cell number using the xCELLigence system. The cell index was calculated from five repeated experiments and data.

seen in the 5000–20,000 cells/well experiments reflects cell cycle effects, while the concentration of 40,000 cells/well was not suited for further experimentation, possibly because of a too high cell density and the resulting contact inhibition.

3.2. *Monitoring of cytotoxicity in real-time using xCELLigence system*

Next, we used the 20,000 cells/well concentration in the xCELLigence assay to examine the toxic effects elicited by the tooth-filling compound monomers/comonomers, as the 20,000 cells/well concentration displayed the lowest variation (Fig. 3). By repeated xCELLigence measurements (5 experiments/substance) of drug-treated HGFs fitted dose–response CI curves were obtained (Fig. 4) in which the highest EC_{50} value was found for HEMA during 24 h of exposure (EC_{50} 11.20 ± 0.3; mean [mmol/L] ± S.E.M.; $n = 5$; [a,b,c] significantly different to HEMA). This was significantly different to TEGDMA[a] (3.61 ± 0.2), UDMA[a,b] (0.20 ± 0.1), and BisGMA[a,b,c] (0.08 ± 0.1). BisGMA was significantly ($p < 0.05$) higher effective compared to UDMA, TEGDMA, and HEMA (Table 2).

A comparison of the above values with those EC_{50} values obtained previously with the end-point XTT assay [25] revealed a close match between the xCELLigence and XTT data (Table 2).

4. Discussion

The aim of this study was to investigate the RTCA SP device of the xCELLigence system as a test platform for both dynamic monitoring of cell proliferation of HGFs during 18 h, and the investigation of the cytotoxicity of the monomers/comonomers: BisGMA, HEMA, TEGDMA, and UDMA during 24 h. For evaluation, we compared this experiment with the one of the commonly used XTT-based viability end-point assay, which previously proofed informative with the tested chemicals [25]. In the human physiological situation gingival fibroblasts are highly exposed to monomers/comonomers after release from composites in the oral cavity [32]. Therefore, in this study human gingival fibroblasts were used.

As shown in Table 1 the CIs of the wells with 20,000, 10,000, and 5000 HGF cells/well correlated with the cell number. The CI increases depending on the number of attached cells on the electrodes (Fig. 1), but the CI of the highest cell concentration (40,000 cells/well) failed to display changes during the following incubation time, suggesting a satura-

Table 2 – EC_{50} values (mmol/L) and relative toxicities of HEMA, TEGDMA, UDMA and BisGMA derived from the presented xCELLigence system and from previously described XTT assay [25].

Substance	xCELLigence		XTT [25]	
	EC_{50} (mmol/L)	Relative toxicity	EC_{50} (mmol/L)	Relative toxicity
HEMA	11.20 (0.30)	1	11.53 (0.60)	1
TEGDMA	3.61 (0.20)[a]	3	3.46 (0.20)[a]	3
UDMA	0.20 (0.01)[a,b]	56	0.10 (0.05)[a,b]	109
BisGMA	0.08 (0.01)[a,b,c]	140	0.09 (0.01)[a,b,c]	133

The half maximum effect concentrations (EC_{50}) of monomers/comonomers were obtained based on the dose–response curves of cell index during 24 h exposure in HGFs and calculated from repeated experiments ($n = 5$) with the real-time xCELLigence system. The EC_{50} values of monomers/comonomers in HGFs with the end-point XTT assay were published previously [25]. Data are presented as means (mmol/L) ± S.E.M. in parentheses.

[a] Significantly ($p < 0.05$) different to HEMA.
[b] Significantly ($p < 0.05$) different to TEGDMA.
[c] Significantly ($p < 0.05$) different to UDMA.

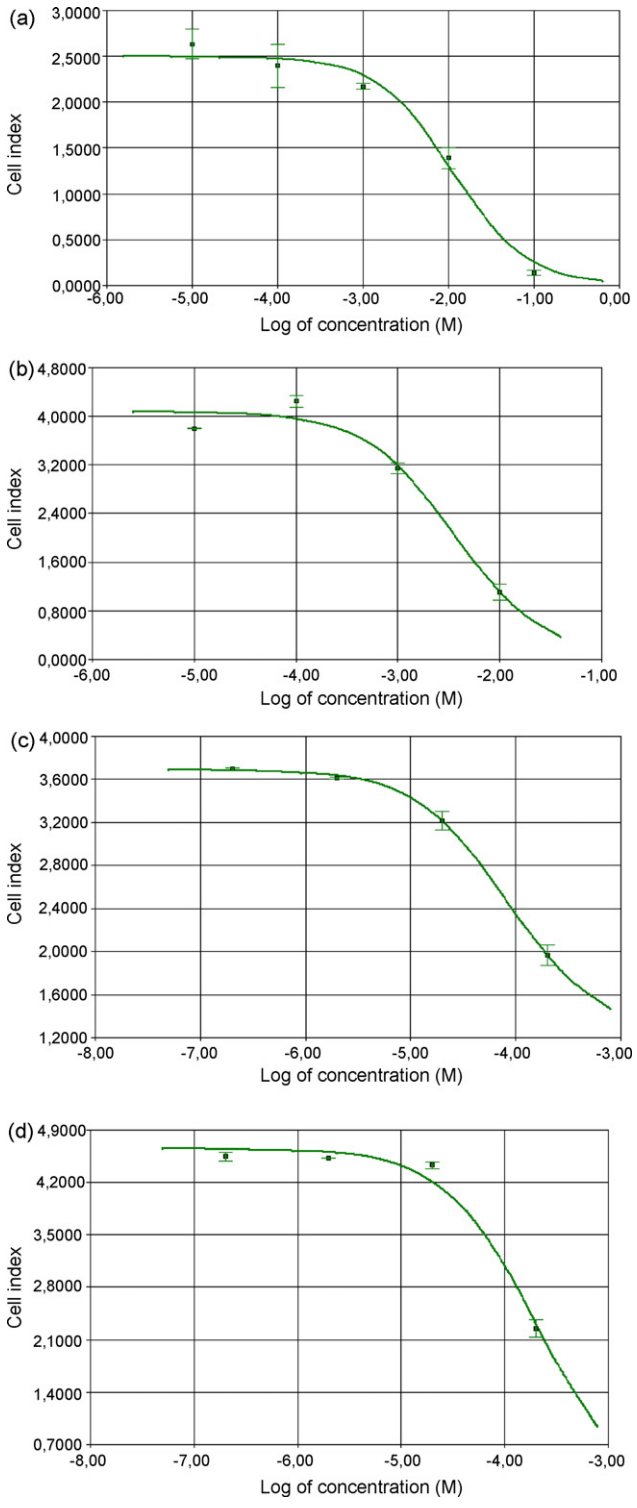

Fig. 4 – Effect of HEMA (a), TEGDMA (b), BisGMA (c) and UDMA (d) during 24 h exposure on the viability of HGFs was measured based on the dose–response curves of the cell index by the xCELLigence system. Data points represent mean values ± S.E.M. ($n = 5$).

tion effect likely by contact inhibition of the cell cycle by densely attaching cells. As shown in Fig. 2 the steep increase of the CI of each cell concentration of the HGFs up to 2.5 h may be characterized by its similar adhesion as well as the time. After 2.5 h in the wells HGFs with 20,000, 10,000, and 5000 cells/well entered a lag phase up to 6 h, in which they likely fully spread but were not actively proliferating. While HGFs with 20,000 cells/well assumed growth after 6 h, the cells with 10,000 and 5000 cells/well remained in the lag phase up to 11 h, and only thereafter entered the growth phase. In contrast, the HGFs with 40,000 cells/well never entered growth phase. This early confluence response of the 40,000 cells/well can be explained due to contact inhibition among the HGFs in the E-Plate 96. Hence, this cell concentration was excluded for further experiments. Interestingly, cell concentrations of 20,000 and 10,000 cells/well displayed similar growth phases as the 5000 cells/well between 10 and 18 h. The slowed growth of the 5000 cells/well could be the reason of reduced cell number, which likely will delay the optimum saturation period. Our data suggest that 10,000 and 20,000 cells/well are the optimum concentrations for a dynamic monitoring of cell proliferation by the xCELLigence system, and can be applied as a conventional end-point in *in vitro* assays.

Compared to conventional end-point cell-based assays, dynamic monitoring of cell response, such as cell adhesion, spreading, proliferation, and also cell death is one of the advantages of the xCELLigence system to optimize the cell concentration for *in vitro* assays and also allows both cell and assay conditions to be constantly obtained before and during the time of the experimentation. Further, the response of live cells to an, e.g., chemical exposure can be monitored in real-time, which is impossible to achieve by the currently established end-point assays, e.g., the XTT-based viability assay [25].

4.1. *The xCELLigence assay in toxicology testing of dental composite compounds*

Our toxicology testing of tooth-filling resin components revealed EC_{50} values for HEMA, TEGDMA, UDMA, and BisGMA that are comparable to the end-point XTT-based viability assay data [25], demonstrating that the new xCELLigence system is in real-time, a dynamic assay format for live cell cytotoxicity assessment.

Additionally, the voltage applied to the electrodes during the impedance measurement on the RTCA station of the xCELLigence system is about 20 mV (RMS). This value was demonstrated in the previous experiments as a low electric field, which would not affect the physiological cell status during the assay [33–35].

The main advantages of the xCELLigence system compared to the other market instruments with impedance technology are once design of the circle-on-line-electrodes, and requirement of fewer frequencies, which can allow a better sensitivity during the measurement. Further, the 80% electrode coverage area in the wells of the xCELLigence system enables a better reproducibility, whereas the other market instruments include much smaller electrode area with less effectiveness and higher variability [33].

Furthermore, the integrated software of the xCELLigence system allows users to obtain parameters such as: average value, SD, EC_{50} and IC_{50}. Since these values are recorded and expressed in CI units, it can be both analyzed on the xCELLigence system and exported to Excel for any type of mathematical analysis, or graphics, facilitating the scientific analysis of the obtained data.

Moreover, the xCELLigence system allows a label-free assessment of the cell viability as well as the cell death. While labeling reagent and electron-coupling reagent were added for the measurement of cell viability and death by the end-point XTT-based viability assay, the real-time xCELLigence system illustrates this process without any stain or label [33,36].

In comparison to the predecessor instrument RT-CES [27–29,33] the xCELLigence system has several important improvements providing the xCELLigence system with better electrical measurements due to gold incorporated circle-on-line-electrodes, and also it is much more user-friendly respecting its new graphic user interface and mobile format. Our results thus demonstrate that in cytotoxicity assays, the xCELLigence system can be used to optimize parameters such as cell number, period of the exposure, and compound concentrations. Once these parameters are optimized using the xCELLigence system, they can be applied in toxicology testing. Finally, dynamic monitoring with the xCELLigence system allows for the calculation of optimized EC_{50} values in real-time.

5. Conclusions

In conclusion, the xCELLigence system with dynamic monitoring can be used as a rapid diagnostic tool both to analyze cellular behavior and to explore the effect of compounds in cell-based *in vitro* assays.

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