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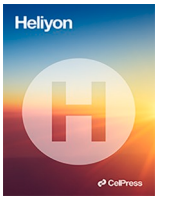

Review article

Label-free biomolecular and cellular methods in small molecule epigallocatechin-gallate research

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ABSTRACT

Small molecule natural compounds are gaining popularity in biomedicine due to their easy access to wide structural diversity and their proven health benefits in several case studies. Affinity measurements of small molecules below 100 Da molecular weight in a label-free and automatized manner using small amounts of samples have now become a possibility and reviewed in the present work. We also highlight novel label-free setups with excellent time resolution, which is important for kinetic measurements of biomolecules and living cells. We summarize how molecular-scale affinity data can be obtained from the in-depth analysis of cellular kinetic signals. Unlike traditional measurements, label-free biosensors have made such measurements possible, even without the isolation of specific cellular receptors of interest. Throughout this review, we consider epigallocatechin gallate (EGCG) as an exemplary compound. EGCG, a catechin found in green tea, is a well-established anti-inflammatory and anti-cancer agent. It has undergone extensive examination in numerous studies, which typically rely on fluorescent-based methods to explore its effects on both healthy and tumor cells. The summarized research topics range from molecular interactions with proteins and biological films to the kinetics of cellular adhesion and movement on novel biomimetic interfaces in the presence of EGCG. While the direct impact of small molecules on living cells and biomolecules is relatively well investigated in the literature using traditional biological measurements, this review also highlights the indirect influence of these molecules on the cells by modifying their nano-environment. Moreover, we underscore the significance of novel high-throughput label-free techniques in small molecular measurements, facilitating the investigation of both molecular-scale interactions and cellular processes in one single experiment. This advancement opens the door to exploring more complex multicomponent models that were previously beyond the reach of traditional assays.

1. Introduction

The need for labeling arises from the limited sensitivity of conventional methods, which struggle to detect biomolecules due to their light elemental composition, small mass, and lack of fluorescence [1]. Techniques such as those based on electrons, X-rays, visible light, and mass spectrometry, which have been effective in physics and chemistry, are much less powerful in biology. Attaching a biomolecule to an artificial entity with a much greater electron density, optical polarizability, or mass, the molecule becomes “visible”

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to the technique. However, the excitement surrounding this sudden detectability can sometimes overshadow the possibility that the labeling process may interfere with the biological effect under investigation [2].

New technologies that are sensitive enough to detect biomolecules without labeling them have opened up new possibilities for label-free detection [3–8]. This is particularly useful for natural compounds, which often have small molecular weights and binding pockets, making labeling difficult or impossible [1]. Label-free biosensors are more and more important in investigating the mode of action of bioactive molecules (even with low molecular weight), and they offer advantages over classical techniques that require labels or dyes, which can disturb the samples. These methods are generally straightforward, cost-effective, and rapid. Moreover, the diversity of natural products is still essential for drug discovery [9,10]. In the research of active compounds found in traditional (Chinese) medicines, novel label-free methods can be applied to investigate the effects of these compounds on cell behavior without the use of dyes or labels [9,11–14]. These methods help to study the biological properties of small molecules, investigate various biological coatings and adhesion assays, and motility tests can be performed in a high-throughput format [9]. These label-free methods, especially optical waveguide-based, highly sensitive biophysical tools [11,14,15], have great potential in drug discovery as they provide a comprehensive view of receptor-ligand binding in living cells. The emergence of these label-free assays has made them increasingly popular in research and development areas focusing on the biological roles of drug candidates [9].

Label-free biosensors help measure various cellular processes (such as adhesion, spreading, proliferation, differentiation, migration, receptor–ligand binding, signal transduction analysis, and cytotoxicity) and investigate interaction kinetics [16]. Detection capacity used to be considered a limitation of label-free detection [17], but fortunately, recent developments have significantly improved these capabilities [16]. Quartz crystal microbalance (QCM) [18–20], cellular dielectric spectroscopy (CDS) [21,22], surface plasmon resonance (SPR) [23,24], and optical waveguide lightmode spectroscopy (OWLS) [25], usually apply a low number of sensing elements, but novel ones are having high-throughput [16]. These biosensors can detect biomolecules as small as 1–200 Da, meaning typically below 5 pg/mm^2 surface adsorbed mass changes, and can produce up to 460,000 data points/hour [16]. Examples of such biosensors include electric cell–substrate impedance sensing (ECIS) [26], photonic crystal-based sensors [27,28], and resonance waveguide grating (RWG) [15,16,29,30]. Optical waveguide-based sensors can also detect dielectric or nanoparticles and self-assembled layers [16,31,32]. Label-free, surface-sensitive methods are beneficial for monitoring the assembly kinetics and layer structure of nanoscale thin films [33]. These biosensors are crucial in biomedicine, analytical chemistry, and maritime industries.

The waveguide-based methods are considered to be innovative phenotypic assays that offer great promise for drug discovery [11,12,14,34,35]. This is due to their ability to reveal the intricate nature of drug actions and interactions and provide a comprehensive understanding of receptor-ligand interactions in live cells. Waveguide-based sensors utilize the so-called evanescent field generated, which is then influenced by the surface-adhered living cells, proteins, and drugs. A layer of material, which could be a phospholipid

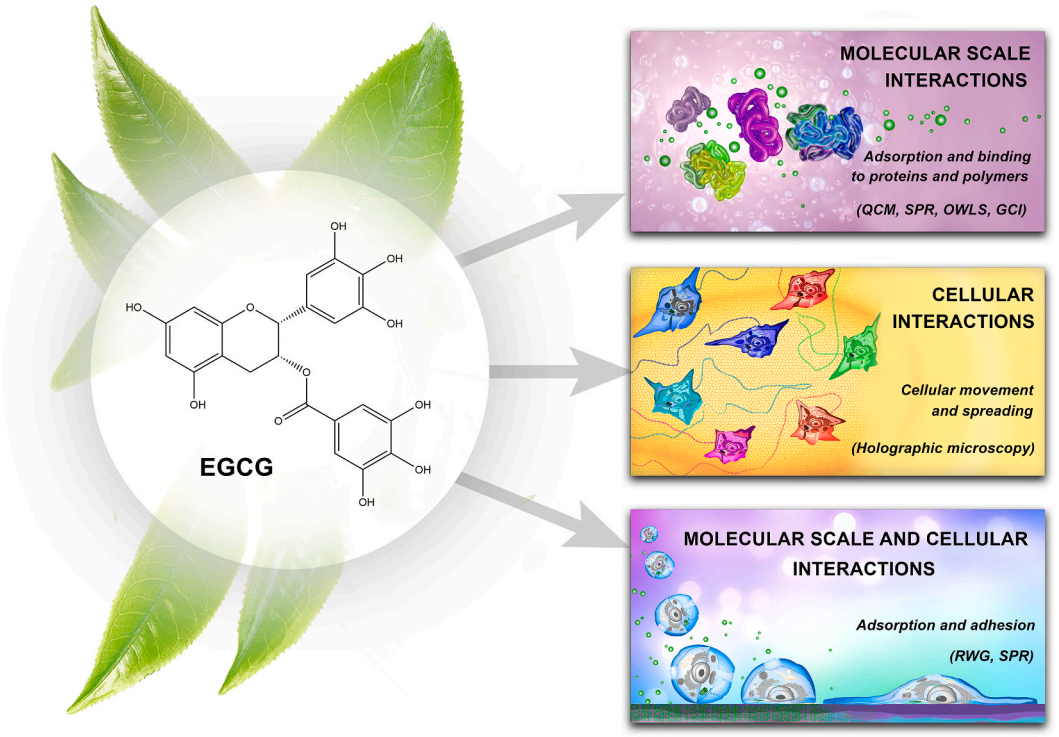

Fig. 1. Label-free biomolecular and cellular methods in EGCG research. On the left: chemical structure of EGCG. This compound is isolated from the Camellia sinensis leaves. On the right: the label-free methods are summarized and categorized into three groups. These techniques and the results of the EGCG research are detailed in further chapters.

membrane, extracellular matrix proteins, or tissue, is initially placed on the planar optical waveguide. This layer is then exposed to a medium containing the analytes, such as the natural compounds [1,33,36]. The molecular events that occur in or on this adlayer alter the characteristics of the light that propagates through the waveguide.

Label-free biophysical methods are highly valuable for tracking biological processes [33,37–39], even at the single-cell level [40]. As a result, these techniques are increasingly popular in fields such as drug discovery and the study of the biological effects of small molecules [35]. Additionally, these methods can be easily adapted to measure changes in cell shape [41] and can be utilized for cell adhesion and migration assays without the use of labels.

The *in situ* monitoring of cellular crawling along the endothelium is an essential process in the action of both cancer and immune cells [13,42]. However, the techniques of measuring this biological process in real time are limited and have essential drawbacks. The label-free digital holographic microscopy might solve these problems in an elegant way [43]. The technique is perfectly suitable for measuring migration, motility, and morphological changes of living cells in a high-throughput manner [44]. Cellular motility, migration, proliferation rate, and morphological features (optical thickness, volume, area) can be easily measured, even for relatively large cell populations. Digital holographic microscopy is available in a miniaturized format, compatible with a humidified incubator [13,44,45]. From the captured images, a continuous movie can be created.

This review focuses on novel label-free biophysical methodologies applicable in small molecule research. Techniques capable of investigating molecular scale and cellular interactions, or both, are all reviewed systematically. We choose green tea polyphenol EGCG as an example compound to highlight the capabilities. First, we overview its most essential characteristics and highlight the wide range of effects investigated at the molecular and cellular levels. Next, we summarize relevant classical techniques to identify the need for label-free investigations. After, we classified the label-free methods according to their measurement focus; the molecular or cellular level analysis (see Fig. 1). Notably, less studied compounds are also mentioned for every label-free technique as further examples.

2. The most important characteristics of EGCG

Applying natural compounds, typically from herbs, has an ancient and remarkable history. Even today, several modern pharmaceutical agents are based on or inspired by natural substances [1]. For example, the famous and traditional beverage green tea is made from *Camellia sinensis* leaves and contains a significant amount of polyphenols. In addition, the water extract of green tea leaves contains a massive amount of epigallocatechin gallate (EGCG) molecules (around 16.5 wt%). This component is unique to *C. sinensis* and is not found in any other plant [9,46]. Tea catechins, particularly EGCG, have proven to benefit human health positively. It has activities against multiple sclerosis, Alzheimer and Parkinson's disease [47]. Furthermore, it has antioxidant, anti-inflammatory, anti-diabetic, anti-cardiovascular, anti-metastasis, anti-cancer, anti-neurodegenerative, and anti-bacterial properties [9,48–50]. EGCG has significant applications in various prosthetic implants, wound healing, and tissue engineering [47]. Furthermore, it has been reported that EGCG exhibits antiviral activity by inhibiting the infection of the live SARS-CoV-2 virus and its variants by inhibiting binding to the cell surface ACE2 receptor [51]. EGCG is the ester of epigallocatechin and gallic acid. Due to its polyphenolic structure, EGCG is a good donor for hydrogen bonding and binds strongly to biomolecules [52]. Understanding the pharmacokinetics of EGCG, sausage receptor binding, and chemical interactions is crucial for interpreting epidemiological data and extrapolating results from *in vitro* or *in vivo* animal studies to humans. Among tea polyphenols, EGCG is the strongest antioxidant and interacts heavily with reactive oxygen species [46]. However, Kim et al. have also reported pro-oxidant actions of EGCG [53]. The antioxidant properties of EGCG are linked to its phenolic groups, which are susceptible to oxidation and can for quinone, the oxidized derivative of aromatic compounds [54]. Of note, the interactions and differences between the normal and oxidized forms of EGCG have only recently been studied. For example, the interactions with serum albumin, a transport protein in human blood, have been investigated from this perspective [55].

EGCG can synergize the therapeutic activities of conventional drugs (e.g., together with caffeine can improve the anti-obesity activity of EGCG). Micro and nanoformulations of EGCG have shown desirable therapeutic effects. Nanosystems produced from different nanomaterials (for instance, nanotubes, nanofilm, and lipid nanoparticles) have been applied to address the disadvantages of several bioactive compounds. In the case of EGCG, the limitation of usage may be the fast oxidation, low stability, and bioavailability in physiological conditions. To minimize these drawbacks, formulation or modification of this compound can be a good solution. For example, palmitate-modified EGCG can be created to improve the metabolic stability and bioavailability of the compound [47]. In the metal-organic framework formulation, the collagen accumulation and re-epithelialization levels in diabetic wounds were higher compared to the control experiments [56,57]. The catechin-biomaterial combination can be a choice for scarless wound healing. Mg^{2+}EGCG-coated titanium network formulation can improve osseointegration of the bone implant [47]. In a recent study, the tea-polyphenol/β-cyclodextrin/NaCl inclusion complexes were created and applied in low-salt Sichuan-style sausages. These complexes inhibited the protein and lipid oxidation, reduced residual nitrite, and blocked the growth of pathogens as well [58].

EGCG is also one of the most extensively studied substances in human medicine, and its interactions with normal and cancer cells regarding adhesion have been actively analyzed. Cellular adhesion is a fundamental biological process in which living cells organize into tissues [59]. Cellular adhesion and movement are critical processes in various physiological and pathological events, including embryonic development, tissue repair, immune response, and cancer metastasis [13]. Natural compounds can affect cellular adhesion. Usually, label-based methods are used to investigate the effects of compounds on cell adhesion and movement, etc., *in vitro*. The emergence of modern biophysical techniques has made it possible to examine the mode of action on living cells with throughput and resolution beyond conventional assays.

To gain insight into potential therapeutic options, it is crucial to observe the adhesion and movement of live cells in specific environments [13]. Cell migration occurs within or on the extracellular matrix (ECM) surface, and the lamella of the cell must adhere to the matrix components to generate traction forces for movement [60]. Focal adhesions act as anchoring sites during cell spreading and

migration, connecting the actin filaments of the cells to the substrate through the cell membrane [61]. Adhesion and migration are closely related, as adhesion is crucial in achieving cellular movement. Thus, when measuring cell migration, information about cell adhesion can also be collected [1].

Monitoring the motility and migration of cancer cells is a crucial study area since cancer metastasis is driven by cell migration [43]. Previous research has shown that highly tumorigenic cells move significantly faster than nontumorigenic cells, making it necessary to monitor individual cells rather than just populations [42]. Consequently, the development of novel types of drugs inhibiting metastasis has become a key focus of many research projects [43]. Additionally, while traditional herbal extracts are becoming more popular for treating these diseases, there is a lack of systematic quantitative studies in the literature [13]. Currently, significant research is being conducted on the influence of EGCG on cell motility and migration [62,63]. Furthermore, the motility and stiffness of cancer cells are strongly related to their metastatic capabilities [13].

The processes of spreading and adhesion are vital for maintaining cellular homeostasis, as well as for many other complex functional processes [64,65]. There are various methods available for measuring the adhesion strengths (forces), which can be divided into single-cell and population-level research. In single-cell studies, detachment events are initiated by breaking molecular bonds using techniques such as optical tweezer and micropipette-based live cell manipulation. In population cell studies, detachment can be achieved through static adhesion or dynamic adhesion [61,66,67]. Measuring migration and adhesion *in vitro* results in valuable data about novel compounds [16]. However, it is important to note that this environment is significantly simplified compared to the *in vivo* case.

The identification of cellular adhesion is extremely useful for both diagnostic and basic research purposes. Alterations in cell adhesiveness can be indicative of various diseases, such as alternations in the variety of integrins during tumor transformation [68]. Furthermore, analyzing the impact of bioactive molecules on cancer cell adhesion can be a valuable tool for developing antitumor medications. Modulating the interaction of a cell and its ECM can also impact cell behavior and function [69]. *In vivo*, cell adhesion is a complex and tightly regulated process involving various components, such as proteins, carbohydrates, cell surface receptors, and other factors like ions and molecules with low molecular weight. However, due to experimental challenges, most quantitative data obtained from experimental models of cellular adhesion may oversimplify the *in vivo* situation [16].

Tea is one of the most consumed beverages in the world, so it may have a relevant impact on human health which is interesting to study. Furthermore, among the tea catechins, EGCG is the most abundant, relevant, and studied. The field of natural compound research is huge, but we selected EGCG research, a very small part of it to be in focus. This is a very interesting compound that is worth investigating due to its special properties. Thus, we summarize the new results of epigallocatechin-gallate research measured by novel, label-free techniques. We mention the new results on the effect on cellular adhesion and migration of the EGCG-treated cells and we show the findings on the adsorption processes on different coatings and molecular interactions. Furthermore, we advise label-free techniques for performing EGCG measurements for investigating both molecular-scale interactions and cellular processes. The wider application of the reviewed methodologies may open up new ways in cell adhesion and natural compound research; our main goal is to outline the present trends in label-free biosensing regarding the small molecule compound EGCG.

3. Classical techniques for cell lines and compounds

Labeling techniques are still popular and reliable methods to investigate the effects of compounds *in vitro*. The commonly used labeling techniques are the enzyme-linked immunosorbent assay (ELISA), Western blot, and flow cytometry [1]. ELISA applies antibodies linked to enzymes that cause a color change (for instance, by altering a dye) to label the compounds of interest. The Western blot is a basic method today using protein-binding bodies [70]. However, these two techniques cannot be used for direct cell viability measurements. In contrast, Western blot can be used to investigate cellular death. Flow cytometry is also a label-based method widely employed for counting and sorting large numbers of living cells. The employed labels are fluorophores attached to the antibodies that recognize the molecules or living cells targeted [71,72]. Of note, label-free flow cytometers also exist using impedance (Coulter counters) [1,73].

Labels were intensively used in EGCG research or investigating other compounds with natural origin. Typically MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and the trypan blue exclusion test were used. But, Wang et al. showed that the MTT- and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)-based assays influenced the results when the antiproliferative effect of EGCG was analyzed. The authors suggested that the increased activity of mitochondrial dehydrogenase is behind these findings. This is because the presence of EGCG reduces MTT and MTS but increases formazan production. Therefore, a critical view of results is needed when EGCG (or potentially other active natural compounds) is used in label-based cell viability and proliferation assays [72,74].

Cytotoxicity and cytostatic assays quantitatively measure the viability of living cells. The most crucial difference between these is the length of treatment (short-term (<4 h) may adversely affect metabolism markers or ATP content before measurable membrane integrity changes, and long-term exposures (24 h <) may cause underestimation of cytotoxicity due to degradation of marker enzyme activity after its release into the extracellular environment [75–77].

Most cell viability assays can be divided into three categories; monitoring the loss of membrane integrity, directly measuring metabolic markers or ATP levels, and those that assess metabolic activity. Metabolic assays mainly focus on ATP or the reduction of resazurin dyes or tetrazolium. Proliferation affects metabolite ratios (NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD), capable of reducing tetrazolium salts to formazan products. The most common stainings/labels are the crystal violet, resazurin (7-hydroxy-3H-phenoxazin-3-one), tetrazolium salts, and BrdU ((5-bromo-2′-deoxyuridine) [1].

Other techniques focus on cellular movements. These are mainly migration studies and also have significant drawbacks. For

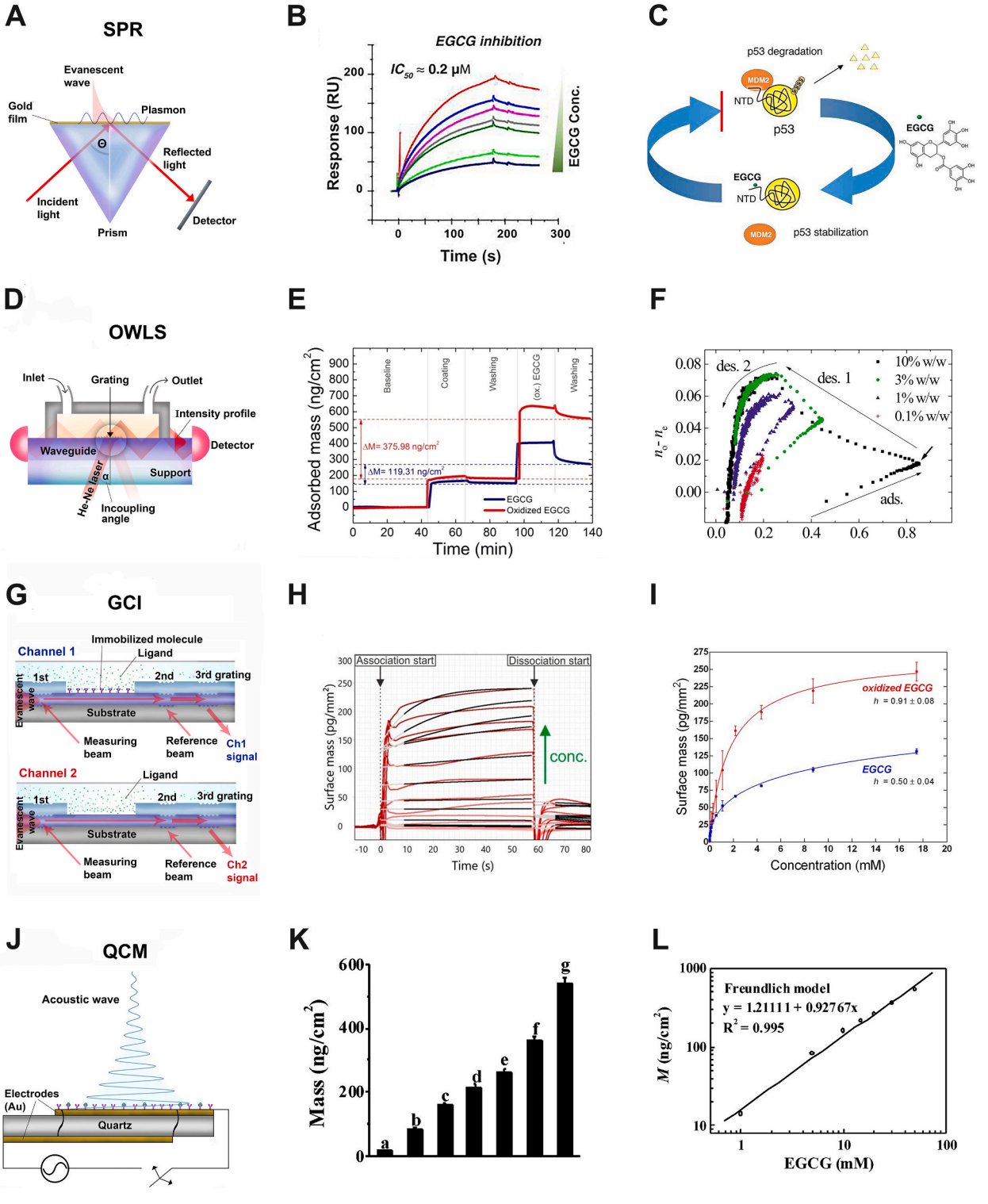

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Fig. 2. *Label-free methods for investigating molecular interactions with EGCG and the collection of the results.* ***A****. Working principle of the SPR method. Based on ref* [33]. ***B****. SPR data of full-length p53–MDM2 binding in the presence of various concentrations of EGCG (0–*500 nM*). The concentration of p53 is* 250 nM. *Based on ref* [85]. ***C****. EGCG disrupts p53–MDM2 interaction due to its binding to the N-terminus of p53. The EGCG–p53 interaction disrupts p53 interaction with its regulatory E3 ligase MDM2 and inhibits the ubiquitination of p53* by *MDM2, likely stabilizing p53 for anti-tumor activity. Based on ref* [85]. ***D****. Working principle of the OWLS method* [33]. ***E****. The in situ OWLS measurement data when EGCG and oxidized EGCG were adsorbed at the previously assembled PP layer. Based on ref* [16]. ***F****. Plots of the birefringence (n_o–n_e) vs adsorbed mass (M) for mucin adsorbed from the bulk. Based on ref* [91]. ***G****. Working principle of the GCI method. Based on ref* [33]. ***H****. Fits of GCI biosensor kinetic data using the homogenous model for oxidized EGCG. Based on ref* [55]. ***I****. Fitting of EGCG and oxidized EGCG data using the Hill model. Based on ref* [55]. ***J****. Working principle of the QCM method. Based on ref* [33]. ***K****. Changes of mass values of EGCG adlayer on BSA surfaces at different EGCG concentrations (*1 mM-50 mM*). Based on ref* [103]. ***L****. Adsorption isotherm of EGCG onto BSA coating fitted to the Freundlich model. Based on ref* [103].

example, filter assays detect migration over a membrane due to chemoattractants (Zigmond and Dunn, Boyden chambers) [13]. Of note, these assays are incompetent with certain cell types. Timelapse microscope imaging is also used to monitor cell movements. However, when combined with fluorescent markers, the life cycles of cells can be disturbed, and due to photobleaching, the imaging time is strongly limited.

4. Label-free methods for investigating molecular interactions

Traditionally, the first label-free biosensing methods were developed to measure the interaction of biomolecules with each other or solid surfaces. The most important methods - overviewed here by the prospect of EGCG research - are surface plasmon resonance (SPR), optical waveguide lightmode spectroscopy (OWLS), grating-coupled interferometry (GCI), and quartz crystal microbalance (QCM) [9,33]. Although it is not a biosensing method, we also consider Fourier-transform infrared spectroscopy a valuable platform in molecular-scale EGCG research, especially concerning measurements of human plasma.

4.1. Surface plasmon resonance (SPR)

SPR is the first commercialized label-free biosensor technique. It uses the optically generated collective oscillations of free electrons, the so-called surface plasmons, at the surface of a gold film [4,23,24,33]. The most common ways of exciting these surface plasmons are by employing a prism coupler (Fig. 2A), a waveguide coupler, or a grating coupler [23]. During propagation of the surface plasmon, so-called evanescent optical fields are generated at the solid-liquid interface which monitors the adsorption of biomolecules in real-time [23,24,33].

In short, the conditions of generating the surface plasmons, and consequently the monitoring evanescent optical fields, depend on the optogeometrical parameters (layer thicknesses, refractive indices of the system). When adsorption occurs on the sensor, the local refractive index shifts at the sensor surface and this changes the conditions to generate surface plasmons [23,24,33]. The evanescent optical fields that are generated at the solid-liquid interface monitor the adsorption of biomolecules in real time. The most used metal in SPR is gold, however, other metals, like platinum [78,79] or silver [80,81] can also be employed.

Using SPR, Tachibana et al. highlighted the cell surface laminin receptor (67LR) as the receptor of EGCG [82,83]. This particular work opened up a new research line in the understanding of EGCG's molecular-scale actions. Moreover, other SPR studies demonstrated EGCG binding to chemokines, whereas the binding of (−)-epicatechin (EC) or (−)-epigallocatechin (EGC) was considerably weaker. These findings highlighted EGCG as a potent therapeutic agent for inflammatory diseases [83].

SPR was also used to characterize EGCG-α-amylase binding affinity [83]. Saeki et al. concluded that EGCG can inhibit caries formation by inhibiting the formation of fermentable carbohydrates. Therefore, EGCG-salivary protein interactions are potentially important in oral health [83,84].

In another work, the specific binding of EGCG to full-length p53 and p53 disordered N-terminal domain (NTD) was reported [85]. The authors discovered that NTD is the major mediator of the p53–EGCG interaction (Fig. 2B and C). Thus, p53 NTD was identified as a target for cancer drug discovery [85].

EGCG was also tested for its ability to block STAT3 binding to its phosphopeptide ligand [86]. SPR experiments showed that EGCG directly competes with pY-peptide for binding with STAT3, having importance in cell survival, proliferation, and movement [86].

The binding affinity of EGCG to human neutrophil elastase (HNE) was determined by SPR as well. Xiaokaiti et al. highlighted that EGCG directly binds to HNE and inhibits its enzymatic activity [87]. This mechanism finally reverses the neutrophil elastase-induced migration of A549 cells.

Another popular beverage compound, the coffee extract 5-*O*-Caffeoylquinic acid, was also tested by SPR, which has anti-cancer activity [88]. In another study, extracts from five selected medicinal plants were studied. Among them, *Onopordon acanthium* L. extract and its isolated compound (onopordia) were identified as ACE (angiangiotensin-converting enzyme) potent inhibitors. Thus, according to these results, SPR biosensors can be used for screening natural product extracts [89].

4.2. Optical waveguide lightmode spectroscopy (OWLS)

Optical waveguide lightmode spectroscopy (OWLS) employs thin-film planar optical waveguides with a coupling surface relief grating (Fig. 2D) [90]. By monitoring the incoupled angle of laser light into the waveguide, one can follow surface processes with a time resolution of seconds [32]. Similar to SPR, OWLS also uses evanescent optical waves, but here the propagating waveguide modes

generate these surface-localized optical fields. Unlike in SPR measurements, which use only the transverse magnetic polarisation, in OWLS two types of orthogonally polarized modes are employed [32]. This is an important feature for the measurement of both the layer thickness and refractive index of adsorbed biological films [25,33].

OWLS was successfully used to monitor the interaction of EGCG with thin polymer layers [16]. The layers were fabricated from PLL-*g*-PEG/poly (L-lysine)-*graft*-poly (ethylene glycol) (PLL-g-PEG, [PLL (20)-g (3.5)-PEG (2)]) (hereafter PP)/and its RGD functionalized counterpart PLL-*g*-PEG-RGD/PLL-g-PEG/PEG-GGGGYGRGDSP (PLL-g-PEG-RGD [PLL (20)-g (3.5)-PEG (2.3)/PEG (3.4)-RGD]) (hereafter PPR)/polymers, and then the binding of EGCG and oxidized EGCG inside these coatings was observed. Previous works highlighted that PP has repellent properties. In contrast, the authors found that the PP coatings interacted with EGCG with an irreversibly bound amount of 100 ng/cm^2. By considering both the molecular weight and size of EGCG they suggested that this value greatly exceeds the monolayer surface coverage of EGCG (around 30 ng/cm^2). Similar OWLS experiments found that the bound amount of the oxidized EGCG was more than three times the adsorbed amount of EGCG (Fig. 2E). The authors performed molecular simulations and concluded that EGCG and oxidized EGCG interact with the PEG chains by H-bonding. The discovered binding mechanism can also be important in EGCG-biomolecule interactions [16].

OWLS was also used to study EGCG-mucin interactions in real time. It has been shown by Horvath et al. that the EGCG triggers a striking adsorption-desorption hysteresis, reflecting sequential adsorption-desorption (Fig. 2F) [91]. The authors used the two waveguide modes of the biosensor to deduce the optical anisotropy of the adsorbed films, which parameter highlighted the structural differences in the adsorbing and desorbing mucin layers [91,92]. In another work, OWLS data demonstrated reduced fibroblast cell attachment and spreading on mucin-coated substrata, but the measured reduction could be countered by introducing EGCG [11].

4.3. Grating coupled interferometry (GCI)

In general, interference–based detection methods have outstanding sensitivity [33,93,94]. GCI is an optical waveguide interferometry-based label-free sensor technique that has just recently started to be employed in high-resolution kinetic measurements of molecular-scale interactions. Fig. 2G presents the cross-section drawing of a GCI setup together with the intensity signal channels [33]. One of these channels can be used for reference measurements [33,55]. A typical GCI sensor chip has three grating regions for convenient in and outcoupling of the interfering light beams (1st and 2nd grating in Fig. 2G) and their final interference signal (3rd grating in Fig. 2G). To measure the phase shift of the sensing waveguide mode, a liquid crystal modulator (LCM) is placed in the reference light path before coupling the beam into the waveguide. Simply, the phase modulation results in a time-varying intensity signal. Refractive index changes inside the evanescent field alter the phase of the guided optical wave, changing the characteristics of the time-varying intensity signal. These signal characteristics are sensitively recorded using a liquid crystal modulator [33].

GCI experiments characterized the interactions of bovine serum albumin (BSA) and EGCG (and oxidized EGCG) and determined the dissociation constant (K_d) of the binding equilibrium [55]. Of note, the binding behaviors of oxidized EGCG to albumins have never been investigated before. The authors found that over stoichiometric binding and oxidization, stoichiometric binding and oxidization increased the bound amount (Fig. 2H and I). K_d values in the mM range were determined. Notably, the authors discovered that EGCG (and also oxidized EGCG) adsorbs to all types of sensor layers with approximately the same amount, independently of surface hydrophilicity/hydrophobicity. This strong and general binding capability is essential information for those working with EGCG. According to the GCI measurements, one BSA molecule can bind up to 35 EGCG or 63 oxidized EGCG molecules, respectively. An additional important feature of GCI is that it was recently combined with a novel high-throughput and sample-saving way of measuring binding kinetics and affinity [55]. The so-called Repeated Analyte Pulses with Increased Duration (RAPID) was recently demonstrated to measure the binding affinities on lignans, which is potentially applicable in cancer therapy [95].

It is important to note that GCI is a well-established technique already applied in several fields, such as plant biology as well [96–101].

4.4. Quartz crystal microbalance (QCM)

QCM typically employs a quartz crystal sensor disk coated by two gold electrodes on its faces (see Fig. 2J) [33]. Applying AC (alternating current) voltage, mechanical oscillations of the crystal can be generated (with the fundamental resonance frequency only or together with several overtone oscillations). The resonant frequencies of these oscillations are sensitive to any added mass on the sensor. The technique is extensively employed in soft biomaterial research and has become a standard today. QCM also gained popularity in the characterization of heavily hydrated polymer layers in combination with the above-mentioned optical methods. However, according to our experiences, it is less useful for high-resolution kinetic investigations, presumably due to its higher sensitivity to mechanical noises, often originating from the sample handling fluidical system itself [33].

For example, Ali et al. demonstrated QCM in EGCG research [102]. Particularly, EGCG-BSA and crude green tea extract-BSA interactions have been monitored. The authors fitted Langmuir and Freundlich isotherms to their QCM data. They found that the Langmuir model better fitted the measurement data in all concentrations investigated. This possibly indicates that the aggregation during the adsorption process is negligible. They reported a lower adsorption rate for the green tea extract, possibly due to competitive mechanisms. The estimated mass saturation is 58 % higher for the crude extract compared to EGCG only (7.9 ng/cm^2 vs 5 ng/cm^2). This suggests that additional components of the extract also bind to BSA, and the competitive binding is probably not significant [102]. In another work, Wang et al. also examined the EGCG adsorption to BSA [103]. The QCM-D (quartz crystal microbalance with dissipation monitoring) showed that the bound EGCG increases the elasticity and viscosity of the BSA layer. The cross-coupling mechanism by H-bonding discovered recently may be also a dominant effect in this case [27]. However, Wang et al. did not

investigate the oxidized form of EGCG. Interestingly, their data are better fitted by the Freundlich model [103]. (Fig. 2. K, L).

Tea compound and saliva interactions and their correlations with aftertaste were also tested with QCM-D. Huigan is an essential sensory parameter used as a quality indicator of tea goods. There is a correlation between this sensory perception and the lubrication behavior of the saliva-tea compound mixture. QCM-D studies show that the interaction between saliva of the sensitive group and EC/EGC ((epicatechin/epigallocatechin) formed a high mass of a complex rigid layer on the gold sensor surface and showed a significant effect on Huigan intensity [104]. Other results indicated that QCM-D could detect different families of tannins with different concentrations and can be miniaturized for portable analysis [105]. With the QCM-A (quartz crystal microbalance-admittance) biosensor, other authors used fucoxanthin isolated from *Undaria pinnatifida* as an α-amylase inhibitor and monitored the interactions with α-amylase [106].

4.5. Fourier-transform infrared spectroscopy (FTIR)

FTIR is a well-known technique to record the infrared spectrum of absorption or emission processes in a solid, liquid, or gaseous media.

Using FTIR measurements, Araújo et al. demonstrated that EGCG consumption led to measurable alterations in plasma composition [107]. Of note, this effect of EGCG on human plasma needs further attention when EGCG is consumed longer. This effect of EGCG and the mechanisms behind it should be further investigated. Notably, the FTIR measurements could be performed using only a drop (i.e., 10 μL) of blood. Therefore, the developed methodology could be employed in larger-scale human research to understand the influence of natural compounds [107]. FTIR can be used in screening procedures, too. For example, in the study of Ramya et al., phytochemical screening and FTIR profile of bioactive natural products (alkaloids, anthraquinones, carbohydrates, coumarins, flavonoids, etc.) from *C. cyminum* have been performed [108].

5. Label-free imaging techniques for investigating cellular processes

5.1. Transmission digital holographic microscopy

The development of modern computer technology launched the possibility of quantitative phase imaging, where a computer calculates the object image from a digitally recorded hologram using a numerical reconstruction algorithm. In transmission digital holographic microscopy (TDHM), a laser beam is split into two parts, one passing through the sample and the other serving as a reference beam. The two beams then interfere with each other, producing a hologram that contains information about the phase and amplitude of the transmitted light through the sample (Fig. 3A). Unlike traditional microscopy techniques, TDHM does not require

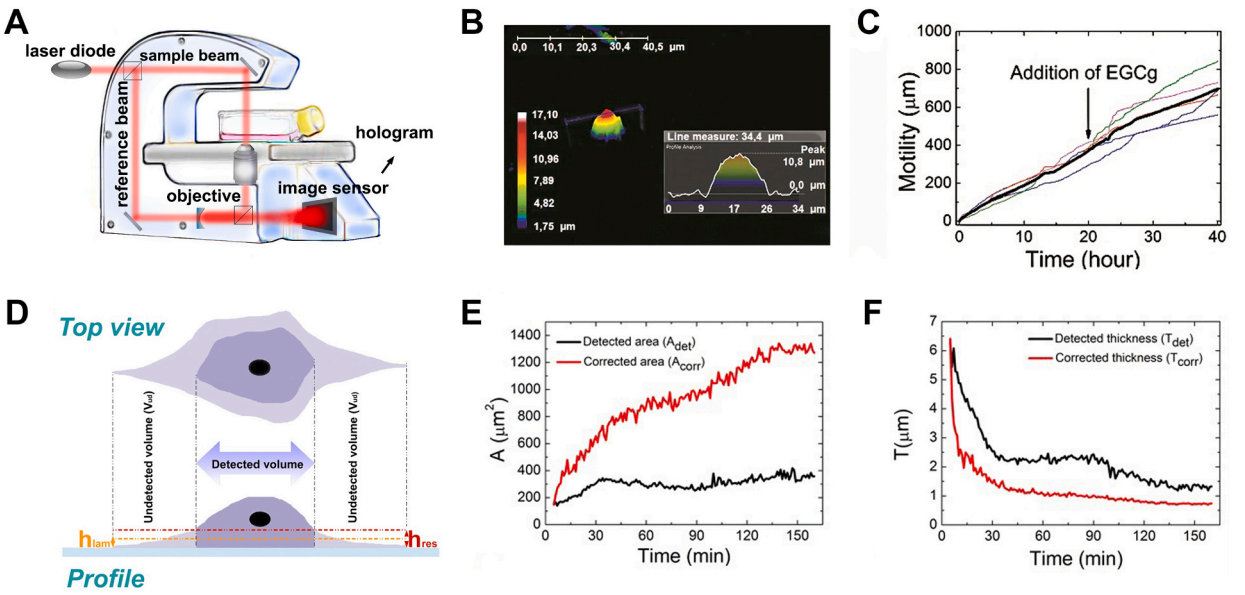

Fig. 3. *Holographic microscope method and the collection of the results.* ***A****. Working principle of the HM4: A laser beam is split into two identical beams (sample and reference), the sample beam transmitting through the study object and interfering with the reference beam creating a hologram. Based on ref* [44]. ***B****. Profile analysis of a single HeLa cell by HM4. This figure is based on the work of ref* [13]. ***C****. HeLa* cell *motility analysis when EGCG is added to the HeLa cancer cells. The black lines represent the mean of the values averaged for five typical cells. Based on ref* [13]. ***D****. Schematic illustration of the detected and corrected data of cells. Sometimes the lamellipodia, the very thin parts of the cells, cannot be sensed perfectly by the instrument. Based on ref* [13]. ***E****. Detected (black) and corrected (red) area value. Based on ref* [13]. ***F****. Detected (black) and corrected (red) thickness values. Based on ref* [13]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

staining or labeling of the sample. Commercially available digital holographic microscope HoloMonitor M4 (HM, PHI AB, Lund, Sweden) allows the imaging of live cells under physiological conditions in an incubator environment in real- and long periods [13,44]. Each image captured contains data on more than 30 cellular parameters in every single cell (Fig. 3B). HM has essential applications in biology, providing quantitative information on living cells. It has been used to study the morphology and dynamics of biological cells, including the invasiveness of cancer cells [45], adhesion, proliferation, migration, and death [44,109,110].

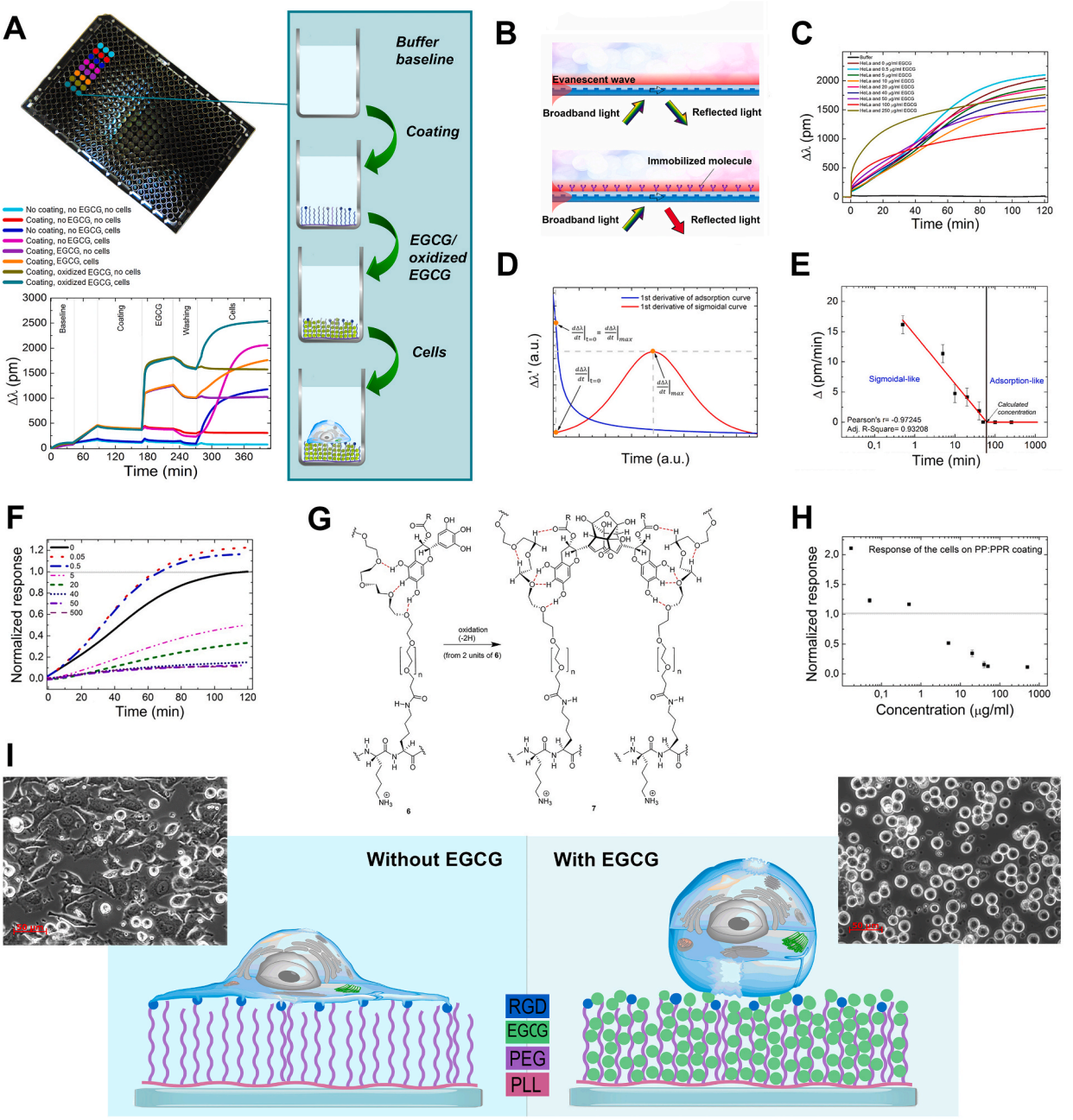

Fig. 4. *The RWG biosensor method and the collection of results.* ***A****. The biosensor microplate (left), the online RWG data (bottom), and the measurement steps (right). Based on ref* [16]. ***B****. Working principle of the RWG biosensor. Based on ref* [33].***C****. Cell adhesion kinetics recorded by RWG method. Increasing the EGCG concentration decreases the sigmoidal character. Based on ref* [72]. ***D****. The first derivative of the sigmoidal and adsorption-like biosensor kinetic curves are used to characterize viability. Based on and more details in ref* [72]. ***E****. Plotted and fitted Δ values calculated from the first derivatives. Decreasing Δ suggests decreasing viability in full accordance with classical assays. Based on and more details in Ref.* [72]. ***F****. Cell adhesion on the polyphenol-treated surfaces. Based on ref* [16]. ***G****. Proposed cross-coupling mechanism of EGCG using strong H-bonding capability. Based on ref* [16]. ***H****. Normalized cell adhesion signals. Based on ref* [16]. ***I****. Illustration of the discovered effect of EGCG. A complete blocking of cellular adhesion can be achieved at high polyphenol concentrations (right). Based on ref* [16].

Understanding diseases requires studying cell migration and motility, crucial factors in cancer progression. The effect of EGCG on cancer cell motility and migration was investigated using a digital holographic microscope HM4. The software of this system calculates motility as the distance the cell moves from start to end of the analysis and migration as the shortest distance between the two points. Adding EGCG (500 μg/mL) to HeLa cells resulted in decreased migration, as well as motility (Fig. 3C) and motility speed.

HM4 creates sharp 3D images of cells, but due to its limited vertical resolution, very thin parts of the cell lamellipodium may not be detected, leading to an underestimation of cell contact area and volume and an overestimation of cell thickness (Fig. 3D) [13]. A simple method has been proposed to correct this issue.

The HM4 has lateral and vertical resolutions of 1 μm, although this may be limited by cell density and culture vessel factors. Parts of the cell may also blend into the background surface, resulting in unrealistic reductions in detected cell volume and underestimation of the spread cell contact area. Pathophysiological conditions can lead to the slow development of anisotonic state in cells, but this usually has little impact on the shift in cell volume. As a result, the vertical resolution of the system is relatively low. Therefore, it cannot accurately detect lamellipodia. The thickness of the lamellipodia (h_{lam}) is approximately 0.5 μm. An anisotonic state that develops under pathophysiological conditions occurs gradually, continuously allowing volume-restoring mechanisms to maintain cell volume [13]. The resulting change in cell volume is insignificant [111]. Consequently, the measured cell volumes in experiments are often unrealistically reduced, and the determined spread of cell contact areas is underestimated. The difference between the directly measured and corrected parameters (area and thickness) are shown in Fig. 3E and F. The recorded HM data can be corrected using the following method:

$$T_{corr} = V_0/A_{corr}$$

where T_{corr} is the corrected thickness, V_0 is the volume at the first time point, and A_{corr} is the corrected area (Fig. 3 E, F) [13].

With this technique, other plant compounds, shikonin [112], berberine [113,114], protopanaxadiol [115], and *Baccharis densiflora* flavonoid [116], were also analyzed.

5.2. MALDI-mass spectroscopy imaging (MALDI-MSI)

Mass spectrometry imaging (MSI) is an analytical method allowing for the label-free determination of the distribution of ionizable biomolecules in tissues based on their specific mass-to-charge ratios [117]. There are several types of MSI techniques, including matrix-assisted laser desorption/ionization imaging (MALDI-MSI), desorption electrospray ionization imaging (DESI-MSI), and secondary ion mass spectrometry imaging (SIMS-MSI). Each technique has advantages and limitations, and the choice of technique depends on the sample type and the research question being addressed. MALDI-MSI [118] has numerous applications in various fields, including biology, medicine, pharmacology, and materials science. For example, it can be used to identify biomolecules such as proteins, lipids, and metabolites in tissues, to study the localization of drugs and their metabolites in cells, and to characterize the chemical composition of materials such as polymers and coatings [119]. In addition, the matrix selection is critical for ensuring highly sensitive imaging of the target compound, especially in detecting small molecules [120]. However, Kim et al. have established a 1, 5-diaminonaphthalene (1,5-DAN)-based MALDI-MSI technique for visualizing the polyphenol EGCG and its metabolites within mammalian tissue micro-regions after oral dosing [121]. The tissue sample is first coated with a thin layer of 1,5-DAN matrix solution in this technique. The matrix solution helps to desorb and ionize the EGCG molecules when irradiated with a laser beam. The sample is then analyzed using a mass spectrometer, which measures the mass-to-charge ratio of the ions produced from the desorbed EGCG molecules [121]. Despite limitations of the detection sensitivity, the label-free 1,5-DAN-MALDI-MSI method is promising for better understanding the metabolism of polyphenols or other biomolecules and their biological or pharmaceutical roles [122].

6. Label-free methods for both molecular scale and cellular interactions

This chapter provides two techniques for investigating molecular scale and cellular interactions. Both methods can monitor the adsorption process and provide kinetic data of cellular adhesion in real-time. The main advantage is that the whole kinetic process can be observed, not just explored endpoint data can be explored.

6.1. Resonant waveguide grating (RWG)

Applying these techniques has become crucial for systematically studying the effect of active substances on cell behavior and exploring various biological materials in a high-throughput format (Fig. 4A) [16]. The Epic BT instrument (Corning Incorporated, NY, USA) is a resonant waveguide grating-based imager biosensor using 96- or 384-well biosensor microplates. Their bottom is a planar optical waveguide, a thin, high refractive index, low loss transparent dielectric layer (waveguide layer, made of Nb_2O_5) on a glass substrate. At the center of each well a 2×2 mm^2 grating structure is embedded into the waveguide layer for light coupling. As a consequence of this, separate biosensors are created. Light beams coupled into the waveguiding film undergo total internal reflections, monitoring the waveguide covering media at each reflection. Simply, material deposited on the surface of the waveguides shifts the phase of the reflected light. The overall phase shift depends on the refractive index of the medium above the waveguide. Due to the exponentially decaying character of the electromagnetic field during the total internal reflection process (called an evanescent optical field), only an approximately 150 nm thick surface layer is monitored. Notably, the incoupling effect only happens at a certain resonant wavelength (λ). Molecular adsorption and cellular adhesion alter the refractive index inside the evanescent field and shift the

monitored resonant wavelength (Fig. 4B) [12,16]. The instrument has a minimum detectable surface bound amount of 0.078 ng/cm^2 [16].

Natural compounds can self-associate into colloidal aggregates in an aqueous buffer [123]. and this can cause false results during the initial phases of drug discovery. On the contrary, RWG-based assays are relevant tools that can effectively distinguish between real and false responses [1,123].

With this technique, it has been discovered that the kinetic curves rapidly and extremely sensitively quantify cell viability *in vitro* in accordance with parallel MTT and flow cytometric cell viability data [1,72]. For untreated and low-dosed living cells, sigmoid kinetic curves were monitored. The higher dose of EGCG resulted in the absence of this character, and adsorption-like curves were recorded (Fig. 4C). A method was proposed to quantify cell viability based on the kinetic curves using their first derivatives (Fig. 4D). These investigations resulted in a critical EGCG concentration of 60 ± 40 μg/mL (Fig. 4E). At this concentration, the sigmoid-to-adsorption-like transition happens. Of note, this result was in full accordance with MTT data. Using the developed methodology, the RWG can nowadays also be used as a label-free, non-invasive, and relatively cheap technique to measure cell viability. It should be highlighted that the RWG technique also gave reliable data at low concentrations, where classical methods failed. For more details, see Fig. 4E [72].

In a recent study, it was demonstrated that EGCG could block a crucial cell adhesion ligand, the RGD (Arg-Gly-Asp) motif, and can also tune matrix properties via H-bonding. Moreover, in this study, the fabrication of a polymer coating, its treatment with EGCG and its oxidized form, and the observation of cell adhesion have been studied *in situ* using RWG. In this study, PP and its RGD functionalized counterpart, PP-RGD (PPR) synthetic polymer films, were fabricated, and the subsequent interaction of EGCG and its oxidized form with these coatings was monitored [16]. Consequently, cellular adhesion to the fabricated layers was also measured (solutions can be mixed to decrease ligand-to-ligand distance and to increase cellular adhesivity [12]. Thus coating solution with RGD-motifs and PP was made by mixing the two types of solutions in a 1:1 ratio (hereafter PP:PPR)). No significant cell signal was observed on the treated films. Thus, these measurements proved that the PP coating remains repellent. Cell adhesivity on the RGD-displaying surfaces was strongly influenced by EGCG pre-treatment. Remarkably, increased cellular adhesivity was even observed, presumably due to the change in layer rigidity (Fig. 4F–H). [16]. The authors concluded that EGCG dimers dominate the above interactions. Fig. 4I summarizes the observations. The authors supported their finding with semiempirical quantum chemical modeling and highlighted the H-bonding capability of EGCG (Fig. 4G) [16].

The extracellular matrix component fibronectin is crucial in the adhesion, growth, differentiation, and migration of living cells. The polyphenol and its oxidized form can bind to fibronectin creating multilayers. This binding reduced the subsequent cellular adhesion, showing a stronger reduction in the case of the oxidized EGCG [124]. Again, the cross-coupling capability and strong binding ability of the polyphenol play an important role. Moreover, the authors highlighted the interactions of EGCG with the disulfide bonds of fibronectin [124–126].

Rhodiola rosea [123] and Chinese herb extracts and natural products [127,128] were also tested and analyzed by RWG biosensors recently.

6.2. Surface plasmon resonance (SPR)

SPR can monitor not just molecular interactions but also interactions between molecules and cells. EGCG interactions in connection with laminin receptor 67LR-mediated cellular adhesion were monitored by SPR [129]. The authors constructed 67LR-overexpressed HepG2 cells. Fujimura et al. found that the EGCG binding to the overexpressed cells was higher than the control cells transfected using the empty vector. The authors also found that EGCG reduced the proliferation of the receptor-overexpressed cells. In summary, 67LR is important in the anti-tumor activity of EGCG at physiological concentrations [129].

Table 1
Summary of the label-free methods focusing on EGCG interactions with coatings (proteins, receptors) and cell types.

METHOD	TYPE OF ASSAY	COATING/PROTEIN/RECEPTOR	REF
SPR	Biomolecular	Chemokins	[83]
		67LR	[82,83]
		α-amylase	[83]
		p53	[85]
		STAT3	[86]
		HNE	[87]
OWLS	Biomolecular	PP and PPR	[16]
		mucin	[91,92]
GCI	Biomolecular	BSA	[55]
QCM	Biomolecular	BSA	[102]
QCM-D	Biomolecular	BSA	[103]
		Saliva	[104]
FTIR	Biomolecular	Human plasma	[107]
Holographic microscopy	Cellular (HeLa)	PPR	[13]
MALDI-MSI	Cellular (Tissue covered with 1,5 DAN matrix)	–	[121]
RWG	Biomolecular and cellular (HeLa)	PP and PPR	[16]
		Fibronectin	[124]
SPR	Biomolecular and cellular (HepG2)	67LR	[129]

SPR-based sensors are extensively employed to monitor whole cells (bacteria, mammalian) and their interactions with surfaces.

Infrared Surface Plasmon Spectroscopy [130–133] has an advantage compared to conventional SPR. Operating at a fixed wavelength and at a variable angle of incidence, this setup allows the wavelength and the angle of incidence to be varied simultaneously. This feature is very useful for studying biological molecules (for example glucose [131) in living cells. MP-SPR (Multi-parametric Surface Plasmon Resonance) method is capable of measuring changes in polymer films that are thicker than the apparent scanning depth of the conventional SPR field. The data gained by this technique can be applied to identify three different modes of change in the films with different kinetic timescales, which are not visible in the *in vitro* measurements. These results can be used, for instance, to optimize the drug-release profile of diverse film formulations for pharmaceutical purposes [134–136]. These methods can be also excellent tools for investigating drug-cell interactions or other compounds.

In Table 1, we summarized the methods focusing on EGCG interactions with coatings (proteins, receptors) and cell types. The mentioned techniques are capable of investigating EGCG or other natural small molecule binding, furthermore, some of them are capable of monitoring their effects on cells (movement and adhesion) real-time without using any labels.

7. Conclusions

Our review aimed at collecting data on a specific research question, namely the EGCG measurements performed by label-free biosensor methods in cellular adhesion and migration studies, including the characterization of the methods and the compound itself. We have elucidated the main points of interest (adhesion and migration process, cell viability, classical assays with dyes, label-free techniques, EGCG characterization, and findings by label-free methods) with inclusion and exclusion criteria. We have made a careful and systematic search of the literature using the following keywords: epigallocatechin-gallate, green tea, label-free, a natural compound, biosensors, cell adhesion, migration, motility, movement, viability, cytotoxicity, flow cytometry, dyes, oxidation). Approximately 130 relevant papers were selected. Very recent (2023) and old references (articles from 1993) were evaluated. Our report covers the knowledge on this topic from the last 30 years. Our criteria were that the compound must be EGCG measured by label-free methods. Further, recent articles that monitor other natural compounds by label-free techniques were also selected as the outlook for other substances (15–20 % of these references).

The present review highlighted that novel label-free techniques could provide pivotal information (especially kinetic information) about the molecular or cellular scale effects of natural extracts without using dyes or labels, affecting measurement data. Affinity measurements of small molecules below 100 Da M weight in a label-free automatized manner using small amounts of samples became possible only recently. These recent developments were also reviewed. Moreover, we overviewed novel label-free setups with excellent time resolution. This is important in kinetic measurements of biomolecules and living cells. In the latter case, molecular scale information can be obtained from the in-depth analysis of cellular label-free kinetic signals. Unlike traditional measurements, label-free biosensors made such measurements possible without isolating the receptors of interest. Throughout our work, we reviewed the label-free methodologies focusing on the EGCG as an example compound, being the most well-known and studied natural compound in the literature.

We highlight that in most of the publications, important information about the oxidative state of the EGCG is missing; there needs to be more information about its preparation and storage conditions. Since EGCG oxidizes quickly even at room temperature and the oxidized form has significantly different properties, this can result in misleading conclusions [9,27]. Only a few studies examined the difference between the two forms. However, the oxidized EGCG has a more intensive blocking effect on cellular adhesion and movement [27]. Thus, the other advantage of applying label-free methods is that the dyes are not required in the measurements. Therefore the redox reactions of EGCG have not affected them (EGCG reduces MTT and MTS and increases formazan formation [74]). Furthermore, if the measurement takes a long time with classical methods, EGCG can oxidize in the meantime. Consequently, the measurement result will also change because it will be valid for the oxidized form. This is often not described in the articles. Considering these factors, EGCG analysis is not as simple as it seems. Label-free methods may resolve these problems in EGCG research due to their simplicity and rapidity compared to classical measuring techniques.

Techniques capable of monitoring molecular and cellular scale interactions were all reviewed with special particular attention to novel biophysical tools applicable to investigate both molecular and cellular scale effects of small molecules in one experiment with statistics.

RWGs are important in biomedical research today, such as in early or second-stage drug candidate testing [65,72]. The application of RWG was even extended to test cell viability in a high-throughput manner. These developments could be employed in investigating drug-resistant tumor cells.

The direct influence of small molecules on living cells and biomolecules is relatively well investigated in the literature using traditional methods. However, the indirect influence of these molecules on the cellular nano-environment is overlooked. Multicomponent measurements with RWGs made such measurements straightforward. As we mentioned before, the essential novelty of the mentioned studies is that the polymer coating fabrication, its treatment with small molecules, and the observation of cell adhesion could all be studied online in triplicates and with different EGCG and oxidized EGCG concentrations at the same time [16]. The wider application of the reviewed methodologies may open up new ways in cell adhesion and natural compound research.

In summary, applying label-free methods in small molecule EGCG research is a novel way with advantages; fast and relatively simple methods without using dyes, meanwhile the oxidation process may not affect the results of the experiments. In some instances, the high-throughput format allows many parallel measurements simultaneously to be applied for rapid drug screening on cells. Cell viability can also be estimated from the adhesion kinetic data. It should be however noted that label-free investigations require careful experimental design with adequately chosen controls. This is because the specificity of label-free is less compared to that of traditional

labeling assays. For example, phenomena like nonspecific binding have the utmost importance and should be avoided by employing engineered surfaces [137].

The outlined knowledge is expected to be increasingly important in human therapeutics due to the recent developments involving natural compounds. Label-free sensing and imaging techniques may open up new application directions in the future as well due to the rapidly progressing technical results of advanced sensing and sample handling systems [138–147]. Significant developments are expected in the study of the population-scale behavior of single cells and their connection to developing novel types of personalized and, more precisely, targeting medicines [40,148–152].

Data availability statement

All data are included in the manuscript.

CRediT authorship contribution statement

Beatrix Péter: Writing – review & editing, Writing – original draft, Visualization, Resources, Funding acquisition, Data curation, Conceptualization. **Inna Szekacs:** Writing – original draft, Supervision, Conceptualization. **Robert Horvath:** Writing – original draft, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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