On-chip sample pretreatment using a porous polymer monolithic column for solid-phase microextraction and chemiluminescence determination of catechins in green tea

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A porous polymer monolithic column for solid-phase microextraction and chemiluminescence detection was integrated into a simple microfluidic chip for the extraction and determination of catechins in green tea. The porous polymer was prepared by poly(glycidyl methacrylate-co-ethylene dimethacrylate) and modified with ethylenediamine. Catechins can be concentrated in the porous polymer monolithic column and react with potassium permanganate to give chemiluminescence. The microfluidic chip is reusable with high sensitivity and very low reagent consumption. The on-line preconcentration and detection can be realized without an elution step. The enrichment factor was calculated to be about 20 for catechins. The relative chemiluminescence intensity increased linearly with concentration of catechin from 5.0 × 10⁻⁹ to 1.0 × 10⁻⁶ M and the limit of detection was 1.0 × 10⁻⁸ M. The proposed method was applied to determine catechin in green tea. The recoveries are from 90% to 110% which benefits the actual application for green tea samples.

Introduction

Rapid development in biotechnology has led to advances in the fields of pharmacy, materials and environmental sciences, while current separation technology struggles to meet the demands of a fast, efficient, high flux method of separation. In recent years, sample pre-treatment technology was focused on solvent-free and miniaturized methods. Solid-phase microextraction (SPME) with its advantages of solvent-free extraction, low sample consumption, simple and flexible operation has been widely used. Since being introduced by Pawliszyn in the early 1990s, the technology and application of SPME have advanced rapidly. Polymer monoliths as a new type of structural material used in sample preparation have several merits of simple preparation, high permeability, and good biocompatibility. Extraction materials currently include magnetic beads, membranes, monolithic columns, etc., of which the monolithic column with its good permeability, high gathering efficiency, and convenience has been widely used as a solid phase extraction material. The glycidyl methacrylate (GMA) monolithic column has epoxy groups that are easily modified to various functional groups, and has been used in sample concentrating.

The microfluidic system, which features analytical automation, integration and micromation, has considerable advantages in reducing reagent consumption and shortening the analysis time. In order to achieve an on-line sample pretreatment method in a microfluidic chip, an integrated microfluidic system was desired. The monolithic column method is suitable for complex sample pretreatment in a microfluidic analysis system. In recent years, several groups have designed many microfluidic devices that integrated the monolithic column in chips as a sample preparation technique.

Chemiluminescence (CL) is the phenomenon of light emission from the reaction between two or more than two compounds. An external light source is unnecessary for CL, which eliminates stray light and the instability caused by fluctuations from the light source. This reduces noise and thus improves the signal to noise ratio. Therefore, there are many reports concerning the application of CL as a detection system in microchips, e.g., CL resonance energy transfer-based detection for microchip electrophoresis, microfluidic CD₄⁺ T-cell counting, CL lensless imaging for personalized diagnostics through multiplex bioanalysis and CL flow-through DNA microarray analysis. These successful applications of CL as a highly sensitive detection method in microchips encourage us to develop a more simple microfluidic device which can not only concentrate the target compounds on the chip channel, but also detect at the same channel using CL. However, in many cases, the CL detection suffers from the effect of reagents and low selectivity. A method which can concentrate the target compounds in a solid phase and then react with CL reagents directly is desirable.

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In this work, we try to use a monolithic column for the solid-phase microextraction of our selected sample. The CL reagent KMnO₄ was used to react with the target compounds in the monolithic column. Both solid-phase microextraction and CL detection were integrated into a microfluidic chip. The analysis of tea polyphenols was used as an application example in the present work. Based on the reported KMnO₄-catechins CL reaction,24–26 their CL signals can be detected when a KMnO₄ solution is pumped into the chip channel. A reusable catechins analysis system was achieved with high sensitivity and low reagent consumption. The on-line preconcentration and detection can be realized without an elution step.

**Experimental**

**Chemicals and materials**

Glycidyl methacrylate (GMA, 97% pure), ethylene dimethacrylate (EDMA, 98% pure), ethylenediamine (99% pure), cyclohexanol, 1-dodecanol, 3-(trimethoxysilyl) propyl methacrylate (γ-MAPS, 95% pure), and epichlorohydrin (EC), catechin (CCN), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG) were obtained from J & K Scientific Ltd. (Beijing, China). Standard solutions (C, EC, ECG, EGCG) were prepared in 2.0 M sulfuric acid solution. The diluted solutions were made just prior to use. Silicon wafers were from Xilika Company (Beijing, China). The CL solution of KMnO₄ was prepared in 2.0 M sulfuric acid solution. The diluted solutions were made just prior to use. Silicon wafers were from Xilika Crystal polishing Material Co., Ltd. (Tianjin, China). Negative photoresist (SU-8 2050), and developer were purchased from Microchem Corp. (Newton, MA, USA). Poly(dimethilsiloxane) (PDMS) and curing agent were obtained from Dow Corning (Midland, MI, USA). Glass slides were obtained from Fisher Scientific (Pittsburgh, PA, USA). All of the other reagents not mentioned above were of analytical grade.

**Apparatus**

The batch CL experiment using a 3 mL glass cuvette and the flow CL experiment generated on the chip were performed with an BPCL ultrawake chemiluminescence analyzer (Institue of Biophysics, Chinese Academy of Science, Beijing, China). Fourier transform infrared (FT-IR) measurements were carried out with a Perkin Elmer 100 FT-IR spectrometer (Massachusetts, MA, USA). The transmission electron microscope was operated at 100 kV, JEOL 6301F (Japan).

**Fabrication of microfluidic chip**

The microfluidic chip for chemiluminescence detection of tea polyphenols was fabricated from poly (dimethilsiloxane) (PDMS) produced by standard soft lithography techniques.13,27 As shown in Fig. 1a and 1b, the microchannel designed was 20 mm in length, 250 μm in width and 80 μm in depth. The radius of the circle in the center of the channel for placing the monolithic column was 5 mm. The layout of the chip was fabricated through coating negative photoresists SU-8 2050 on a silicon wafer cleaned by piranha solution. After photolithographic patterning of a photoresist coated silicon wafer, a mold that carried a relief of the desired microstructure was generated. A 10 : 1 premixed PDMS prepolymer was prepared according to the manufacturer instructions, degassed in a vacuum chamber for 1 h and then poured on the mold and cured in a 70 °C oven for 2 h. The PDMS was cut from the mold with a surgical scalpel and then the mold was carefully peeled off. The channel inlet and outlet were punched by a flat-tip syringe needle. The channels were sealed with a glass cover slip after oxygen plasma treatment for 90 s.

**Preparation of generic poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolithic column**

A custom quartz glass tube (3 cm × 5 mm, i.d.) was first activated by 1.0 M NaOH and 1.0 M HCl for 30 min, respectively. A 3-(triethoxysilyl) propyl methacrylate ethanol solution (2 : 8, v/v) was used to fill the activated quartz glass tube. After sealing the two ends of the quartz glass tube with silicon rubber, the reaction was allowed to perform at 40 °C for 6 h. Then, the residual solution was driven out and the quartz glass tube was washed thoroughly with methanol. Nitrogen gas was driven through the capillary to dry the inner surface before further use. A polymerization mixture containing 24% (w/w) GMA (functional monomer), 16% (w/w) EDMA (crosslinker), 15% (w/w) cyclohexanol (porogens), 45% (w/w) 1-dodecanol (porogens), and 1% AIBN initiator (w/w with respect to the total monomer content) was sonicated to obtain a homogeneous solution, and then purged with nitrogen for 15 min. The quartz glass tube was filled with the polymerization mixture using a syringe and sealed with rubber septa at both ends. The polymerization was carried out in a water bath at 60 °C for 12 h. The column was then rinsed with acetonitrile to remove the porogens.

**Amine-modified monolithic column**

The monolithic capillary obtained was modified with diethylamine to introduce weak amino-exchange groups at the surface of the polymeric skeleton. The reaction scheme is presented in Fig. 2. The column material was prepared by soaking in ethylenediamine solution and placed in a 70 °C water bath for 4 h. This completed the preparation of the diethylenimine-modified poly(GMA-co-EDMA) monolithic material used for SPME.

**CL measurements**

The configuration of the on-chip SPME-photomultiplier tube (PMT) used for the study is shown in Fig. 1c and 1d. Because potassium permanganate has a strong oxidability, in acidic medium it is capable of redox reacting with a majority of organic compounds, in the course of which the energy transfer is often accompanied by a chemiluminescence reaction. Therefore, in the experiment we used concentrated sulfuric acid to control the pH of the potassium permanganate solution, rather than other buffer solutions, since other components in the buffer solutions will interfere with the chemiluminescence reaction. We placed
a photomultiplier tube which connected to a BPCL ultra-weak luminescence analyzer directly under the PDMS microfluidic channel for CL detection. The CL profile and intensity were displayed and integrated for a 0.1 s interval while the voltage of PMT was set at 1.3 kV. The sample solution was connected to the SPME column by a black tube. A solution of KMnO4 (pH 2.0), which was prepared fresh before each experiment, was then flowed through the microfluidic channel after finishing the extraction.

Pre-treatment of tea sample

Commercial green tea leaves harvested in China were obtained from a nearby supermarket. The dried tea leaves (1.0 g) were extracted with 100 mL of hot water at 80 °C for 10 min and the leaves were filtered out by filter paper. The solution was diluted to an appropriate concentration (10 times) with water before detection and the sample pre-treatment was carried out just before analysis.

Results and discussion

Preparation of poly(GMA-co-EDMA) monolithic column

The preparation of the poly(glycidyl methacrylate-co-ethylene dimethacrylate) (GMA-EDMA) monolith is shown in Fig. 2. The principle was based on the report from Svec’s group.28 It was polymerized in situ from GMA and EDMA by heating in the presence of porogenic solvent and initiator. The epoxide groups of the polymerized glycidyl methacrylate were allowed to react with diethylamine according to the reaction. The setup of the SPME in the microfluidic channel is shown in Fig. 1a and 1b. Monoliths were prepared at the desired location in the quartz glass tube which was integrated into the microfluidic channel. A cross-linker provides the material with high mechanical stability and contributes to the formation of the macroporous structure. Special care was taken to eliminate oxygen (a typical inhibitor in free radical polymerization) during preparation. Therefore, the prepared prepolymer solution was degassed by sonication to eliminate the dissolved oxygen. To remove the cross-linker, porogens and initiator in the SPME, the prepared SPME was rinsed thoroughly with acetonitrile and water.

Porogenic solvents are another important part of the polymerization mixtures. Their function is to dissolve all monomers and initiator to form a homogeneous solution and to control the phase separation process during polymerization in order to achieve the desired pore structure. A porogenic mixture consisting of methanol and hexane was considered to be suitable for the preparation of the monolithic concentrators.

This porogenic solvent was developed via an extensive study of a large number which will be published elsewhere. They are characterized by very large pores that provide the monoliths with a low flow resistance and allow the use of a high flow rate. Flow rates of up to 50 μL min⁻¹ can be achieved without causing mechanical damage to the monoliths.
Since the tea polyphenols are hydrophobic and aromatic with a benzene ring and a phenolic hydroxyl, the poly(glycidyl methacrylate-co-ethylene dimethacrylate) was modified on the hydrophobic skeleton to form a strong bonding between the amino functional group and the analyte. The role of hydrophobic function, $\pi-\pi$ interactions and hydrogen bonding, can increase the adsorption of the monolithic column.

Fig. 3a is the appearance of the scanning electron micrograph prepared from the poly(GMA-co-EDMA) monolithic column. We can see from this figure, the overall size of the column has a regular and uniform shape. The surface of the whole column shows the structure of particle clusters. Microsphere particles interconnected into a continuous skeleton and between the skeleton there were interconnected macroporous structures. These micron-scale macropores provide good permeability. It is conducive to increase the extraction capacity and accelerate the exchange of analytes in the material. From Fig. 3a we can also see that there is no obvious cleft on the surface of the monolithic material.

The chemical state of the modified amino monolithic column surface was characterized by FT-IR spectroscopy. As the comparison shows in Fig. 3b, the modification of the monolithic column surface by the ethylenediamine solution gives a newly shown in Fig. 3b.
developing absorption peak at 3300 cm\(^{-1}\) and 3570 cm\(^{-1}\), which is assigned to \(-\text{NH}_2\) groups and \(-\text{OH}\) groups respectively. The shift of the peak was caused by the \(-\text{NH}_2\) group being modified onto the monolithic column. It suggests that the \(-\text{NH}_2\) groups have been successfully grafted onto the monolithic column after modification. And the vibrations of C\(=\text{O}\) and C\(-\text{O}\) (1730 cm\(^{-1}\) and 1150 cm\(^{-1}\) respectively) were observed. They were covalently bonded to the monolithic column frameworks enhancing the stability and hydrophobicity of the monolithic column in aqueous systems.

Sample pretreatment using the prepared material for SPME

The extraction capacity of the polymer monolithic column was tested with series of standardized catechin solutions, the concentration range of which varied from \(1.0 \times 10^{-9}\) to \(1.0 \times 10^{-3}\) M. The results indicated that the extraction capacity increased with increasing concentration of the analyte. It demonstrated that the polymer monolithic column exhibited high extraction potential for the target analytes. The large extraction capacity was due to the extraction mechanism, which was mainly nonspecific adsorption derived from the interaction of polar groups between the analytes and functional monomers in the polymeric sorbent.

The monolithic column was synthesized inside a quartz glass tube (3 cm \(\times\) 4 mm i.d.). An activation step of the polymer monolithic column involves methanol and water (1 : 1, v/v) flowing through the extraction column with a speed of 20 \(\mu\)l min\(^{-1}\). Then a rinse step with acetonitrile and water (1 : 1, v/v) was added to the polymer monolithic column with a speed of 20 \(\mu\)l min\(^{-1}\) after the CL reaction step in order to wash off salts and/or superfluous catechins remaining on the surface of the column. These rinse solutions were subsequently analyzed for the presence of catechins. Catechin was not detected in any of these solutions indicating that no detectable amount of catechins remained on the column after the CL reaction step.

To test the long-term stability and reuse of the polymer monolithic column, a large number of the measurements described above were carried out using a single microfluidic chip containing the same polymer monolithic column. The monolithic column used over a period of 2 months with 30 injections of different concentrations of catechins showed no evident oxidation due to K\(\text{MnO}_4\), indicating the reusability of the column.

The extraction efficiency of the polymer monolithic column for catechins was studied. The extraction efficiency can be represented by the enrichment factor,\(^{29}\) which is the ratio of the peak area obtained with on-chip polymer monolithic column SPME (Fig. 4a) to that of the corresponding analyte in the extraction area obtained with on-chip polymer monolithic column SPME for the KM\(\text{MnO}_4\)-hydroxyl CL detection system as shown in Fig. 5a. The CL intensity increased steadily with the increasing KM\(\text{MnO}_4\) concentration from 0 to \(1.0 \times 10^{-6}\) M and then the CL intensity began to decline from \(1.0 \times 10^{-6}\) M to \(1.0 \times 10^{-4}\) M in the flow-injection system. Therefore, \(1.0 \times 10^{-6}\) M of KM\(\text{MnO}_4\) was selected in the CL system.

As shown in Fig. 5b the CL intensity was increased with increasing the pH value and reached a maximum at pH 2.08 in the flow-injection system. There was a much weaker CL signal when the pH was above 4.30. The pH of the KM\(\text{MnO}_4\) solution was demonstrated to have a significant effect on the CL intensity, and pH 2.08 was selected for the following CL reaction.

As shown in Fig. 5c, the flow rate of sample solution from 0 \(\mu\)L min\(^{-1}\) to 50 \(\mu\)L min\(^{-1}\) was tested in our experiment and the peak value of the CL intensity was the largest in the 30 \(\mu\)L min\(^{-1}\) infuse rate experiment. But the noise was also increased with increasing rates. Considering analytical precision and solution consumption, the optimal flow rate was 30 \(\mu\)L min\(^{-1}\).

Time control of monolithic column sample extraction is necessary. The effects of extraction time were determined in the range from 1 to 30 min. The results shown in Fig. 5d indicated that 5 min was effective for our experiments.

Effects of catechin type on CL intensity

Under the optimized experimental conditions, the CL behavior of CCN, EG, EGCG, ECG and EGCG single standard solutions at the concentration of \(1 \times 10^{-7}\) M with the proposed method was studied, respectively. As shown in Fig. 6, the variability of CL intensity from different catechins was small, indicating that the CL doesn’t depend on the catechin type. This study demonstrated that the total catechin amount can be measured by this CL reaction method.
Analytical performance of on-chip CL method

Under the optimum experimental conditions, the CL intensity increased linearly in the concentration range from $5.0 \times 10^{-9}$ M to $1.0 \times 10^{-6}$ M for catechin (Fig. 7). The detection limit of this method is $1.0 \times 10^{-9}$ M catechin at S/N ratio of 3. The relative standard deviation (RSD) of ten parallel measurements (for single column) at $1.0 \times 10^{-9}$ M catechins in the CL system was 4.8%. The column-to-column RSD was found to be 5.7% ($n = 5$). The results indicate that the proposed CL method has good linearity and high sensitivity and precision.

Interference studies

Compounds of similar structures to catechins which could interfere in the detection were evaluated. The interferences were added into $1.0 \times 10^{-8}$ M catechins and examined by the proposed method. Most ions did not interfere with the determination of high concentration levels of catechins solution. The
effects of some components of green tea, such as glucose and sucrose, caffeine, albumin, glutamic acid were also investigated and their interference could be excluded. Glutamic acid with the concentration beyond $2.0 \times 10^{-4}$ M could affect and quench the CL. But their interferences could be neglected due to the low concentration in samples and the appropriate dilution. The results are summarized in Table 1, which demonstrated the high selectivity of the present method for the determination of green tea.

**Application**

The proposed method was applied to the determination of green tea (Fig. 7). Different kinds of green tea were purchased from a market. Samples were prepared as described above. The results showed that the coexisting substances in the samples did not interfere with the determination. Recovery tests were performed to evaluate the accuracy of this method. The results for the contents and recoveries are summarized in Table 2. The recoveries from 90% to 110% indicate that the method outlined in this experiment is a direct and simple way to analyze the catechins in green tea.

Aside from the inherent advantage of CL detection, the high sensitivity was also due to the existence of large numbers of recognition sites for catechins in the column. Hence, all the catechins adhered on the monolithic column and can react with KMnO₄ solution. In addition, this strategy can be also utilized in the detection of other tea samples or analytes, since any aptamer-target binding event can be translated to an adsorption modification on the monolithic column principally. Using this method, only 5 min was required to finish the sample extraction, while the CL detection can also be accomplished in less than 5 min. Consequently, due to the features of microfluidic devices, the time and sample volume consumption were significantly decreased compared to other methods for catechin detection, and it was exactly in conformity with the desire of sample detection.

**Conclusions**

In conclusion, a simple and highly efficient microfluidic chip was designed by the combination of solid phase micro-extraction and chemiluminescence. As to demonstrate the applications of the microfluidic chip, polyphenols were extracted from green tea and detected via on-line chemiluminescence using it. A reusable catechins analysis system was achieved with high sensitivity and

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**Table 1** Assessment of the interference by metal ions and organic compounds in the CL method for green tea

<table>
<thead>
<tr>
<th>Species added</th>
<th>Mole ratio ($C_{\text{species}}/C_{A\text{A}}$)</th>
<th>Variation of the CL peak height (%) $n = 5$</th>
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</thead>
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<tr>
<td>K⁺</td>
<td>1000</td>
<td>0.12</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>1000</td>
<td>1.02</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>1000</td>
<td>0.69</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1000</td>
<td>1.12</td>
</tr>
<tr>
<td>Glucose</td>
<td>200</td>
<td>-1.48</td>
</tr>
<tr>
<td>Sucrose</td>
<td>200</td>
<td>2.32</td>
</tr>
<tr>
<td>Caffeine</td>
<td>200</td>
<td>4.12</td>
</tr>
<tr>
<td>Albumin</td>
<td>150</td>
<td>2.62</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2</td>
<td>-4.61</td>
</tr>
</tbody>
</table>

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**Table 2** Result of catechins determination and recoveries in three green tea samples

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Added ($\times 10^{-7}$ M)</th>
<th>Observed ($\times 10^{-7}$ M)</th>
<th>Recovery (%)</th>
<th>RSD (%) $n = 3$</th>
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<tr>
<td>1</td>
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<td>—</td>
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<tr>
<td></td>
<td>0.40</td>
<td>0.63</td>
<td>103</td>
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<td>110</td>
<td>5.8</td>
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<td>0.10</td>
<td>0.31</td>
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<td>—</td>
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<td>0.40</td>
<td>0.57</td>
<td>93</td>
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<td></td>
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<td></td>
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<td>90</td>
<td>6.2</td>
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<tr>
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<td>—</td>
<td>—</td>
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<td></td>
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<td>1.22</td>
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<td>0.76</td>
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<td>0.20</td>
<td>0.58</td>
<td>105</td>
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very low reagent consumption. The online preconcentration and detection can be realized without an elution step. Good stability and reusability of SPME was achieved which benefits the actual application for green tea samples. And the high sensitivity of the CL was successfully introduced into the detection of catechins in samples, which can be a cheap and effective method for the quality detection of green tea.

Acknowledgements

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References