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An ionogel composite including copolymer nanowires for disposable electrochemiluminescent sensor configurations

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I.F. Díaz-Ortega[a], J. Ballesta-Claver[a], M. Cruz Martín[a], S. Benítez-Aranda[a] and L.F. Capitán-Vallvey[a,b].

Aniline derivatives such as luminol and benzidines can be electropolymerized for electrochemiluminescent sensors preparation. However, the development of these sensors requires formulations to improve the chemical stability and photoemissive and electrochemical properties. The use of ionic liquids confined in gels (ionogels) can provide a material for electrochemiluminescent sensor preparation and more specifically by means of hybrid organic–inorganic composites which provide flexibility, thermal stability, and ionic transport. By using this configuration we prepared nanowires of luminescent polymers by electropolymerization in the pores of a hydrophilic silica ionogel with chitosan offering a biocomposite that can be prepared in disposable screen-printed gold electrodes. The properties of the prepared biocomposite have been characterized by emissive luminescence, cyclic voltammetry, electrochemical quartz crystal microbalance and scanning electron microscopy. The results show that the electrochemiluminescent copolymer grows efficiently as nanowires in the pores of the biocomposite forming sensing films that produce an intense luminescent blank signal due to the influence of the ionic liquid. Using hydrogen peroxide as analyte, which acts as a scavenger reducing the emissive signal and resulting in a novel electrochemiluminescence configuration for sensing. This configuration broadens the field of electrochemiluminescence sensors due to the biocompatibility of the developed ionogel membrane.

1. Introduction

Electrochemiluminescence (ECL), considered as the generation of light-emitting species by means of strong oxidizing and reducing agents[1], has great potential as an analytical technique for detection in immunoassays, DNA probe assays, sensors and biosensors, mainly in flow and optical fibre formats, high throughput analysis, microfluidic devices and chromatographic and electrophoretic systems[2-5]. Proof of the growing interest in the scientific community in this technique is the exponential growth of ECL papers devoted to analytical chemistry (3,056 papers in the last 20 years, doubling every 7 years).

The ECL can be carried out mainly by two ECL pathways: hot electron-induced and co-reactant. In hot electron-induced pathways, the ECL emission is initiated by hot energetic electrons from a thin insulator film covered in the conductor/insulator/electrolyte surface during cathodic pulse polarization[6]. Sun et al.[7] demonstrated that screen-printed carbon electrodes instead the common Al/Al_2O_3 electrode can generate hot electrons; proposing procedures for quercetin and dissolved oxygen determination[8].

However, more common is the co-reactant ECL pathway where one-directional potential scanning on the electrode in the presence of luminophore and co-reactant is applied, generating co-reactant intermediates that are either strong reducing agents (in oxidative–reduction ECL) or strong oxidizing agents (in reductive–oxidation ECL)[9].

When this co-reactant approach is used for disposable format sensors, it is needed the immobilization of sensing chemistry, including luminophores, by using different immobilization strategies depending on the analytical scheme utilized and the type of luminophore.

In case of ruthenium complexes as luminophore, mainly Ru(II)-bis(2,2'-bipyridyl), the common procedures for immobilization consist of cationic exchange polymer, such as Nafion[10]; inclusion in a self-assembled monolayer of ruthenium complex-aminopropyl imidazole on a gold-deposited screen printed cell containing carbon nanotubes[11] or adsorption in electrochemically oxidized graphite on screen printed cells[12].

However, the most popular chemistry is that based on luminol, for which different forms of immobilization have been proposed including entrapment in membranes, ion-exchange, covalent and electropolymerization[13]. The ability of luminol to undergo electropolymerization by cyclic voltammetry is an interesting option to produce electrochemiluminescent polymers on the transducer surface. It has been prepared on graphite, glassy carbon, platinum, gold, screen-printed and indium tin oxide electrodes, typically in acid conditions[14-20].

In previous papers we studied the improvement in luminol immobilization as a copolymer with 3,3′,5,5′-tetramethylbenzidine[13] or biotinylated pyrroles[21] for disposable ECL sensors preparation by means of electrochemical copolymerization in terms of operational

and temporal stability because the polymerization of luminol alone has some drawbacks such as bad mechanical properties, luminol adsorption in the polymer, short reusability and poor electroactivity in basic medium [18;22].

In order to improve the immobilization of reagents using the electropolymerization process, two approaches can be used such as: a) hydrogels and b) ionogels. In the case of hydrogels i.e. gels based in water, can be cited the hybrid described by Montilla et al.[23] where silica gel is used to produce polyaniline nanowires by electrodeposition generating a polyaniline/silica composite material. With the aim of improving the ability of an ECL sensing film to host biorecognition elements and better hydration conditions, recently Loïc Blum et al.[24] have developed electrochemiluminescent biosensors based on hybrids of electrogenerated polyluminol nanowires and hydrogels such as silica and calcium alginate for hydrogen peroxide and choline detection.

The second approach for reagent immobilization consist on the preparation of ionogels, a new class of hybrid materials, characterized by the incorporation of ionic liquids (ILs) in the gel. ILs are salts of organic cations such as ammoniums, phosphoniums, imidazoliums, or pyridiniums[25]. These salts are characterized by their high ionic conductivity and their wide electrochemical potential window (up to 6.0 V). At this time they are widely considered as "green" materials due to their possibility recyclability in most applications[26] and for their chemical stability. One of the reasons that ionogels can be suitable for ECL analysis is the improvement in both electrochemical and luminescent properties by the easy diffusion of analytes into the immobilized IL phase[25].

To date, ionogels consist of ILs hybridized with other components to improve the membrane properties, which may be: 1) organic, 2) inorganic or 3) hybrid organic–inorganic (e.g. polymer and inorganic fillers)[27].

The first case includes two modalities: a) organic ionogels where low molecular weight gelators are used such as L-glutamic or aspartame acids and b) polymer gels, which combine the mechanical flexibility of a polymer and the characteristic conductivity, like the described compatibility of imidazolium salts with poly(methylmethacrylate), among others.

The second case is based on inorganic ionogels called ''bucky'' gels, such as carbon nanotubes dispersed in ILs where a gelation effect occurs (crosslinking effect) and silica-based ionogels. Silica is a suitable matrix because its properties, like pore size, orientation, and shape, can be easily tuned during synthesis[28]. In this case two modalities we be distinguish: a) dispersion of silica nanoparticles into ILs, where bare silica colloids were shown to be unstable in ILs and b) sol–gel processing, where the IL can be used as a template that is removed at the end of the gelation or as an enhancer of the conductivity, e.g. monolithic materials.

With respect to the possibilities of ECL sensor preparation using polymers, we focused on the third case, composite (hybrid organic–inorganic) ionogels that consist of the association of molecular organogelators (polymers) and inorganic nanoparticles providing flexibility, thermal stability, and ionic transport. As an example Leroux et al.[29] prepared polymer nanocomposites by incorporating silica nanofillers of silicon tetraethyloxide covalently bonded to the polymer chains as poly(methylmethacrylate) with imidazolium salts as bis-(trifluoromethanesulfonyl)imide.

Due to the potential of this last type of ionogel, this paper explores the feasibility of preparing ionogels using different ILs on screen printed cells for sensing purposes combining organic materials such as the copolymer luminol with 3,3′,5,5′-tetramethylbenzidine as luminophore and chitosan with inorganic silica by means of electrochemical growing of copolymer nanowires in the pores of the ionogel matrix. To date, this configuration has not been used and is proposed in this work.

2. Experimental

2.1 Chemicals

As monomers for electropolymerization, stock solutions of 1 mM of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), 97% and 3,3',5,5'-tetramethylbenzidine, 97% (TMB), both supplied by Sigma (Sigma-Aldrich Química S.A., Madrid, Spain https://www.sigmaaldrich.com/spain.html) were used. A stock buffer phosphate solution containing 0.5 M phosphate (Na_2HPO_4) and 0.25 M NaCl adjusted to the necessary pH by adding NaOH or HCl of appropriate concentrations was prepared. Different ionic liquids (ILs) were used, such as triethylsulfonium bis(trifluoromethylsulfonyl)imide ([TES][TFSI], 97%), 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4], 97%), 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM][BF_4], 97%), 1-decyl-3-methyl-imidazolium chloride ([decyl-MIM][Cl], 96%), 1-hexyl-3-methyl-imidazolium hexafluorophosphate ([HMIM][PF_6], 97%), 1-dodecyl-3-methyl-imidazolium iodide ([dodecyl-MIM][I], 95%); 1-hexyl-3-methyl-imidazolium tetrafluoroborate ([HMIM][BF_4], 97%), 1-methyl-3-octyl-imidazolium chloride ([MOIM][Cl], 97%), 1-buthyl-3-methyl-imidazolium hexafluorophosphate ([BMIM][PF_6], 97%). Hydrogen peroxide solutions were prepared daily from H_2O_2 33% w/v (110 vol.) that was supplied from Panreac (Panreac Química S.L, Barcelona, Spain. http://www.panreac.es/) and was standardized iodimetrically. Other reagents and materials also used were: 1, 1'-ferrocenedicarboxylic acid (97%) and Nafion 5% wt/wt solution in a mixture of aliphatic alcohols (Sigma-Aldrich). Chitosan was prepared by dissolving and shaking for 4 hours 1 g in 100 ml water containing 1 ml of acetic acid (95.5 %, Panreac); the prepared dilution of 1 $g \times l^{-1}$ stock solution of chitosan was prepared by dilution with 1% acetic acid. Tetraethyl orthosilicate (TEOS) stock solution 2.4 M was prepared by mixing, under vigorous stirring at room temperature, 4.46 ml of TEOS, reagent grade 98% (Sigma-Aldrich), 1.44 ml of water and 6 µl of 4.0 M HCl in a closed vial for 2 h. After that, 1 ml of this solution was mixed carefully with 1 mL of water and submitted to rotaevaporation periodically controlling the weight loss until some 0.62 g due to the elimination of ethanol by alkoxide hydrolysis reaction. The composite was prepared by using the stock solutions indicated above (see Fig. 1) obtaining the ionogel solution. Reverse-osmosis type quality water (Milli-Q Plus185 from Millipore, Molsheim, France) was used throughout.

2.2 Apparatus and software

The ECL emission from screen-printed cells was measured using an H8529 photomultiplier (PMT) interfaced to a C8855 USB photo counting unit, both from Hamamatsu (Hamamatsu Technologies K.K., Shizuoka, Japan), connected to a PC. The potentiostat used was an Autolab PGSTAT 128N with F120 module for chrono-coulometric measurements (Metrohm Autolab B.V., Utrecht, The Netherlands). The arrangement used for ECL studies has been described in previous works[30] and basically consists of a black box that contains two black methacrylate piece holders, where one piece

fits on top of the other, one to insert the electrochemical cell in a fixed position and the other to hold the PMT.

An electrochemical quartz crystal microbalance (EQCM) (Metrohm Autolab B.V.) was used to study the polymerization process by recording the change in resonant frequency of a 6 MHz EQCM Crystal Au/TiO_2 quartz crystal oscillator. A Cary Eclipse fluorescence spectrometer (Varian Australia Pty Ltd.) was used for luminescence measurements. As the conventional Scanning Electron Microscopy technique induces an instantaneous dehydration of the sample due to the high vacuum needed, we used Environmental Scanning Electron Microscopy (ESEM), which makes it possible to examine any specimen, wet or dry, insulating or conducting in situ and close to its natural state. An ESEM FEI model Quanta (FEI Co., Hillsboro, Oregon, USA) 3.5 nm was used for imaging with increases ranging from 7x to 1,000,000x and equipped with a ZEISS DSM 250 electronic microscope prepared to perform energy dispersive X-ray spectroscopy (EDX) microanalysis. Samples were mounted on Peltier cooling stages set at 2ºC with a working distance of about 10 mm and a humidity of 80 %. A Crison digital pH-meter (Crison Instruments, Barcelona, Spain) with a combined glass-saturated calomel electrode was used for pH measurement.

Software programs used were: Statgraphics centurion software package (Manugistics Inc. and Statistical Graphics Corporation, USA, 2007); Microsoft Office Suite 2013 (Microsoft Corp., Redmond, WA, USA); CSW32 v.1.3.3 (2001); CSWAIA v 1.7.3 (2001) (Dataapex software, Czech Republic); NOVA v.1.8.17 (2005-2012) (Metrohm Autolab B.V.) and GPES v.4.9 (2007) (Ecochemie, The Netherlands).

2.3 SPE cells and pre-treatments

Low temperature (LT) gold screen-printed cells (SPE) were selected for sensor preparation due to our previous studies for a convenient electropolymerization of aniline derivatives[13]. These cells were supplied by Dropsens S.A. (Oviedo, Spain http://www.dropsens.com/). The cells consist of a round-shaped working electrode, a counter electrode, both of the same material, and a silver pseudo-reference electrode on a ceramic support. Before being used, the electrochemical cells were tested for uniform behaviour. In order to prepare a receptacle on the disposable electrochemical cell, we printed the round form on the working electrode by screen printing using a plastisol ink containing 30% Puff additive, which expands during heating (170 – 200 ºC), giving a raised print used as a receptacle of some 50 µl capacity.

A first pre-treatment of gold SPE cells was carried out by immersing them in 0.2 M H_2SO_4 and applying three voltammetric sweeps between -0.2 and 1.0 V. Then the SPE cells were dried for 10 min at room temperature. After the addition of the ionogel solution to the SPE cell (as shown in the following section), a second pre-treatment was carried out by immersing the cell in the same 0.2 M H_2SO_4 solution and applying 40 voltammetric sweeps between -0.2 and 1.0 V. In both cases, a 0.1 V × s^{-1} was set.

2.4 Ionogel sensor preparation

A ionogel solution was prepared by mixing 20 µl TEOS stock solution 2.4 M, 20 µl of 1.0 g × l^{-1} chitosan solution and 30 µl of [TES][TFSI] stock solution in an ultrasonic bath. This ionogel solution can be used for a week if kept at -20 ºC degrees. To prepare the composite and prompt the gelation, 70 µl of pH 7.4 PBS solution containing 0.2 M phosphate and 0.25 M NaCl was added finally to the inonogel solution. 5 µl of this mixture was dropped onto the working electrode of the gold LT SPE cell, waiting for two minutes at room temperature after use.

After the second pre-treatment, the luminol-TMB copolymer was grown in the pores of the prepared composite on the SPE cell by cyclic voltammetry working between -0.2 and 1.0 V, at 0.1 V×s^{-1}, with 20 cycles from a solution of 1 mM luminol and TMB (1:1 ratio) in 0.2 M H_2SO_4 (standard conditions). Then, the sensor was washed with 0.2 M pH 7.4 phosphate buffer solution to eliminate adsorbed luminol or TMB traces. The prepared cells were stored in dark conditions at 4ºC until use (see Fig. 1).

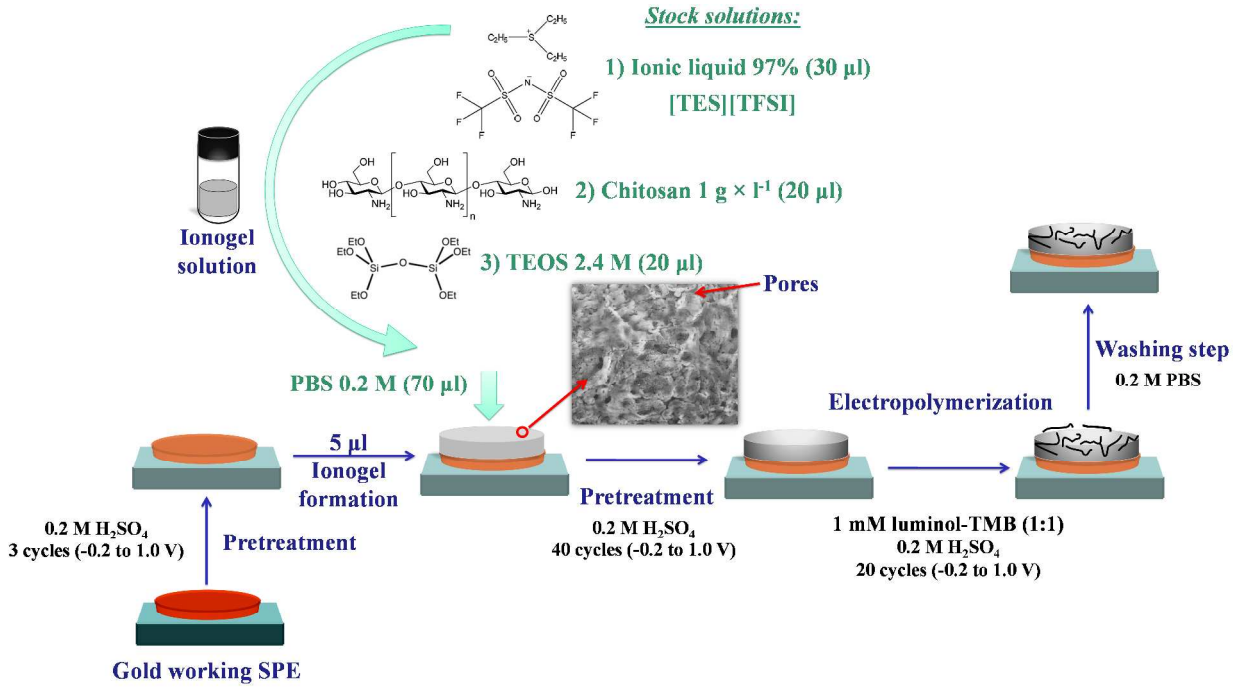

Fig.1. Schematic illustration concerning to the preparation steps of the composite on the disposable sensor.

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2.5 ECL procedure and signal measurements

The experimental system for ECL measurement has been described elsewhere using the instrumentation showed in apparatus and software section[30]. Aqueous standard solutions of different concentrations of H_2O_2 at pH 9.0 adjusted with stock solution saline phosphate buffer were prepared. 50 µl of the H_2O_2 solution was deposited into the receptacle of the SPE, which was then placed in a homemade holder and covered with a lid holding the PMT. The ECL signal obtained for analytical purposes was the integral signal over the course of 2 min coming from consecutives pulses of 0.6 V for 1 s each with a dwell time of 10 s.

3. Results and discussion

The goal of this study is the preparation and characterization of SPE cells with an ionogel composite[31] with biocompatibility and biodegradability properties for electrochemiluminescent detection. This biocomposite is prepared by a ionogel solution that consist on the biopolymer chitosan, TEOS stock solution and an IL, inserting ECL nanowires into the pores of the solid ionogel prompted by 0.2 M PBS solution by in situ copolymerization of luminol and an aromatic amine: 3,3',5,5'-tetramethylbenzidine. A study of biocomposite electro-preparation and ECL characterization on different SPE cells was performed.

3.1 SPE sensor composition

Sensing biocomposite layer

In previous works, our research group has studied the influence of reagents and the composition of the ECL transduction layer in sensor preparations using chitosan and polymers to enhance the mechanical and electrochemical properties[13;32;33]. However, the use of sol-gel membranes can improve the biocompatibility but lacks stability and conductivity and undergo dehydration and cracking over time. A combination of different polymers such as the synthetic anionic polymer nafion and the cationic polysaccharide chitosan can improve the above characteristics.

In the first instance, a sol-gel configuration was used by immobilizing the electroformed poly(luminol-TMB) as nanowires obtaining an intense ECL signal with H_2O_2 (see Table 1). However, the membrane was brittle and cracks some minutes after preparation due to the rapid dehydration of the membrane, resulting in an unstable sensor. To overcome the observed deficiencies it was necessary to include other components in the membrane in order to produce a hydrogel. Nafion, an anionic sulfonated perfluorinated vinyl ether polymer, widely used as an ion-exchange polymer, is described as a hydration agent in the preparation of sol-gel sensors by water adsorption at the hydrophilic ionic groups forming ionic clusters[34]. In this case, its use for composite preparation generates a reduction in the ECL signal of some 60% against the cell containing only sol-gel and a retraction in the membrane with fast cracking in a period of minutes. This behaviour can be attributed to the fact that nafion decelerates the electron transfer as well as the diffusion of luminol[35].

Moreover, chitosan, a positively charged natural polysaccharide, generates a sol-gel that is highly porous and structured materials creating a hydrophilic environment[36]. The biocomposite is prepared by entrapping the chitosan in the porous silica matrix when the pH of the solution containing sol nanoparticles of silica and chitosan is moved to neutrality, starting the crosslinking. In this biocomposite, the electron mobility is reduced as is, consequently, the ECL signal to some extent: some 21.5% with respect to the SPE cell containing only sol-gel, although the biocomposite membrane shows good mechanical properties.

Table 1 ECL analytical signal of the different composites to obtain a better sensor configuration. In all cases, the analyte was hydrogen peroxide 5.0 µM and electropolymerized nanowires of poly(luminol-TMB) has been included in all the composites.

Composite	Layer Composition	ECL analytical signal a.u. × s	%
Sol-gel	TEOS	397	84.2
Hydrogel	TEOS/nafion	77	16.3
	TEOS/chitosan	311	66.1
	TEOS/chitosan/nafion	155	32.8
	TEOS/chitosan/nafion/ferrocene	11	2.3
	TEOS/chitosan/ferrocene	20	4.2
Ionogel	TEOS/chitosan/[TES][TFSI]	472	100.0

The combined use of chitosan and nafion along with sol-gel in a hydrogel does not provide any differential characteristics, only some increase in the ECL signal with respect to that of nafion (50.4%) and a decrease with respect to that of chitosan (50.3%). With the goal of improving the ECL signal in the chitosan/sol-gel material, we studied the effect of the incorporation of electronic mediators and ionic conductors. The inclusion of a typical electronic mediator such as 1,1´-ferrocene dicarboxylic acid, generally used as an electronic transporter in sensing membranes[37], dramatically reduces the ECL emission, centred at 440 nm, some 95% compared to the sol-gel layer, due to the absorption of ferrocene at 450 nm.

On the contrary, the incorporation of ionic liquids (ILs) produces an ionogel that produces a large increase in the ECL signal, the reason for why they are used for biocomposite preparation. ILs can be classified, according to their preparation, in three generations[38]: 1) those based on 1-alkyl-3-methylimidazolium salts with tetrachloroaluminates or halides (based on eutectics); 2) those obtained by replacing the anion by tetrafluoroborate or other anions (discrete anions); 3) covalent inclusion of a functional group in the cation, anion or both. In this study we tested different ionogels with silica and chitosan prepared from ILs belonging to three generations (see Table 2). The results show that ILs from the first generation prevent the preparation of the biocomposite due to solubility problems, an aspect that is improved in second generation ILs due to their hydrophilic properties, which produce a water-stable composite. However, the produced ECL intensity reaches only 21% of the maximum. The use of the third generation IL triethylsulfonium bis(trifluoromethylsulfonyl)imide ([TES][TFSI]) dramatically increases the ECL intensity.

Table 2. Preparation of ionogels using different ILs, grouped by generations. All composites prepared contain 1 mmol of IL and were tested against hydrogen peroxide 5.0 μM with electropolymerized nanowires of poly(luminol-TMB) included into them. The ECL analytical signal was recovered.

Composite	Generation	IL	ECL analytical signal a.u. × s	%
Ionogel (TEOS/ chitosan/IL)	First	[decyl-MIM][Cl] [dodecyl-MIM][I] [MOIM][Cl]	solubility problems	---
	Second	$[HMIM][PF_6]$	---	---
		$[EMIM][BF_4]$	74	15.7
		$[BMIM][BF_4]$	87	18.5
		$[HMIM][BF_4]$	---	---
		$[BMIM][PF_6]$	98	20.9
	Third	[TES][TFSI]	472	100.0

The different behaviour of ionogels containing the studied ILs can be justified by their fluorescent properties. The ILs containing bulky imidazolium cations show absorption bands around 350 nm with a fluorescence emission near 480 nm, although their confinement in the silica matrix modifies the ground and excited state involved in fluorescence, shifting their absorption and emission maxima, i.e., the emission peak, from 484 to 579 nm in the case of $[BMIM][PF_6]$, consequently reducing the ECL emission due to absorption by imidazolium ILs[39]. In turn, the [TES][TFSI] shows the absorption band at 373 nm with the emission band at 431 nm, which apparently does not affect the ECL process. Thus, the [TES][TFSI] containing triflimide anion was selected. This IL is widely used for its relatively low viscosity and high ionic mobility and thus for the electro-formation of conducting polymers[40] such as polypyrrole[41] and poly(3-methylthiophene)[42].

A cyclic voltamogram on a gold SPE cell using 0.2 M H_2SO_4 solution (Fig. 2A(a)) shows an oxidation peak at 1.0 V corresponding to the oxidation of gold by water, and subsequently a reduction peak at 0.75 V due to the reduction of Au oxides formed in the anodic reaction on the working electrode. The placement of a sol-gel layer on the working electrode (Fig. 2A(b)) dramatically reduces the oxidation and reduction peaks due to the low conductivity of the material. The inclusion of chitosan in the sol-gel layer does not apparently improve the electrochemical properties (Fig. 2A(c)). The contribution of ionic liquid to the conductivity is highlighted in Fig. 2A(d), showing left-shifted gold oxidation and reduction peaks that result in a modified SPE electrode that improves the electrochemical characteristics of the bare gold SPE electrode.

Preparation and characterization of ECL nanowires

After the selection of the different elements in the preparation of the sensor, we proceeded to study the preparation and characterization of the biocomposite containing the ECL copolymer nanowires that is evidenced at first by small dark blue dots that can be seen on the surface.

To study the electro-formation of the copolymer, first a bare Au SPE cell was used with luminol and TMB solutions working under the same electropolymerization conditions as previously studied by our group[13] (between -0.2 and 1.0 V, at 0.1 $V \times s^{-1}$, from 1 mM solution of both luminol and TMB (1:1 ratio) in 0.2 M H_2SO_4), obtaining the results in Fig. 2B(a).

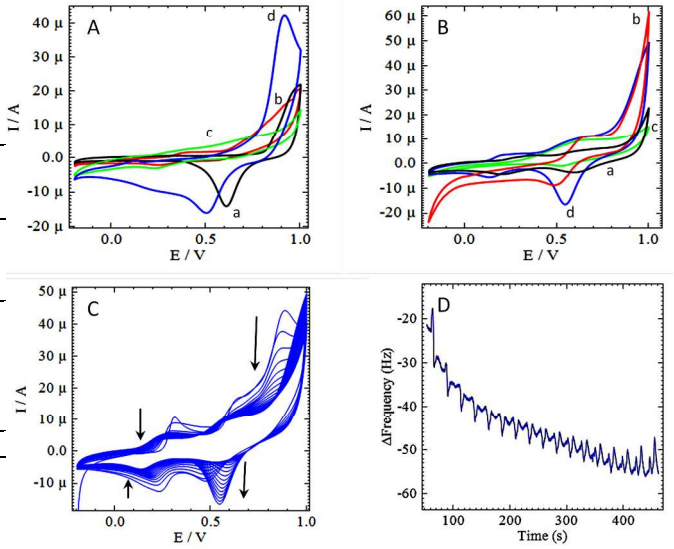

Fig. 2. Preparation of poly(luminol-TMB) in the biocomposite using gold SPE cell. (A) Cyclic voltammograms (last cycle of pre-treatment): Black(a): bare cell; Red(b): sol-gel; Green(c): TEOS/chitosan; Blue(d): TEOS/chitosan/IL. (B) Cyclic voltammogram (last cycle of copolymerization process): Black(a): bare cell; Red(b): sol-gel; Green(c): TEOS/chitosan; Blue(d): TEOS/chitosan/IL. (C) Cyclic voltammograms of luminol-TMB copolymer formation. Arrows in the figure indicate the direction of the successive cycles. (D) EQCM study of luminol-TMB copolymer formation. The monomer concentrations are 1mM in 0.2 M H_2SO_4, and the pre-treatment was carried out at 0.2 M H_2SO_4.

A study of luminol-TMB copolymer formation by cyclic voltammetry in the ionogel TEOS/chitosan/IL biocomposite is shown in Fig. 2C.

An increase in the intensity and left-shifted anodic and cathodic peaks is observed for copolymerization on a sol-gel layer on an Au SPE cell (Fig. 2B(b)). However, this membrane experiences a quick cracking effect due to poor hydration. The polymerization in the hydrogel TEOS/chitosan biocomposite produces a dramatic decrease in the intensity of the voltammetric peaks that appear at the same voltage (Fig. 2B(c)). Finally, the inclusion of [TES][TFSI] IL in the biocomposite improves the formation of the copolymer (Fig. 2B(d)).

A study of luminol-TMB copolymer formation by cyclic voltammetry in the ionogel TEOS/chitosan/IL biocomposite is shown in Fig. 2C. The same previously described pattern can be observed[33]: TMB oxidation peak at 0.60 V; luminol oxidation peak at 0.89 V; copolymer oxidation at 0.63 V and reduction at 0.54 V. The EQCM study corroborates the electro-formation of the copolymer (Fig. 2D), showing a progressive increase in the mass by decreasing the resonance frequency by cycling. Using the Sauerbrey equation[43], the calculated mass of the deposited copolymer after 20 cycles was 674.8 $ng \times cm^{-2}$.

The superficial topology of the porous biocomposite (Fig. 3B), obtained after the second pre-treatment, prepares the composite for the growth of copolymer strands in the porous structure (5 μm of pore size), giving rise to a massive growth of poly(luminol-TMB) nanowires (Fig. 3C). An interconnected filament shaped structure is observed when enlarging the image (Fig. 3D) in which the EDX spectrum confirm the presence of carbon from the copolymer and sulphur that can arise from either hydrogen sulfate or triflimide

anions. In the latter case, this is the product of the ionic liquid that has been described as a dopant in polypyrroles[41].

SPE cell and ionogel cell pre-treatments

A first pre-treatment of bare gold SPE cell generates a functional surface for polymerization removing organic ink constituents and contaminants, stressing the surface roughness and preparing the gold particles for a redox response[19]. In this case, the pre-treatment consists of three voltammetric sweeps from -0.2 to 1.0 V at 0.1 $V \times s^{-1}$ in 0.2 M H_2SO_4[33].

Once the electrode with the biocomposite was prepared and before the electropolymerization process, a new pre-treatment step was necessary to improve the immobilization of the copolymer and the stability of the ionogel membrane. The morphology of the composite (Fig. 3A) is considerably modified after pre-treatment, increasing the number and distribution of the pores (Fig. 3B) and improving the nanowire formation on them.

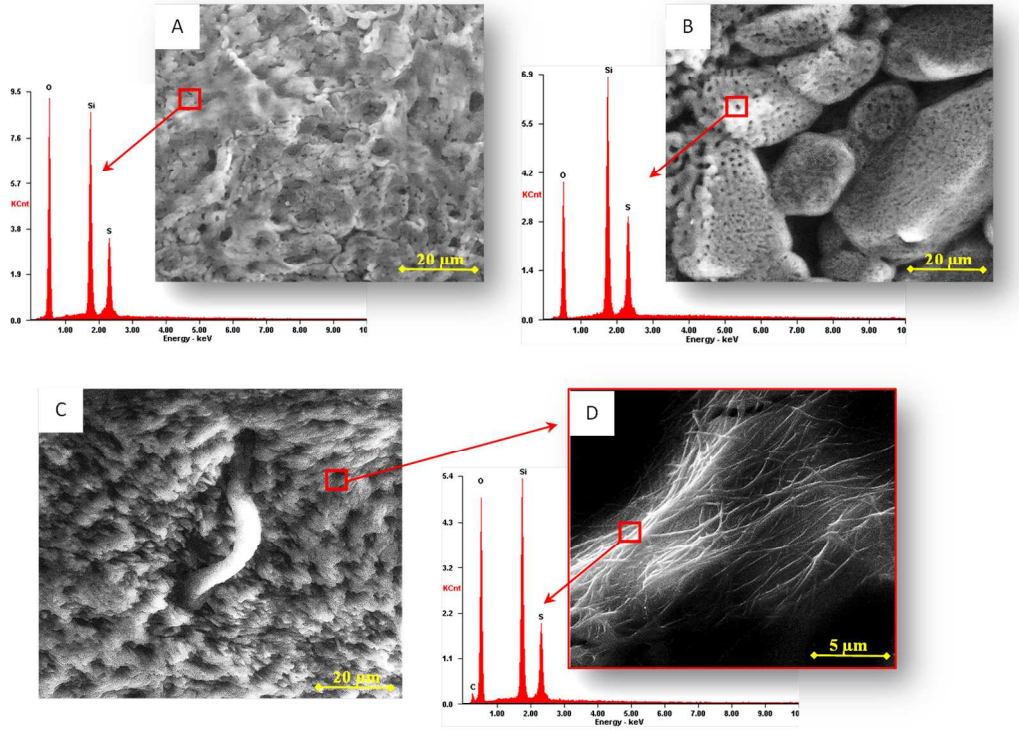

Fig. 3. ESEM images of biocomposite layer on gold SPE at different resolutions. (A) Bare biocomposite matrix; (B) pre-treated biocomposite matrix; (C) nanowires of poly(luminol-TMB) in the biocomposite; (D) filament shaped structure of poly(luminol-TMB) nanowires. EDX microanalysis spectra are included. The ECL polymer was prepared using the same monomer concentration of 1 mM in 0.2 M H_2SO_4.

After 40 cycles in the same previous conditions, a better electro-formation of the copolymer occurs after the second pre-treatment. This second pre-treatment can have three functions: a) a polishing effect that eliminates traces or extra components of the composite; b) a removing effect that permits the pore walls to strengthen on ageing because some removed IL acts as a template[27], as we can observe by an EQCM study where a constant loss of mass after each new pre-treatment cycle, obtaining a mass loss of 858.9 $ng \times cm^{-2}$ (after 40 cycles); and c) an interchange of the counter ion acetate present in chitosan by hydrogen sulphate. This is sustained by the EDX study that shows the presence of sulphur that was not present earlier in the composite structure after pre-treatment (see Fig. 3A and 3B). The increase in hydrogen sulphate in the membrane favours polymerization due to the fact that it acts as dopant agent in copolymer formation[44].

Optimization of the sensing biocomposite layer

The composition of the biocomposite layer formed by TEOS, chitosan and the selected IL was optimized individually. The TEOS concentration was maintained constant at 0.35 M as a starting point by using the TEOS stock solution previously described. Different concentrations of chitosan ranging from 0.01 to 1.10 $g \times l^{-1}$ along with 1 mmol [TES][TFSI] were used to prepare the sensing layer. The poly(luminol-TMB) was prepared in all cases by means of 40 cycles with the same electrodynamics conditions. The ECL signal increases with chitosan concentration up to 0.14 $g \times l^{-1}$, decreasing afterwards (Fig. 4A). This result comes from the increase in composite pore size described by El-Kadib and Bousmina[45] when the TEOS/chitosan ratio decreases, which involves an increase in the amount of copolymer formed as shown by cyclic voltammetry. A further increase in the amount of copolymer reduces the emitted ECL due to autoabsorption from the copolymer[33].

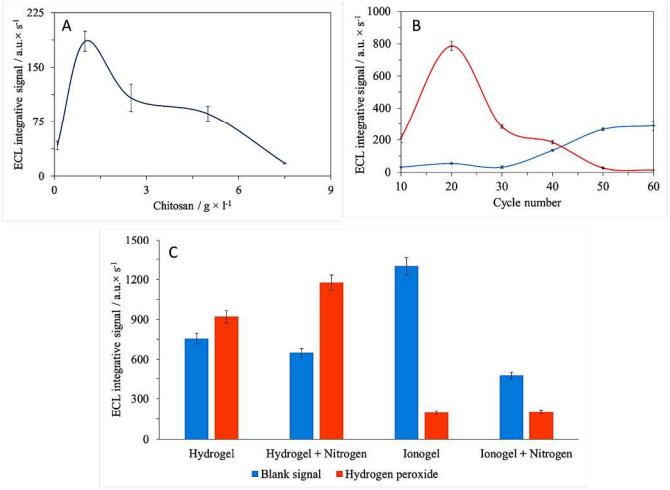

Figure 4. Optimization of composite layer formation. (A) Influence of chitosan concentration on biocomposite layer on ECL signal. Condition: 1 mM monomers (1:1 ratio), 0.7 M [TES][TFSI] and 0.35 M TEOS, ranging from 0.01 to 1.10 g × l^{-1} chitosan; (B) optimization of cycle number: Blue (lower): without [TES][TFSI], Red (upper): with [TES][TFSI]. Conditions: 1 mM monomers (1:1 ratio), 0.14 g × l^{-1} chitosan, 0.7 M [TES][TFSI] and 0.35 M TEOS. (C) In this bar chart, two sensors has been studied: Hydrogel (TEOS/chitosan) and ionogel (TEOS/chitosan/[TES][TFSI]). These sensors has been exposed to nitrogen atmosphere in order to observe the dissolved oxygen effect. Effectively, IL enhances the blank signal due to the stabilization of superoxide anion. The addition of hydrogen peroxide enhances the ECL signal in hydrogel sensor but conversely the ECL signal decrease in ionogel sensor. In this study, 5.0 µM of H_2O_2 was used.

The influence of the [TES][TFSI] concentration on the composite was studied between 0.2 and 1.2 M with 0.14 g × l^{-1} chitosan. The ECL values show low variation between 0.2 and 0.7 M in IL, decreasing afterwards due to the same reason discussed above: an increase in the autoabsorption due to an increase in the amount of copolymer formed and thus in the colour intensity. The final composition selected for the biocomposite was 0.14 g × l^{-1} chitosan, 0.7 M [TES][TFSI] and 0.35 M of TEOS.

Cycle number

Working at 1 mM (1:1 monomer ratio) in the optimized conditions discussed above, the analytical signal grows up to 20 cycles, decreasing afterwards (see Fig. 4B, red line). This is attributed to an increase in the mass of the formed nanowires along the number of cycles, which generates a self-absorption of the ECL emission (emission wavelength at 450 nm) produced by the increased formation of diimine TMB^{2+} cations (absorption wavelength at 450 nm) on the electrode surface[46]. Fig. 4B shows that in the absence of IL, the ECL signal decreases probably due to a less efficient electro-formation process. An increase in the cycle number is necessary in cells prepared without IL to achieve similar signals (up to 60 cycles).

3.2 Sensor measurement conditions

As could be expected in a ECL sensor based on a luminol-TMB copolymer, the luminol reactivity against H_2O_2 increases with the basicity of the medium (pH > 8.0) due to the contribution of hydroxyl groups in catalysing the ECL reaction[47]. As in previous works, the blank signal increased with the pH but its standard deviation grew more strongly than the signal at a pH higher than 9.5[13]. For this reason, pH 9.0 (phosphate buffer) was selected as the working pH. The volume of sample placed in the receptacle of the SPE cell has an effect on the ECL signal, with the ECL being higher, the higher the volume, up to 50 µl (receptacle maximum capacity).

To optimize the value of the electrode potential applied, a study using cyclic voltammetry was performed obtaining the maximum ECL emission at 0.6 V, a behaviour similar to that previously observed for poly(luminol-TMB) on bare gold SPE cells[13], suggesting that this new biocomposite maintains its electrochemical properties thanks to the use of chitosan and IL.

3.3 Mechanism of the ECL reaction

Luminol exhibits a blank ECL signal in the presence of oxygen due to the radical oxidation of luminol with the superoxide anion generated from the dissolved oxygen (eq. 1 and 2)[48;49]. The stabilization of the superoxide anion by the ionic liquid [TES][TFSI] due to the strong ionic interactions between cationic IL and anionic superoxide ($O_2^{\cdot-}$), according to Mjalli et al.[50] increases the blank signal (see the expanded image of Fig. 5 and the bar chart of Fig. 4C). The consumption of the dissolved oxygen is observed when several electrochemical cycles are given, achieving a decreasing signal each time (Fig. 5).

$$O_2 + e \rightarrow O_2^{\cdot-} \quad E^0 = -0.16\ V \qquad \text{(eq. 1)}$$

$$\text{Luminol} + O_2^{\cdot-} \rightarrow 3APA^* \rightarrow 3APA + h\nu \qquad \text{(eq. 2)}$$

Moreover, imidazolium containing ionic liquids generates an unstable superoxide because the superoxide anion reacts with the imidazolium cation, giving the corresponding 2-imidazolones[50], the reason for which the low ECL signal in imidazolium containing ionic liquids in the preliminary studies in section 3.1 was reinforced.

In the presence of hydrogen peroxide, the usual increase of the ECL signal due to the chemical reaction of hydrogen peroxide and diazaquinone coming from luminol is observed in typical ECL sensors and less in hydrogel (TEOS/chitosan). On the contrary is not evidenced in the ionogel sensor (TEOS/chitosan/[TES][TFSI]) (see bar chart in Fig. 4C) because a decrease in the ECL intensity due to the addition of hydrogen peroxide is observed, which decreases with the concentration. One reason for this could be the conversion of the electroformed superoxide to oxygen with the addition of H_2O_2 following the Haber–Weiss reaction[51]. Generally this reaction needs Fe(III) as a catalyst (Fenton reaction), but it can be electrochemically catalyzed at 0.48 V (pH 7.0), according to Koppenol[51], with the consequent elimination of the superoxide anion from the media (eq. 3).

$$O_2^{\bullet-} + H^+ + H_2O_2 \rightarrow O_2 + HO^{\bullet} + H_2O \qquad 0.48\ V = \Delta E^\circ \quad \text{(eq. 3)}$$

For that reason, the sensor presented in this paper is sensitive to H_2O_2, acting as a scavenger of the increased blank signal generated by the biocomposite including the [TES][TFSI] ionic liquid as mediator.

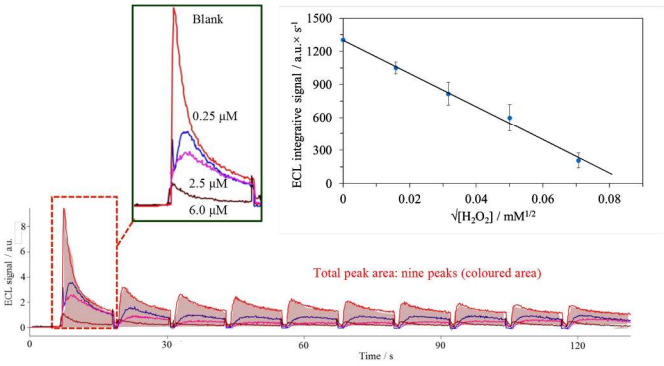

Fig. 5. ECL response of the disposable sensor to hydrogen peroxide. The record shows the ECL signal obtained for 2 min coming from consecutives pulses of 0.6 V for 1 s each with a dwell time of 10 s. The ECL signal used for analytical purposes was the integral signal for this time, overshadowed into the red line that correspond to the profile of a blank; blue line: 0.25 µM H_2O_2; pink line: 2.5 µM H_2O_2; and brown line: 6.0 µM H_2O_2. The insets show an enlargement of the first peak and the linear calibration function.

3.4 ECL sensor analytical characterization

Analytical signal

As the analytical parameter, we studied the evolution of the ECL signal, working in chronoamperometric mode, by recording the height or peak area of the ECL intensity after each pulse. Fig. 5 shows the profile of the blank and hydrogen peroxide signals: a first intense peak that decreases with subsequent pulses. The reproducibility of the ECL emitted from any excitation pulse (peak height) is around 20-40 %, the same as the peak area (one pulse). However, the use of the peak area of a train of pulses produces a more reproducible value, typically less than 10 %. The analytical signal used in this paper was the peak area of all the ECL emissions obtained from nine consecutive pulses lasting 1s with a dwell time of 10 s. This analytical signal was denoted the ECL integrative signal (a.u.×s).

Analytical parameters

The dependence of the ECL integrative signal on the H_2O_2 concentration was obtained. The linear dependence with negative slope observed between 0.0093 µM and 7.4 µM is the proposed analytical function. The ECL integrative signal presents square root dependence with H_2O_2 concentration. The limit of detection obtained by using the standard criteria[52] was 0.8 nM and the limit of quantification was 9.3 nM. The repeatability of the biocomposite sensor was tested by using different screen-printed cells for each measure. Ten blanks produced an relative standard deviation (RSD) of 1.2 % (high blank values) and working at 5.0 µM H_2O_2, the RSD was 8.5% (n=5) (Table 3), acceptable values considering that commercial SPE cells used alone have a precision of around 5%, and, the values are similar to those obtained for poly(luminol–TMB) gold SPE cells previously studied[13].

The lifetime of the disposable sensor was checked using a series of prepared sensors, regularly testing their response to a 5.0 µM H_2O_2 concentration. These sensors, when protected from light and kept at 4 ºC, can be used for a month without substantial change. At room temperature and in the dark, the sensors can be used for a week, without the signal changing below 10% (254 ± 23 a.u.×s). After that, the signal decreases some 20% on each subsequent day. The prepared sensors must be used in disposable format because each sensor cannot be reused more than 10 times, as we could confirm working at 5.0 µM H_2O_2, after which they show a decrease in the signal around 35% for each use.

Table 3. Analytical characteristics of biocomposite sensor for hydrogen peroxide determination.

Parameter	Values
Range, µM	0.0093 - 7.4
Intercept, a.u. × s	1301 ± 16
Slope, a.u. × s × $M^{-1/2}$	(-1.5 ± 0.06)×10^4
R^2	0.996
LOD*, nM	0.8
LOQ*, nM	9.3
RSD* (%) blank (n=10)	1.2
RSD* (%) H_2O_2, 5.0 µM (n=5)	8.5

*LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation.

A comparative study between different electrochemiluminescent and amperometric sensors from the bibliography (see Table 4) shows that these methods offer more than three orders of magnitude for H_2O_2 response, as observed in the sensor presented here (three orders). With respect to the limit of detection, the value found (0.8 nM) is in accordance with the results obtained by other researchers. In the case of the reproducibility, the obtained values from the bibliography using conventional electrodes are better than our results (8.5 %), due that they do not to work in disposable format.

Table 4. Analytical parameters of hydrogen peroxide detection in recent papers

System	Linear range µM	LOD nM	Linearity	Detection	Reproducibility
Hydrogel:[53] Tetramethoxysilane/chitosan	250 to 3400	3000	0.998	Amperometry	4.0 %
Dendrimer:[54] Titanate nanotubes/poly(amidoamine) and luminol	0.001 – 0.9	1.0	0.998	ECL	---
Composite:[55] Titanate nanotubes/nafion and luminol	0.001 – 0.5	0.88	0.998	ECL	2.1 %
Composite:[56] Poly(luminol-benzidine)	0.0002 – 0.1	0.060	0.997	ECL	5.0 %
Composite:[13] Poly(luminol-TMB)	0.0061 – 0.1	2.6	0.984	ECL	10.2 %
Hydrogel and nanowires:[24] Polyluminol/calcium alginate	0.4 – 5000	---	---	ECL	---
Ionogel and nanowires:[(This work)] TEOS/chitosan/IL and poly(luminol-TMB)	0.0092 - 7.4	0.8	0.998	ECL	8.5 %

Conclusions

The use of a ionogel biocomposite prepared by the solution method from chitosan and ionic liquid in silica matrix to host nanowires of poly(luminol-TMB) prepared by in-situ electropolymerization in the pores of a ionogel make it possible to prepare a disposable reagentless electrochemiluminescent sensor with good mechanical, conductivity and luminescent properties, as well as good operational and temporal stability.

The preparation of ionogels with the inclusion of [TES][TFSI] as the ionic liquid containing triflimide anion produces: a) an interesting increase in the conductivity of the biocomposite layer needed for ECL generation, b) an increase in hydration along with chitosan needed for silica matrix stabilization, and c) strong ionic interactions that stabilize the superoxide anion formed from the oxygen present, giving rise to an intense blank ECL emission.

The developed sensor is sensitive to hydrogen peroxide that acts as a scavenger of the stabilized superoxide anion, reducing the ECL signal with the increase in the H_2O_2 concentration, resulting in a reagentless disposable sensor with a linear range of three orders of magnitude but a better resolution than with the usual disposable sensors because the acquisition of luminescence signal is based on the measurement of the total luminescence area using a photomultiplier detector like an enhanced CCD camera. The modified SPE cell studied paves the way for the preparation of biomaterials such as oxidase-based because binding ionogel with ECL polymers can offer new possibilities for disposable ECL biosensors.

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Notes and references

[a] ECsens. Department of Analytical Chemistry, Campus Fuentenueva, Faculty of Sciences, University of Granada, E-18071 Granada, Spain.
[b] Corresponding author. E-mail address: lcapitan@ugr.es (L.F. Capitán-Vallvey).

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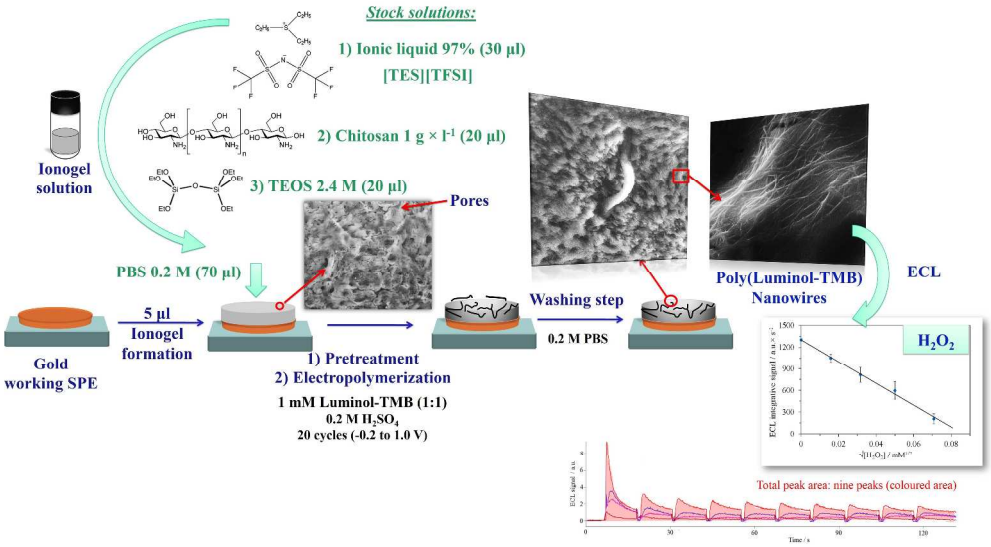

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