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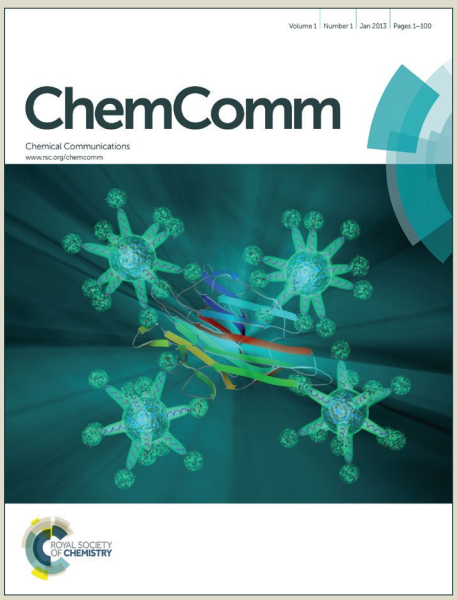




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# A smart material for the *in situ* detection of mercury in fish

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**We have developed a new fluorogenic polymer capable to detect the presence of mercury contamination in fish samples. The modified polymer emits blue light when irradiated with UV light proportionally to the quantity of mercury, as $MeHg^+$ or $Hg^{2+}$, present in fish. The quantitative relation between the concentration of mercury in fish and the increasing of fluorescence in the polymer in contact with fish samples was confirmed, giving rise to quick and reliable results in the measurements of the presence of mercury in fish by a portable fluorogenic polymeric probe.**

Environmental contamination by mercury is a serious concern because of the large amounts of mercury released to the environment by human activities.[1] As a result, emissions to the environment have increased significantly, tripling the mercury content of surface waters compared to pre-anthropogenic conditions.[2] Mercury is a persistent metal in the environment as the volatile mercury metal but also as the water soluble $Hg^{2+}$ and $MeHg^+$ cations, that are strongly interconnected in the environment because of the natural cycle of mercury. $MeHg^+$ is a known neurotoxin and a ubiquitous environmental toxicant that leads to long-lasting neurological and developmental deficits in animals and humans, which is usually accumulated in fish in the aquatic environment. Due to their inherent toxicity, Hg(II) species have to be continuously monitored, therefore many chemical reporters have been studied.[3] There are very few colorimetric or fluorogenic probes for $MeHg^+$, which is in strong contrast to the enormous interest in the detection of $MeHg^+$ in living systems.[4] We have contributed with some sulfur containing chromogenic probes.[5] It is known that $MeHg^+$ acts physiologically by binding to sulfhydryl groups in proteins or cysteine, forming water soluble complexes in tissues.[6] We have designed and tested some new sulfur-containing fluorogenic probes for their ability to selectively interact with Hg(II) species so they could be employed to detect $MeHg^+$ by mimicking its behaviour in cells. In this way, we used modified charge-transfer fluorogenic probes bearing a sulfur-containing functional group that exerted a quenching effect on the initial fluorescence of the core structure by a photoinduced electron transfer (PET). Subsequent interaction of the sulfur-containing group with thiophilic cations increased the fluorescence of the probe, therefore detecting Hg(II) cations. Following this idea we developed new fluorogenic probes for the selective fluorogenic detection and speciation of $Hg^{2+}$ and $MeHg^+$ in aqueous-organic mixtures.[7] The use of those probes permitted the speciation of $MeHg^+$ and $Hg^{2+}$ in samples containing mixtures of both cations.[8] Now, with the purpose of preparing new portable fluorescent sensors for practical applications, we have bonded the best fluorogenic probe to a polymeric hydrophilic matrix to develop a new fluorescent polymer capable to detect the presence of mercury contamination in fish samples. In this paper we report the direct measurements of the presence of mercury in fish samples with the polymeric probe. The film-shaped functional membrane **(Mem)** was prepared by the photochemically initiated radical polymerization[9] of the hydrophilic monomer **2HEA** and **PGM**. **EGDMA** was used as cross-linking agent (Scheme 1). The co-monomer molar ratio **2HEA/PGM//EGDMA** was 95/5//5, respectively. 2,2-Dimethoxy-2-phenylacetophenone (**DMPA**, 1.5% wt) was employed as a photochemical initiator. The photoinitiated bulk polymerization was performed in a 100 µm thick silanized glass hermetic mould upon irradiation with a UV mercury lamp at 20 °C, for 4 h. The water-swelling percentage (WSP) of the membrane was 60% and the DMF swelling 300 %. Then *N*-(4-azidophenyl)-4-(5-(1-oxo-indan-5-yl)pyrimidin-2-yl)piperazin-1-carbothioamide[7] (**JG10**, 0.2 mmol) in DMF was stirred under nitrogen in an orbital shaker with 0.4 g of the polymer membrane **Mem** and 3 mg (5% mol) of $Cu(NCCH_3)_4^+[PF_6]^-$ as catalyst for 72 hours. After that, the reaction was finished and the functionalized polymer **JG25** had a light orange-yellow colour. The polymer was washed with water and left to dry (Scheme 1). The characterization of the membrane was carried out by SEM analysis, with gold and carbon recap. The atomic proportion was taken by X-ray

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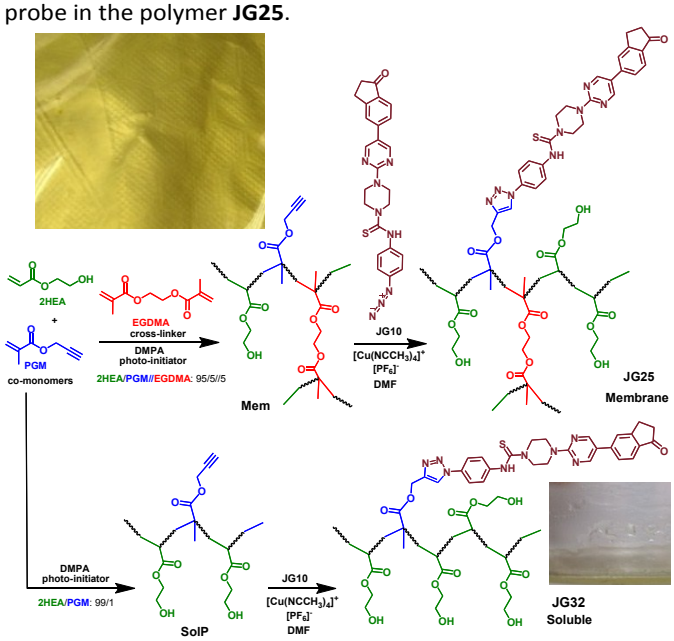
analysis on different areas of the polymer. The proportion between oxygen or carbon and sulfur atoms was very similar to the theoretical results associated to a 100 % stoichiometric reaction. The appearance of IR signals at 1514 and 1598 $cm^{-1}$, that were not present on the IR spectrum of **Mem**, were associated to the presence of the fluorogenic probe, their intensity was low according to the low percentage (5%) of the probe in the polymer **JG25**.




**Scheme 1.** Synthesis of the fluorogenic polymers **JG25** and **JG32**. Insets: pictures of the polymers under white light.

To study the selectivity of the polymeric probe in solution, the water soluble polymer **SolP** was prepared by the photochemically initiated radical polymerization of the hydrophilic monomer **2HEA** and **PGM**, the co-monomer molar ratio **2HEA/PGM** was 99/1, respectively, in the same conditions of the previous case. Then reaction of **JG10**[7] (0.05 mmol) in DMF with the soluble polymer **SolP** (0.7 g) and 1 mg (5% mol) of $Cu(NCCH_3)_4^+[PF_6]^-$ at 30 °C for 24 hours gave the polymer **JG32**, obtained as a slime in 80% yield, that was characterized as in previous case (Scheme 1).

We performed fluorescence tests in the presence of common cations and anions and the polymers. The solutions of the probes were tested by increasing the concentration of cations and anions in solution and checking the variation in colour and/or fluorescence under visible and UV light (366 nm). There was no physical change in the presence of anions but, in presence of cations, $Hg^{2+}$ and $MeHg^+$ produced a very high increase in fluorescence of the polymer **JG25**, and the increase of fluorescence was proportional to the concentration of cations under a UV lamp (366 nm) (Figure 1). It could be also seen an increase in fluorescence for $Ag^+$, albeit in minor extension than for $Hg^{2+}$ and $MeHg^+$, quantified by measuring with a fluorometer (See the Supporting Information). We also tested the azide **JG10** ($10^{-5}$ M in MeOH:$H_2O$ 60:40) showing small increase of fluorescence for $Ag^+$ and strong increase in the presence of $Hg^{2+}$ and $Au^{3+}$. Then a $1.2\times10^{-2}$ g/L solution of the soluble polymer **JG32** in water was prepared (1% fluorogenic probe, $10^{-6}$ equivalents/L), the cations were added in solution and the water soluble polymer showed an increase of fluorescence only in the presence of $Hg^{2+}$ and $MeHg^+$ cations, even in the presence of heavy metal cations (for instance, $Au^{3+}$ acted by quenching fluorescence) (Figure 2) within two minutes, corroborating the selectivity of the probe.

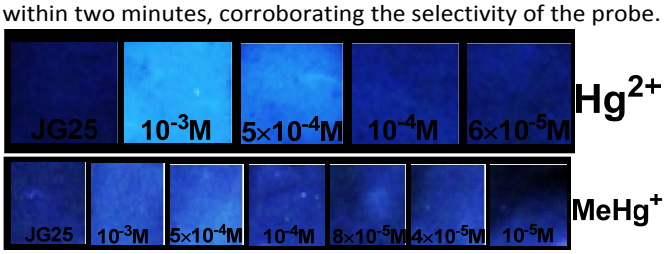



**Figure 1.** (left to right) Upper: Fluorescence changes of **JG25** in the presence of $Hg^{2+}$: 0, $10^{-3}$, $5\times10^{-4}$, $10^{-4}$, $6\times10^{-5}$ M under a UV light (366 nm); lower: Fluorescence changes of **JG25** in the presence of $MeHg^+$: 0, $10^{-3}$, $5\times10^{-4}$, $10^{-4}$, $8\times10^{-5}$, $4\times10^{-5}$, $10^{-5}$ M.

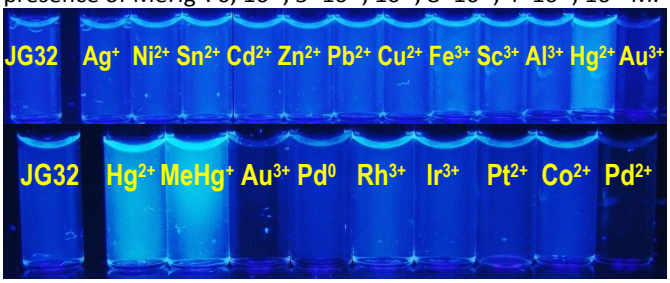



**Figure 2.** (left to right) Fluorescence changes of an aqueous solution of **JG32** in the presence of (upper): 2 equivalents of $Ag^+$, $Ni^{2+}$, $Sn^{2+}$, $Cd^{2+}$, $Zn^{2+}$, $Pb^{2+}$, $Cu^{2+}$, $Fe^{3+}$, $Sc^{3+}$, $Al^{3+}$, $Hg^{2+}$, $Au^{3+}$, and (lower) 3 equivalents of $Hg^{2+}$, $MeHg^+$, $Au^{3+}$, $Pd^0$, $Rh^{3+}$, $Ir^{3+}$, $Pt^{2+}$, $Co^{2+}$, $Pd^{2+}$, all under a UV light (366 nm).

We then performed quantitative titrations in fluorescence of the polymer JG25 in the presence of increasing amounts of $Hg^{2+}$. Preliminary kinetic study showed that the increase of the fluorescent signal in the presence of $Hg^{2+}$ was fast in the first five minutes and then increased slowly for more than one hour until it reached an asymptotic maximum. The changes at the beginning of the additions were linear, therefore we added quantities of $Hg^{2+}$ every 5 minutes so the resulting graphic plot was the expected typical plot for species studied in equilibrium (Figure 3). From the titration data a limit of detection was calculated as $6.6\times10^{-6}$ M or 1.3 ppm of $Hg^{2+}$ in water, with a possibility of false positive and false negative equal or inferior to 5%. The value was reached 15 minutes after the first measurement, so this was the average time that was necessary to detect a noticeable increase of the fluorescence in the solution. We then performed quantitative titrations in fluorescence of **JG25** in the presence of increasing amounts of $MeHg^+$ in water. The preliminary kinetic study showed that the behaviour of the polymeric probe in the presence of $MeHg^+$ resulted to be different to the previous behaviour in the presence of $Hg^{2+}$. In this case, the increase of the emission reached a maximum at 90 minutes for $MeHg^+$ concentrations of 1-2.5 × $10^{-4}$ M and then decreased on time. Therefore, as in the previous case, the titration experiments with the polymer were performed by adding the solution of $MeHg^+$ every 5

minutes so the resulting graphic plot was the expected typical plot for species studied in equilibrium (Figure 3).

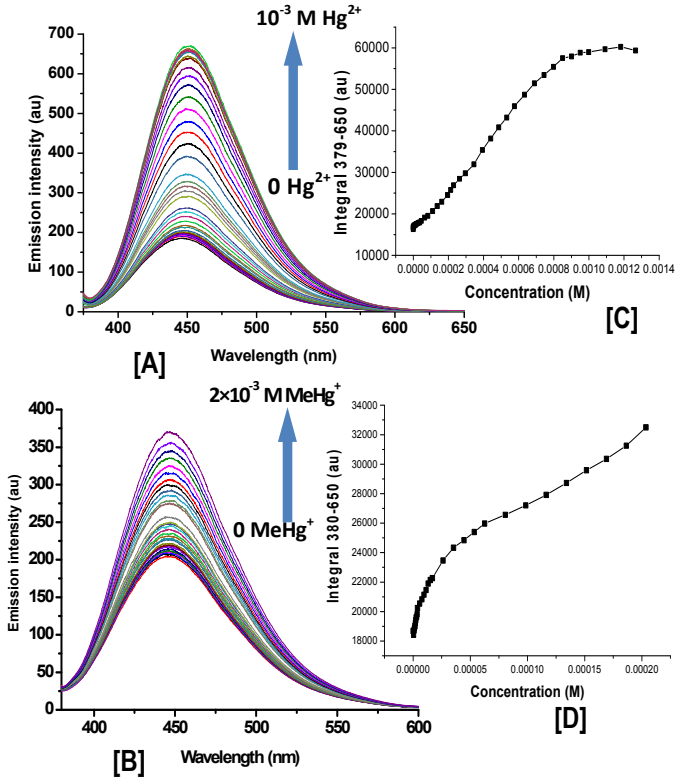



**Figure 3.** Left: Fluorescence curves by addition of increasing concentrations of **[A]** $Hg(ClO_4)_2$ in water to **JG25**, $\lambda_{exc}$ = 369 nm and **[B]** MeHgCl in water to **JG25**, $\lambda_{exc}$ = 364 nm. Right: Titration plot by using integral surfaces of the fluorescence curves between 380-650 nm in response to increasing concentrations of **[C]** $Hg(ClO_4)_2$ in water, $\lambda_{exc}$ = 369 nm and **[D]** MeHgCl in water, $\lambda_{exc}$ = 364 nm.

From the titration profiles of **JG25** and both cations it was clear that for lower concentrations of $MeHg^+$ the fluorescence of the polymer JG25 increased at a higher slope than in the previous case of $Hg^{2+}$, but at higher concentrations the final increase in fluorescence was much lower. By using the data from the titration, the limit of detection was calculated as $1.5\times10^{-6}$ M or 0.3 ppm of $MeHg^+$ in water, this value was reached in less than 20 minutes and reflected the higher initial slope in the titration plot, probably due to the higher lipophilicity of $MeHg^+$ in comparison to the lower initial slope in the titration plot of the more hydrophilic $Hg^{2+}$. The total increase of fluorescence after the titration was 380% for $Hg^{2+}$ and 180% for $MeHg^+$ reflecting the lower activity of the latter cation towards the polymeric probe. Then we performed tests with samples of fish, tuna, swordfish, conger and panga to check the direct detection of mercury contamination in fish samples. Every fish sample, 2 g, was grinded and mixed with 5 ml of water and then left in contact with a piece of polymer **JG25**. After a period of time the polymer fragment was taken from the solution and dried in order to compare the fluorescence of every fragment, showing that there were clear differences between samples. In a representative example, blank and panga showed almost no fluorescence, the reference polymer fragment with added $Hg^{2+}$ showed the highest fluorescence, and tuna, swordfish and conger eel gave an intermediate fluorescence by inspection under the UV light, indicating the presence of mercury in the samples. Because most of mercury in fish is $MeHg^+$,[10] in fact the detection corresponded to the whole contamination by mercury in fish, mainly by $MeHg^+$ with very little $Hg^{2+}$. Albeit the differences in fluorescence could not be evaluated quantitatively by a first sight, they gave a clear qualitative evaluation of the presence or absence of mercury in the fish samples. ICP-Mass showed that samples of fresh tuna, swordfish, conger eel, and panga had mercury amounts of 0.70-0.72 ppm for tuna, 2.8-3.4 ppm for swordfish, 0.44 ppm for conger eel and 0.02 ppm for panga, which clearly correlated with the fluorescence of the polymer samples when the mercury contamination was higher than the limit of detection of the polymer **JG25** for both species of mercury, $MeHg^+$ and $Hg^{2+}$ (Figure 4).

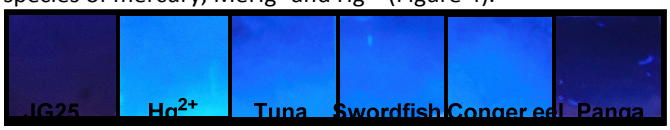



**Figure 4.** From left to right, polymer samples of JG25 in contact with: water (JG25), a solution of $Hg(ClO_4)_2$, $2.5\times10^{-3}$ M ($Hg^{2+}$), fish samples (Tuna), (Swordfish), (Conger eel), (Panga) for 20 minutes in contact with every fish sample or $Hg^{2+}$ solution; all samples under UV light, $\lambda_{exc}$ = 366 nm.

Therefore the polymer **JG25** emitted blue light when irradiated with UV light, 366 nm, semi-quantitatively in relation to the quantity of mercury, as $MeHg^+$ or $Hg^{2+}$ cations, presented in the fish sample. So the polymer can be used as a fast fluorogenic probe to detect mercury derivatives in contaminated fish, acting as a smart label for the fluorescent detection of mercury contamination in fish samples when the amounts are close to a health risk.[11] In order to find the best conditions to have reproducible and reliable measures of the presence of mercury contamination in fish samples, we took several samples from different fish species purchased at the fish market, lyophilized the fish samples, extracted the samples by two common methods, acidic and silica methodologies,[12] and performed fluorescent measurements with **JG25** and the extracts as well as the solid samples, and checked the amount of mercury in extracts of lyophilized samples by ICP-mass analysis. The amount of mercury from ICP-mass analysis of lyophilized samples was then converted to amounts of mercury in fresh samples by taking in account the percentage of water present in fresh fish of every species. As in the qualitative experiments, large predator fish gave the largest amounts of mercury (1.0-1.5 ppm for swordfish, tuna and dogfish, 0.5 ppm for conger eel, and 0.2 ppm for panga; the only exception was the farmed salmon that had no mercury. For measurements from extracts, the fluorescence was measured as the variation of emission intensity from pure water to the extracts in contact with the polymer probe, affording a clear correlation between the ICP values on fish samples and the emission intensities obtained with **JG25** and extracts. We then normalized the graphs, taking as reference maximum the ICP-Mass result from the acid extraction of

dogfish, considered the results of ICP-Mass in ppm and converted the fluorescence values to ppm relative to ICP-Mass values. We then used the original samples used for extractions to compare the results from direct measurements of fish samples and from the extracts. To perform direct measurements, 0.5 g of every lyophilized fish sample was mixed with 2 ml of water. Then a piece of the polymeric sensor was added. To check the difference in fluorescence every polymer fragment in contact with fish samples was measured after one hour in the fluorometer. The results were compared with the corresponding results from extractions by normalizing the bars to dogfish (Figure 5).

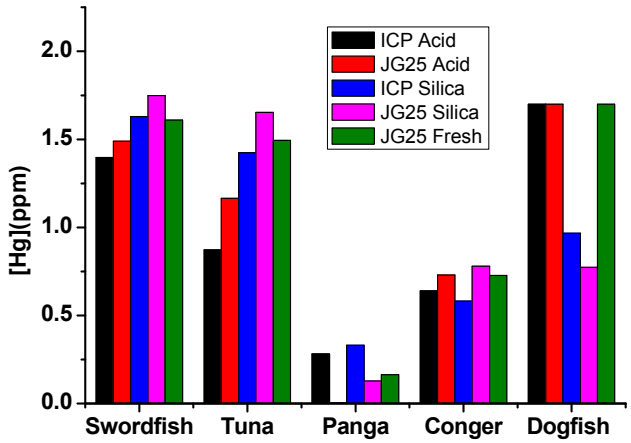



**Figure 5.** Graphical representation of the amounts of mercury detected by emission intensity variation in experiments by direct contact of **JG25** with fish samples (green bar) ($\lambda_{exc}$ = 365 nm, $\lambda_{em}$ = 455 nm) compared with the results from the extracts by ICP-Mass analysis (black and blue bars) and intensity variation of **JG25** in contact with extracts (red and purple bars) of fish samples, $\lambda_{exc}$ = 365 nm, $\lambda_{em}$ = 455 nm.

Therefore, a semi-quantitative relation between the concentration of mercury and the obtained values of fluorescence was confirmed. The fluorescence increase of the polymer in contact with real fish samples was a complex process due to the presence of a high proportion of $MeHg^+$ with some $Hg^{2+}$, which had different behaviour in contact to the polymer. But we have demonstrated that a careful experimental procedure gave rise to quick and reliable results in the measurements of the presence of mercury in fish samples by a portable fluorogenic polymeric probe. In conclusion, we have developed a new fluorogenic polymer capable to detect the presence of mercury contamination in fish samples. The modified polymer emitted blue light when irradiated with UV light proportionally to the quantity of mercury, as $MeHg^+$ or $Hg^{2+}$ cations, presented in the fish sample. With this polymer we have designed a fast and useful fluorogenic probe to detect mercury derivatives in contaminated fish, in a way that the new polymeric sensing material acted as a smart label for the fluorescent detection of both $Hg^{2+}$ and $MeHg^+$ in fish samples.

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