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Contact-active microbicidal gold surfaces using immobilization of quaternary ammonium thiol derivatives

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ABSTRACT

Contact-active auto-bactericidal surfaces were obtained by grafting of specially designed thiol derivatives containing antimicrobial quaternary ammonium moieties on gold substrates. The formation and quality of the self-assembled monolayers (SAMs) were characterized by X-ray photoelectron spectroscopy, cyclic voltammetry and contact angle measurements. The bactericidal activity of the modified gold surface was evaluated against *Staphylococcus aureus* using an original procedure. This activity was demonstrated to be dependent on the length of the alkyl chain borne by the charged nitrogen atom of the quaternary ammonium moiety, and on the contact time.

1. Introduction

Conferring materials with antibacterial surface properties is a fascinating area for research and development in the battle against microbial contamination, particularly nosocomial infections [1]. The use of organic or aqueous solutions of conventional disinfectants or antimicrobial agents is restricted by the toxicity of their residues since they are liquids or gases of low molecular weight [2,3]. Moreover concerning metallic materials, the usual alternative strategy consisting in coating the metal surfaces with naturally antibacterial polymers or synthetic polymers impregnated with antimicrobial agents (including silver) suffers from an important drawback since the potentially active agents are released gradually into the surrounding environment. Consequently, metal surfaces that could kill harmful micro-organisms on contact without releasing antimicrobial agents are in a growing field of research from a long time, the nature of antimicrobial agents immobilized on the metal surfaces and the methods of covalent immobilization being varied [4–6]. For instance, the concept of self-assembled monolayers (SAMs) [7] of thiol and disulfide molecules covalently bound to metals and bearing potentially biocide moieties can be considered [6]. This potentially biocide moieties could be a quaternary ammonium salt exhibiting a thiol function. Indeed, the antimicrobial activity of various quaternary ammonium salts is known for a long time and this class of cheap antibacterial agents is effective against various germs and widely used in the hospital environment [1,8–12]. So, the final purpose of our research project is to associate this SAMs concept and the well known bactericidal properties of quaternary ammonium compounds in order to obtain various metal surfaces with a potential contact-active antimicrobial activity using the most effective quaternary ammonium structure bearing a thiol function. In the present work, we present our first attempts using gold and *Staphylococcus aureus* as model surface and germ, respectively, and the original thiols **1**–**10** described in Scheme 1. This strategy can be summarized as shown in Scheme 2.

Abbreviations: BF, blocking factor; CFUs, colony forming units; CA, contact angle; CV, cyclic voltammetry; MIC, Minimal Inhibitory Concentration; MLC, Minimal Lethal Concentration; MPN, most probable number; SAMs, self-assembled monolayers; TSB, trypticase soy broth; XPS, X-ray photoelectron spectroscopy.

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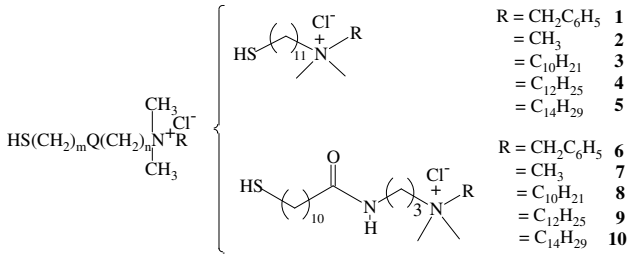


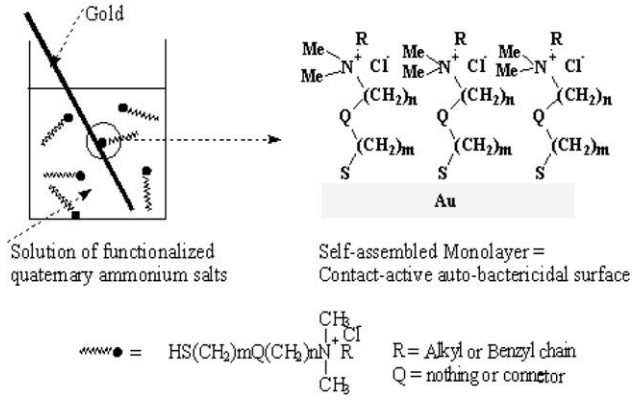
Scheme 1. Quaternized ammonium chlorides used in this work.

2. Results and discussion

This section is organized in three main parts. In the first part, we focus on the choice of the metal substrate as model, the structure of the quaternary ammonium sulphur derivatives used to obtain SAMs as well as the model of micro-organisms tested in this study, *S. aureus*. The second part is dedicated to the characterization and the quality of the monolayers on the gold surface using X-ray photoelectron spectroscopy (XPS), cyclic voltammetry (CV), and contact angle (CA) measurements, while the third part concerns the choice of the method for the evaluation of antimicrobial properties of the monolayers obtained, and the interpretation of the bacteriological results as a function of the structure of the thiol grafted.

2.1. Choice of the metal surface, immobilized structures and micro-organism

The final aim of our research project is to obtain contact-active antimicrobial metal surfaces such as iron, copper, zinc,… Our first attempts to reach this aim were to use gold as model metal substrate for various reasons. First at all, gold is the most widely used metal substrate for fundamental research in molecular assembly. This comes from its remarkable properties [6,13]. For instance, it is easy to produce as thin films by thermal evaporation and is fairly inert under atmospheric conditions. So generally, gold is used as model surface before starting works on SAMs on other non noble metals. Second, gold is suitable for biological applications because of its good biocompatibility, and it can be used as metal surface to protect some medical instruments [14]. Finally, gold also has a high affinity for thiols so that thiol-containing molecules are known to produce good quality thiolate SAMs with remarkable long-term stability [13]. However, because of the sensitivity of the grafting towards surface contaminants a re-treatment of gold substrate must be realized and grafting conditions (concentration, solvent, dipping time,…) must be optimized according to our own previous experiments on SAMs [15–17], particularly on gold [18–20]. Taking into account the low solubility of charged sulphur derivatives in organic solvents, only 10^{-2} M solutions can be used. In order to compensate this relatively low concentration, a large dipping time of 18 h is used. The roughness of the metal surfaces could affect the organization of SAMs and the physicochemical measurements, particularly the values of contact angles. In fact, this factor is negligible for three essential reasons: (i) it is a nanoroughness (see Experimental section), (ii) the roughness is the same on all gold samples and (iii) experiments with all sulphur derivatives have been performed on coupons from the same gold sample.



Scheme 2. Formation of contact-active auto-bactericidal metal surface via SAMs' formation with functionalized thiols.

Concerning the structures to be grafted, **1**–**10**, we have recently shown that: (i) thiols and disulfides bearing internal quaternary ammonium group in their structure can absorb on clean gold surface and form organized SAMs providing that the hydrocarbon spacer between the charged nitrogen and the grafted sulphur atom is long enough to minimize the repulsive interactions between the charged groups when the structure is grafted. In addition, the presence of a H-donor amido group between the quaternized nitrogen atom and the thiol function also compensates the repulsive interaction of neighbouring structures on the surface thanks to the formation of hydrogen bonding [21]; (ii) although the presence of a thiol function at the end of one of the tails slightly decreases their tensioactive properties closely related to their antibacterial effects, these bitailled surfactants, **1**–**10**, keep their microbiostatic and microbicidal activities against the four strains, *Pseudomonas aeruginosa*, *S. aureus, Aspergillus niger* and *Candida albicans,* compared to their unsulfurated homologues. Moreover, the presence of the amido connector between the charged nitrogen and the thiol function (series **6**–**10** *versus* series **1**–**5**) does not largely affect the antibacterial properties of the most efficient of these preservatives [22].

Three principal reasons explain the choice of *S. aureus* as model to test the antibacterial activity of our immobilized ammonium salts: (i) Although the literature data does not show direct relationship between antibacterial activity of free molecules and that of their immobilized forms on various substrates, our previous results on the microbicidal properties of free structures **1**–**10** demonstrate that, among the four micro-organisms studied, *S. aureus* is the one that exhibits the lowest Minimal Inhibitory Concentration (MIC) and Minimal Lethal Concentration (MLC) towards all the compounds **1**–**10** [22]; so we could expect that this germ will present the best chance to give positive antibacterial results when structure **1**–**10** will be immobilized on gold substrates; (ii) *S. aureus* is among the bacteria the most responsible for nosocomial infections [23]; this germ is an important surface contaminant because it possess adhesins that favours the binding on foreign materials and leads to the formation of biofilms in which *S. aureus* develops its resistance ability. This pathogen strain is able to live in dry conditions with water activity (aw) near 0.85 and considered as a xerophile. This ability to survive under such drastic conditions like walls, floors or other dry surface will render *S. aureus* particularly dangerous in Hospital environments; (iii) lastly, *S. aureus* is, with *Escherichia coli*, one of the most used micro-organisms for evaluation of antibacterial surface activity.

2.2. SAMs' characterization

The formation and quality of monolayers obtained with the previously synthesized thiol derivatives **1**–**10** on gold substrates are investigated using XPS, CV curves and CA measurements.

XP survey spectra of the bare Au substrates before and after UV–ozone treatment and US-rinsing, and of the modified Au substrates with sulphur derivatives **3** and **8** (R = $C_{10}H_{21}$) taken as examples are

presented in Fig. 1a–d. Fig. 1a shows the importance of oxygen and carbon contaminants on the crude gold surface and the necessity to proceed to a drastic cleaning. Comparison of Fig. 1a and b along with results presented in Table 1 demonstrates the efficiency of the cleaning treatment; however, Fig. 1c and Table 1 also reveal the presence of residual contamination even after cleaning treatment (the literature reports that this type of carbon is always present on all solid surface and corresponds to a C 1s binding energy around 284.6 eV whatever the pre-treatment and precautions). Comparison of Fig. 1c and d with Fig. 1b shows the increase of the C 1s peak intensities in comparison with the Au 4p3 or Au 4d peaks after dipping in the methanolic solutions of thiols. Because of their low relative sensitivity factor and their low proportions at the surface, the peaks corresponding to N and S atoms do not appear on these survey spectra.

C 1s spectra of bare Au surface after UV–ozone cleaning and US-rinsing, and of the monolayers obtained after dipping for 18 h in methanolic solution of **3** and **8,** taken as examples, are represented in Fig. 2a–c. Fig. 2a confirms that, even after cleaning, the Au surface is polluted by aliphatic and oxidized carbons (C_{cont}). In Fig. 2a and b, the spectra exhibit two main components while three components are present in Fig. 2c. These features are characteristic of spectra observed for series **1**–**5** and **6**–**10**, respectively, due to differences in chemical environment of carbons along the grafted molecules: the main peak is centered around 285 eV and is characteristic of alkyl moieties, one other component appears as a shoulder of the first peak at 286 eV and is characteristic of methylene or methyl carbon bound to electron-withdrawing groups X such as N^+, CO, and NH; the other peak at 288 eV for monolayers **6**–**10** is assigned to oxidized carbons, mainly C of amide function.

Table 1
Theoretical and experimental atomic percentages of Au, C, and O evaluated from the survey spectra for metal substrate before cleaning and after UV–ozone treatment.

Substrates	Au		C		O	
	Calc. %	Exp. %	Calc. %	Exp. %	Calc. %	Exp. %
Before cleaning	100	22	0	37	0	41
After cleaning	100	58	0	37	0	5

Table 2 summarizes the theoretical and experimental percentages of C, S, N and O on cleaned Au after modification with compounds **1**–**10** and dodecanethiol used as model, along with the re-calculated values for C, S, and N considering the theoretical values for oxygen. The theoretical percentage for each atom is the ratio between the number of the considered atom C, S, N or O in the formula of the used thiol and the total number of these atoms in the formula. The experimental values are obtained directly from the peak areas of the XP survey spectra by using classical software included in the treatment program of the XPS apparatus, this software taking into account the sensitivity factor of the different atoms under study (RSF).

For all structures, the experimental percentage of oxygen is upper than the theoretical value, particularly for the monolayers obtained with the shortest alkyl groups (compounds **2** and **7**). Consequently, the number of carbon is systematically underestimated in comparison with the true C number. Nevertheless and excepted for monolayer **10**, the experimental number of carbon is in very good agreement with the theoretical value when the estimation is done considering the stoichiometric values for oxygen.

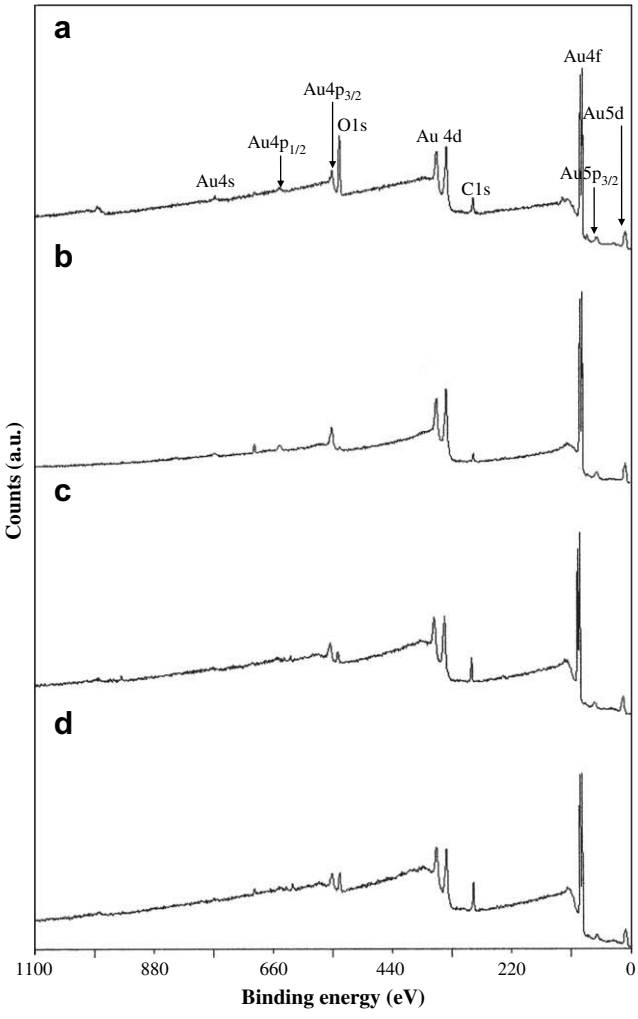


Fig. 1. XP survey spectra of the Au substrates before (a) and after (b) UV–ozone treatment, and after modification with **3** (c) and **8** (d).

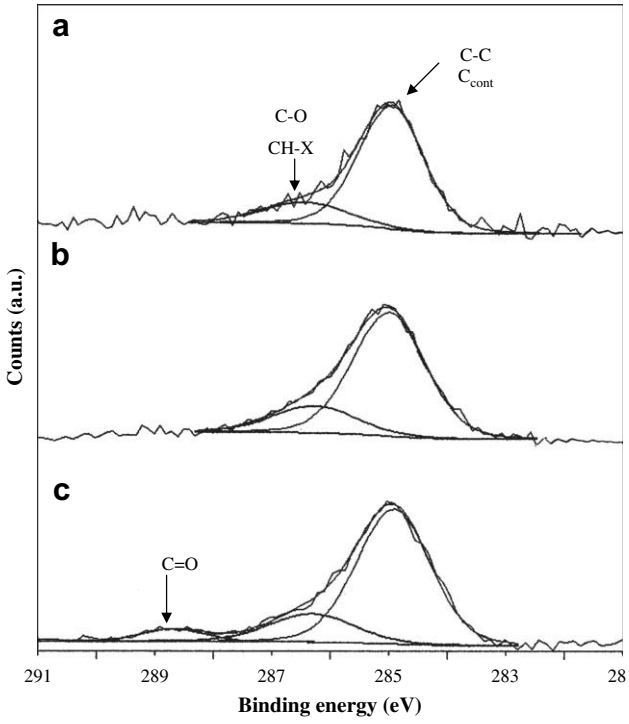


Fig. 2. XP C 1s spectra of cleaned Au (a), and Au substrates modified with **3** (b) and **8** (c).

Table 2
Theoretical and experimental atomic percentages of C, S, N, and O evaluated from the survey spectra of metal substrate grafted with **1**-**10** derivatives and with dodecanethiol ($C_{12}H_{25}SH$).

Modified substrates	C		S		N		O	
	Calc. %	Exp. %	Calc. %	Exp. %	Calc. %	Exp. %	Calc. %	Exp. %
Mod. $C_{12}H_{25}SH$	92	96	8	4			0	0
Mod. **1**	90	79 (93)[a]	5	3 (4)[a]	5	3 (3)[a]	0	15
Mod. **2**	88	68 (92)[a]	6	3 (4)[a]	6	3 (4)[a]	0	26
Mod. **3**	92	82 (93)[a]	4	3 (4)[a]	4	2 (3)[a]	0	13
Mod. **4**	92	80 (93)[a]	4	6 (7)[a]	4	4 (4)[a]	0	10
Mod. **5**	93	75 (89)[a]	3	5 (7)[a]	3	3 (3)[a]	0	17
Mod. **6**	85	71 (83)[a]	4	8 (9)[a]	7	3 (4)[a]	4	18
Mod. **7**	81	67 (82)[a]	5	6 (7)[a]	9	4 (5)[a]	5	23
Mod. **8**	86	77 (89)[a]	3	3 (3)[a]	7	3 (3)[a]	3	17
Mod. **9**	88	72 (88)[a]	3	4 (5)[a]	6	4 (5)[a]	3	20
Mod. **10**	88	88 (95)[a]	3	1 (1)[a]	6	1 (1)[a]	3	10

[a] Percentages evaluated using the calculated values for the oxygen atom instead of the experimental values; for instance, C 93% for Mod **1** (entry 2) is the ratio 79/(79 + 4+3 + 0).

This means that the residual carbon contaminant on the cleaned Au substrate is negligible in comparison with the carbon density due to the grafted molecules, excepted for monolayer **10** for which the number of carbon largely exceeds the theoretical value while the experimental S and N percentages for this surface are underestimated in comparison with the theoretical values. With the exception of compound **10**, this result and the good quality of the SAMs are confirmed by good agreement between the theoretical and the experimental percentages of each component of C atoms summarized in Table 3. As demonstrated by the overestimated number of sulphur (after oxygen percentage correction in Table 2) of all surfaces excepted **10**, the residual oxygen contaminant seems to be due in part to the presence of oxidized sulphur species. Indeed, Fig. 3a–c taken as examples of XP S2p spectra of Au substrates modified with **2**, **3** and **8**, respectively, allow observing, next to the two principal doublets 2p3/2-2p1/2 (for peak fitting procedure, see experimental part) around 162 and 163 eV assigned to thiolates and free thiols respectively, a clump centered around 167.5 eV (particularly in Fig. 3a); deconvolution of this clump leads to two structures which fit well with oxidized sulphur species such as sulfinates and sulfonates.

These XPS experiments seem to show that, excepted for compound **10**, all the thiol derivatives with or without the amide group lead to SAMs of good quality on cleaned Au substrate.

The relative qualities of SAMs are also estimated using the concept of blocking factor (BF) that can be evaluated from CV

Table 3
Percentages of the principal components of the C 1s XP spectra of the **1**–**10** monolayers calculated from the molecular structure and the experimental spectra.

Modified Substrates	CH_2 and CH_3 285 eV		CH_2–X[a] and CH_3–X[a] 286 eV		$C_{oxidized}$ 288 eV	
	Calc. %	Exp. %	Calc. %	Exp. %	Calc. %	Exp. %
Mod. **1**	80	76	20	24	0	0
Mod. **2**	71	63	29	37	0	0
Mod. **3**	83	80	17	20	0	0
Mod. **4**	84	85	16	15	0	0
Mod. **5**	85	80	15	20	0	0
Mod. **6**	70	72	26	24	4	3
Mod. **7**	59	65	35	27	6	8
Mod. **8**	73	76	23	18	4	6
Mod. **9**	73	75	23	21	4	4
Mod. **10**	77	99	20	<1	3	<1

[a] Electron-withdrawing group: N^+, C(O), NH.

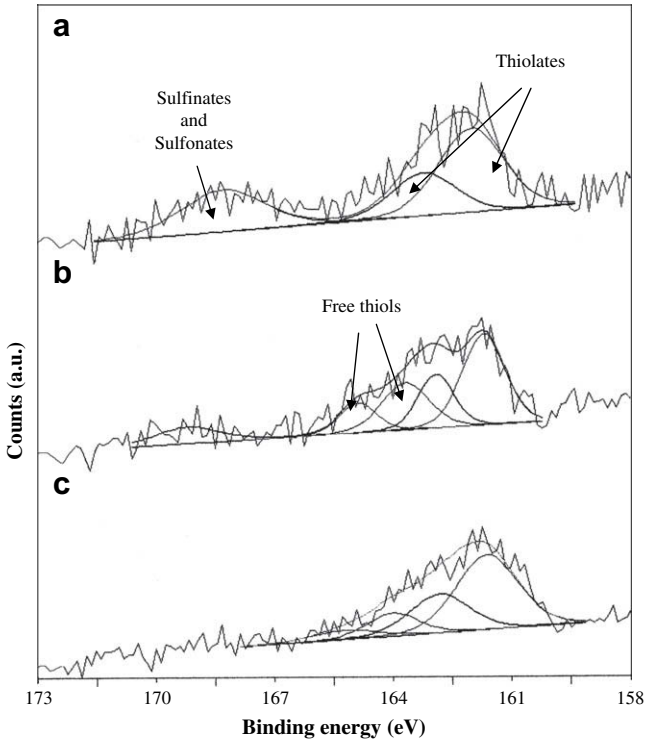


Fig. 3. XP S 2p ($S2p_{3/2} - S2p_{1/2}$) spectra of Au substrates modified with **2** (a), **3** (b) and **8** (c).

measurements [16,17,24]. Indeed when a gold sample is used as working electrode, surface oxidations are different according to the metal surface is grafted with organic molecules or not. So, the ratio between the difference of oxidation area of bare (A_{bare}) and covered (A_{cov}) substrates on cyclovoltammograms, and the oxidation area of bare substrate defines BF: $BF\ (\%) = ((A_{bare} - A_{cov})/A_{bare}) \times 100$. In fact, this BF definition is non totally quantitative because A_{cov} could contain a small part of participation of other oxidative processes such as, for example, the electron transfer by tunneling through blocking films. However, it can be assumed that such participations are very low and that BF is a very good evaluation of the quality of grafted films. The BF results are presented in Table 4. The high BF values confirm the good to very good quality of SAMs obtained as well with both the series **1**–**5** and **6**–**10** as the dodecanethiol used as model in order to validate our grafting process. In contrast with our previous results obtained on semi-fluorinated thiols and disulfides [21], the presence of the H-donor amide group in the series **6**–**10** increases only slightly the organization of the grafted molecules comparatively to the series **1**–**5** since, in the present case, there is no important repulsive force between the hydrocarbon chains of adjacent thiols in comparison with the perfluorocarbon chains of our previous work. For both series **1**–**5** and **6**–**10**, the poorest organization seems to be obtained with the structures **5** and **10** bearing the longest chain $R = C_{14}H_{29}$ as reflected by the slightly lowest values of BF in comparison with those of other structures. This result confirms that obtained from XPS experiments, at least for compound **10**.

In the literature of SAMs, contact angle measurements are also largely used in order to evaluate or to confirm the quality of the SAMs [25–28]; however, this method is particularly used with semi-fluorinated compounds because of the high hydrophobicity and lipophobicity of semi-fluorinated carbon chains that largely increase the values of CA in comparison to hydrocarbon chains for a given roughness of the treated surface [16,17,19,21,29]. In the

Table 4
Water, hexadecane and diiodomethane contact angles (θ_w, θ_h and θ_d), total surface energies (γ_S) and blocking factor (BF) measured for cleaned Au and for Au substrates modified with **1**–**10** derivatives and with dodecanethiol ($C_{12}H_{25}SH$).

Surfaces	Clean Au	Mod. $C_{12}F_{25}SH$	Mod. **1**	Mod. **2**	Mod. **3**	Mod. **4**	Mod. **5**	Mod. **6**	Mod. **7**	Mod. **8**	Mod **9**	Mod **10**
BF (%)	0	98	90	92	87	97	84	92	91	90	95	86
Θ_w (deg)	82	106	64	39	69	75	79	62	33	65	69	77
Θ_h (deg)	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Θ_d (deg)	55	57	45	21	48	50	51	40	17	44	46	49
γ_S (mN/m)	33	29	43	61	39	37	35	44	64	43	40	36

present work, the grafted structures bring a hydrophobic and oleophilic hydrocarbon chain with variable length on the one hand, and a hydrophilic quaternary ammonium function on the other hand. Consequently, it is difficult to use only the variations of CAs in order to discuss on the relative quality of the SAMs. These CAs are summarized in Table 4 along with the total surface energies. As suggested in a recent study by Sharma and Rao on the main approaches for the estimation of surface free energy [30], the Owens, Wendt, and Fowkes model is used in this work to obtain these total surfaces energies [31,32]. It appears that: (i) all the surface are highly oleophilic and more hydrophilic than the clean Au substrate; (ii) the hydrophilicity increases when the length of the hydrocarbon chain decreases due to the loss of hydrophobicity of the alkyl chain and also to the best exposure of the charged nitrogen at the extreme surface; (iii) the estimated surface energies varies in the inverse way than the water CA, the highest values being obtained for the shortest alkyl chain (compounds **2** and **7**) and the lowest for the longest chain (**5** and **10**); (iv) the presence of an amide function in the series **6**–**10** does not greatly affect the CAs and surface energies since for a same R chain the corresponding values of θ_w and γ_S are near along of both series **1**–**5** and **6**–**10**. All these results are in agreement with equivalent qualities of SAMs for all the grafted structures.

2.3. Antimicrobial activity of the modified gold substrates

Recent literature data describe a lot of procedures leading to an evaluation of antimicrobial activity of a solid surface [1,33–35]. No described method seems to be entirely satisfactory for a future industrial routine check because, in our opinion, they do not reproduce usual cases of surface contamination, and we decide to use the Rohm and Haas' internal procedure described in experimental section in which the cells were incubated in dry conditions to simulate xerophilic conditions encountered on contaminated surface where *S. aureus* can survive. The biocidal efficacy data of bare gold sample and modified gold samples against Gram-positive *S. aureus* are presented in Table 5 along with the Minimal Inhibitory Concentration (MIC) previously obtained for bactericidal activities of free thiols against *S. aureus* [22]. The tests were conducted in triplicate in separate experiments and the data presented are the average of three values.

Several observations are apparent from Table 5. First, it appears that only the biocidal surfaces bearing molecules with long alkyl chains, particularly with 10 and 12 carbon atoms, are relatively effective against Gram-positive *S. aureus* after a contact time of 4 h. This observation of the dependence of biocidal activity of quaternary ammonium salts with the length of their alkyl chain is in total agreement with literature data concerning immobilized ammonium salts [1,36,37]. This type of dependence with the length of the alkyl tail linked to nitrogen was previously observed in different studies on solubilized biocides [38–40]. This phenomenon was called "cut-off effect". In our case, the comparison of the biocidal activity of the substrates modified with **2**–**5** on one hand and **7**–**10** on the other hand, with that observed for the corresponding free molecules and expressed by their MIC values reported in Table 5 seems to show that: (i) the effect of the chain length on the "cut-off effect" is similar for the free and the grafted molecules in each series; (ii) the maximum of the parabolic dependence is shifted towards the slightly high chain length for the series with the amide connector as well for the solubilized molecules as the grafted molecules. The origin of the "cut-off effect" is not well explained. Among the various assumptions proposed by Balgavy and Devinsky [39], the concept of free volume could be applied to the quaternary ammonium salts in solution. The assumption is that the polar ammonium heads interact with those of phospholipids and the hydrocarbon chains are parallel to those of phospholipids of the cell. At this level, the density of the hydrophobic area of the bilayer is necessarily modified and a free volume is formed. When the length of the hydrocarbon chain of ammonium salts is smaller than that of phospholipids, the total free volume created in the bilayer is small. When the length of the surfactant tail becomes comparable to that of phospholipids, the free volume decreases and tends towards zero. Molecules bearing chains between these two extremes lead to the most important free volume inside of the bilayer. More the free volume is large more the decrease of hydrophobicity of the membrane is large. Consequently, a largest destabilization of the membrane is expected and the bactericidal activity increases. In agreement with our own results, this "cut-off effect" was also observed on various surfaces grafted with quaternary ammonium compounds such as glass or iron oxide surfaces [1,36], or carbohydrate-based surfaces [37]. Taking into account the relationship observed between the parabolic dependence of the bactericidal activities of the free molecules **2**–**5** and **7**–**10** and those of the modified gold substrates (Table 5), the concept of free volume proposed for the solubilized biocides could be also applied to these immobilized ones. The analysis of the literature data and the present results show that the dependence of the microbicidal activity with the length of the alkyl chains depends as well on the type of quaternary ammonium salts as on the type of grafted surfaces, and today it seems impossible to give a general rule and an exact interpretation of these dependences.

The second observation coming from Table 5 is that the bactericidal efficiency of substrates modified with compounds **8**–**10** seems to be superior to those of surfaces with compounds **3**–**5**. This observation is directly related to the previous one. Assuming that the parameters describing the quality of theses SAMs, such as BF and γ_S are quite similar for **3** and **8**, **4** and **9**, and **5** and **10**, respectively (Table 4), this apparent increase of efficiency for the molecule bearing an amido group could be attributed to the possibility of hydrogen bonds. This is in good agreement with the well established models for the prediction of antibacterial activity of various classes of antibacterial agents which state that hydrogen bonding is one of the more important descriptors [41]. (iii) no activity is observed for all the surfaces after a contact of 24 h; however, after abundant washings of surfaces modified with **2**–**4** and **7**–**10** with ethanol and then water, the activity after 4 h is entirely recovered. This behavior has been previously observed in the literature, for instance for quaternary ammonium salts

Table 5
Bactericidal activity against *Staphylococcus aureus* of gold slides grafted with quaternary ammonium thiol derivatives along with the MIC of solubilized molecules [22].

Sample	Contact time of 4 h			Contact time of 24 h		MIC (μMol/L) for free thiol[b]
	CFUs[a]	Log red.	Bactericidal efficiency (%)	CFUs[a]	Log red.	
Bare Au	3.7×10^6	–	–	>5.3[c]	–	–
Mod. **1**	2.7[c]	0.14	30	>5.3[c]	0	681.6
Mod. **2**	2.7[c]	0.14	30	>5.3[c]	0	56.6
Mod. **3**	1.8[c]	0.31	51	>5.3[c]	0	11.6
Mod. **4**	1.8[c]	0.31	51	>5.3[c]	0	34.7
Mod. **5**	3.7[c]	0	0	>5.3[c]	0	1306.3
Mod. **6**	3.7[c]	0	0	>5.3[c]	0	592.2
Mod. **7**	2.7[c]	0.14	30	>5.3[c]	0	105.4
Mod. **8**	1.5[c]	0.39	67	>5.3[c]	0	17.5
Mod. **9**	1.8[c]	0.31	51	>5.3[c]	0	18.7
Mod. **10**	2.7[c]	0.14	30	>5.3[c]	0	187.7

[a] Colony forming units.
[b] Minimal Inhibitory Concentration of free thiols against *Staphylococcus aureus* according to reference [22].
[c] 10^6.

insolubilized on porous glass surfaces [42]. The loss of bactericidal activity at 24 h is due to a layer built by the dead bacteria consortium on the SAM after 4 h or less. The microbes are negatively charged which could have probably masked the positive hydrophilic head of the immobilized quaternary ammonium compounds and consequently inhibit the uptake of the biocide part inside the cells. In other words, a moiety of immobilized quaternary ammonium moieties is uptake inside the microbes and consequently deactivates the unique layer of immobilized biocides. This phenomenon could be considered as an important drawback for using this strategy to protect metallic surfaces against bacterial contamination, and solutions to circumvent this problem must be investigated soon.

3. Experimental section

3.1. Synthesis of thiol derivatives

The synthesis and characterization of thiols **1**–**10** has been previously described along with their microbicidal properties [22].

3.2. Substrate Preparation

The substrates used in this study were square coupons (17 mm × 17 mm) cut from commercially available gold {111} wafers on silicium (Neyco France Au 99.99 % thickness 150 nm on Cr 20 nm; nanoroughness from Atomic Force Microscopy measurements: Ra = 1.1 nm; Rq = 1.5 nm). The substrates were cleaned by sonication for 5 min in four solvents (dichloromethane, hexane, acetone, methanol) and treated first during 20 min in a UV/Ozone cleaner and then by sonication for 20 min in methanol. After this treatment, the Au substrates were immediately used for the preparation of the monolayer to minimize pollution of the surfaces due to their exposition to the laboratory atmosphere. The exposure time to the laboratory atmosphere of the Au substrates during this transfer operation is estimated to last less than 3–5 s.

3.3. Immobilization of thiols on the gold substrate

The monolayers were formed by immersion of the Au substrates in 10^{-2} M methanolic solutions of thiols **1**–**10** or dodecanethiol for 18 h. This concentration and the dipping time were optimized in a previous work [21]. Highest concentrations cannot be used because the low solubility of charged sulphur derivatives in organic solvents. In order to compensate these relatively low concentrations, a large dipping time of 18 h is necessary to obtain SAMs of good quality.

All thiol solutions were degassed with argon and kept under argon atmosphere during modification. After immersion, all modified samples were rinsed with pure methanol, blown dry under nitrogen gas. The samples were immediately used for XPS characterization and then stored under argon atmosphere to be bacteriological analyzed within 24 h. The storage under argon atmosphere did not modify the quality of the SAMs since the contact angles remain constant when measured immediately after the monolayer formation and after the 24 h storage. Moreover, XPS experiments performed immediately and after storage do not show any significant difference.

3.4. Surface characterization

XPS of monolayers were obtained with a Surface Science Instrument spectrometer using a monochromatic and focused (spot diameter of 600 μm, 100 W) Al Kα radiation (1486.6 eV) under a residual pressure of 10^{-7} Pa, the photoemitted electrons being collected at 35° takeoff angle. The binding energy of the core levels was calibrated against the Au $4f_{7/2}$ binding energy set at 83.9 eV. The spectra were performed with a non linear Shirley-type baseline using the appropriate Scoffield factors. The peaks were analyzed using mixed Gaussian–Lorentzian curves (80% Gaussian character). The peak fitting of S2p ($S2p_{3/2} - S2p_{1/2}$ doublet) was performed with constraint at the level of the intensity of the components ($S2p_{3/2}/S2p_{1/2} = 2$) and of their energy shift ($S2p_{3/2} - S2p_{1/2} =$ 1.2 eV). The binding energies of the core levels of the C, N, O, and S atoms are assigned from literature data [43].

The electrochemical characterization of gold surfaces with and without monolayer was carried out by a conventional three electrodes cell. A calomel electrode saturated with KCl was used as reference electrode, a platinum slab as a counter-electrode and the gold wafers as working electrodes. The cell was connected to an autolab PGSTAT 30 potentiostat from Eco Chemie B.V. equipped with general purpose electrochemical system GPES software (version 4.9 for Windows). CV measurements were carried out in 0.5 N H_2SO_4 aqueous solutions (deoxygenated by argon bubbling for 20 min prior to the measurements) with a potential ranging from 0.3 to +1.6 V per SCE at a sweep rate of 50 mV/s.

CAs were performed using the sessile drop method on a Krüss DSA10 contact angle goniometer. The angles reported here were the averages of at least five measurements. Reproducibility was within ±3°. They were recorded at 20 °C on 10^{-6} dm^3 drops of ultra pure water, diiodomethane and hexadecane as wetting liquids.

3.5. Microbial strains

S. aureus (ATCC 6538) was grown overnight on Trypticase Soy Agar slants at 30 °C then inoculated into 10 mL of M9 G (M9 glucose minimal medium consists of 0.2% glucose, 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 20 mM NH_4Cl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, and 5 μg/mL thiamine) [44] and incubated overnight at 30 °C. The optical density of the suspension was then measured at 660 nm. M9GY medium (Minimal salt medium M9 G supplemented with 0.2% glucose and 0.1% yeast extract) [45] is added to the suspension to adjust the optical density at 660 nm to 0.450 (this corresponds approximately to a population of 10^8–10^9 cfu/mL).

3.6. Evaluation of antimicrobial activity

A 15 μL volume of *S. aureus* bacterial suspension (7×10^8 cfu/mL) was spread on the unmodified and modified gold slides (17 mm × 17 mm). The gold slides were air dried for 1 h at ambient temperature. The slides were incubated at 30 °C for 4 or 24 h at 95% relative humidity. At the end of the incubation, the gold slides were emerged in 10 mL of 0.5 % aqueous solution of Tween 80 for 30 min. The number of viable cells in the suspension was determined by the Most Probable Number (MPN) method [46]. The MPN method is a well established method for viable micro-organisms quantification. It is readily adaptable to small sample sizes and various micro-organisms. For the MPN approach, 40 microliters samples were added to 96-well polypropylene microtiter plates containing 160 microliters of recovery media. Samples were then serially diluted (5-fold) across the microtiter plate using a Biomek™ 1000 Automated Workstation. Statistical tables were used to calculate the most probable number estimate of the microbial population in the sample from a combination of positive growth readings in a set of serial dilutions in the 96-well microtiter plates. Positive readings are made on a growth – no growth basis. Confirmation of growth in each well was determined manually by visual observation of turbidity or cell mass. In all cases, the assessment of growth was based on a comparison to the control wells containing no micro-organisms. MPN tests were set up with either two or four replicates per dilution tested. The limit of detection of the MPN method was 10-30 cells per mL, depending on the number of dilution replicates used. MPN counts for viable bacteria were determined after two days incubation in Trypticase Soy Broth (TSB) medium at 30 °C.

4. Conclusions

We demonstrate that microbicidal contact-active gold surfaces can be prepared from grafting of specially synthesized thiol derivatives bearing a quaternary ammonium moiety and variable hydrocarbon chains. Combining XPS, cyclic voltammetry and contact angle measurements, we have shown that the self-assembled monolayers formed on cleaned gold are of good to very good quality; however, this result could be confirmed by direct titration of ammonium function in a future step of this research. The antibacterial activity of the modified substrate was checked against *S. aureus* using an original procedure. The results support the antibacterial activity of the synthesized molecules after their adsorption on the gold surface, particularly for the derivatives bearing an alkyl chain with ten or twelve carbon atoms on the charged nitrogen and an amido group as connector. However, this activity is relatively low and disappears after a contact of 24 h due to a film formation of killed bacteria on the active surface, and ethanol and water washings were necessary to recover the initial antibacterial activity. This loss of activity after a relatively short time could be considered as a non negligible drawback. The next step of this work will be to try to replace the hydrocarbon chain on the nitrogen atom with perfluorinated alkyl chain with the hope to reduce the adhesion of dead cells on the modified gold surface while keeping and increasing its antimicrobial activity. Indeed, some of our previous works showed that a perfluorinated chain can enhance the bactericidal activity of the free quaternary ammonium salts [47–49]. Moreover, it is well known for a long time that perfluorinated coatings present anti-adhesive properties [50–52], and we can expect that this essential property of perfluorinated chains could decrease the rate of formation of the "biopassivation" layer on the bactericidal SAMs, and consequently lead to an increase of their bactericidal efficiency between 4 and 24 h. After an optimization of the surface activity against *S. aureus*, the procedure will be extended to other usual micro-organisms including Gram (−) strains such as *P. aeruginosa*.

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