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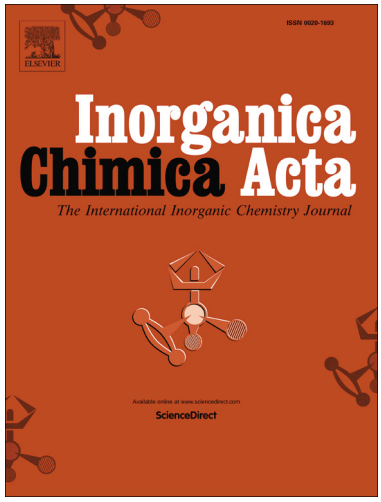

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Conjugation between maleimide-containing Pt(IV) prodrugs and furan or furan-containing drug delivery vectors via Diels-Alder cycloaddition.

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Abstract: Pt(IV) complexes are considered to act as antitumor prodrugs and their *in vivo* activity can be improved exploiting drug targeting and delivery strategies. With a view to such applications, the maleimide-containing ligand 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetic acid was used to produce the cisplatin-based Pt(IV) complexes (*OC*-6-44)-diamminedichlorido(ethanolato)(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetato)platinum(IV) and (*OC*-6-44)-acetatodiamminedichlorido(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetato)platinum(IV). These complexes underwent Diels-Alder reaction with furan, used as a model molecule to set up the experimental conditions, at ambient temperature up to 50 h, with limited decomposition. Finally, the reaction between the maleimide-containing Pt(IV) complexes and silica nanoparticles decorated with furan were successfully used as a proof-of-concept to demonstrate the *clickability* of functionalized vectors for drug delivery.

Keywords: Pt(IV) complexes • click chemistry • drug targeting and delivery • Diels-Alder reaction

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1. Introduction

Despite the fact that Pt(IV) complexes were recognized as anticancer agents in the original paper reporting on the biological activity of cisplatin (*cis*-diamminedichloridoplatinum(II), (*SP*-4-2)-diamminedichloridoplatinum(II), $[PtCl_2(NH_3)_2]$, CDDP) [1], their development was much less intense than those of the worldwide approved Pt(II) drugs. In the recent years, in the quest of the *Holy Grail of the anticancer drugs* (*i.e.*, the search for drugs more selective and less toxic than cisplatin), the higher oxidation state of platinum has experienced a renewed interest because of its peculiar chemical features [2-5].

The 6-coordinated octahedral geometry of Pt(IV) is *generally* characterized by kinetic inertness. This can minimize off-target effects, thus improving the therapeutic properties and allowing oral administration. Additionally, Pt(IV) complexes can be selectively (at least in principle) reduced in the hypoxic tumor environment to the corresponding cytotoxic Pt(II) metabolite with the loss of their axial ligands (*activation by reduction*).[4, 6-8]

To further improve the features of such compounds, part of the research shifted towards the use of Pt(IV) complexes as scaffolds for other purposes. The unique features of the Pt(IV) chemistry may be exploited to build conjugates acting as “guided missiles” in the drug targeting and delivery (DTD) strategies. The active DTD takes advantage from specific interactions between the drug and the biological target to increase a selective recognition of the conjugate by the cancer cell or cancer tissue [8-11]. On the contrary, passive DTD takes advantage of the enhanced permeability and retention (EPR) effect in tumor tissues, using functionalized polymers, nanoparticles, micelles, liposomes or nanotubes bearing the drugs as a cargo. By this effect, macromolecules selectively gather in the tumor interstices, because the tumor vessels are highly disordered and swollen with abundant pores resulting in enlarged gap junctions between endothelial cells [12].

In both cases, controlled drug release from the vectors can exploit the *in vivo* activation by reduction of the Pt(IV) moiety producing its active square planar Pt(II) metabolite selectively at the tumor site.

Finally, very recently, the Pt(IV) chemistry has been used to design dual- or even multi-functional drug candidates that act as combination-therapy single-molecule, also named "combo". One or more adjuvant/synergistic agent/s can be conjugated to the octahedral Pt(IV) assembly so that they and the Pt(II) metabolite may be released simultaneously and may act on different molecular targets/mechanisms in additive or (better) synergistic way [2, 5, 13].

Whatever the strategy employed, it is necessary to have Pt(IV) complexes containing versatile side-chain functionalities to link the vector or the coadjuvant drug (*e.g.*, -COOH [14-17], -C(=O)H [18], -NH_2 [19], -OH [20], -N_3 [16, 21, 22]) [23].

Among the most traditional examples, maleimide has been introduced in Pt(IV) complexes as connecting moiety. This functional group is a popular reagent for thiol-selective modification in the pH range 6.5-7.5: the double bond of maleimides undergoes an alkylation reaction with sulfhydryl groups to form stable thioethers. A set of cisplatin and oxaliplatin-based Pt(IV) complexes bearing a maleimide moiety in axial position was reported by Keppler et al. [24]. This modification promoted selective binding to serum albumin in the bloodstream, leading to prolonged plasma half-life time and preferential accumulation of the drug in the tumor tissue due to EPR effect [24-26]. A similar approach was used to link a Pt(IV) moiety to the epidermal growth factor receptor-targeting peptide LARLLT [27].

The maleimide group can also be used as the alkene derivative in the Diels-Alder reaction, consisting of the covalent coupling of a conjugated diene with an alkene to form a 6-membered ring containing a single double bond. This process is extensively used in organic synthesis, and recently it has been applied to bioconjugation and drug delivery systems, since it proceeds in water, at near room temperature, and with high yields [28-31].

The Diels-Alder reaction is an alternative to the well-known copper-catalyzed Huisgen 1,3-dipolar cycloaddition of organic azides and alkynes (CuAAC), widely used for the connection of molecular entities of all sizes. In the CuAAC click reaction, ascorbate is used as reducing agent to maintain the required Cu(I) oxidation state [32]. However, both Cu(I) and ascorbate may be reducing agents also for Pt(IV), and, hence, this may complicate the application of the basic CuAAC reaction to Pt(IV) complexes [22].

Here we describe the preparation, characterization, and some coupling reactions of Pt complexes containing the maleimide-based carboxylic acid 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetic acid directly coordinated to the metal center (Figure 1 and Figure 2). Moreover, the most promising complexes were loaded onto furan-functionalized silica nanoparticles, after setting the Diels-Alder reaction conditions with NMR-monitored model syntheses.

2. Results and Discussion

2.1. Synthesis of the Pt complexes

After preparing the maleimide-containing ligand 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetic acid, **HL**, according to previously reported procedures [33, 34], different strategies were attempted to introduce such a ligand into the octahedral assembly of a Pt(IV) complex.

The first strategy involved the equatorial coordination of carboxylate **L** (Figure 1). For this purpose, the suitable Pt(II) complex **1** derived from cisplatin upon substitution of one chlorido with ligand **L** was prepared. The presence of two different leaving groups in complex **1** was testified by the splitting in two of the ^{1}H-NMR signal of the ammine groups. As protons *trans* to chlorido show a higher chemical shift than the ones *trans* to carboxylate [35], it was possible to assign the ^{1}H signals at

4.16 and 4.70 ppm to the protons of NH_3 *trans* to **L** and to chlorido, respectively. Moreover, the ^{195}Pt NMR signal falls in a region well-matched with the presence of a Pt(II) coordinated to one chlorido, one carboxylato, and two amine ligands [36].

Any attempt to oxidize complex **1** with common procedures was frustrated by low yield and/or low purity. In particular, both the use of hydrogen peroxide in water (to obtain the dihydroxido derivative) [37] and of *N*-chlorosuccinimide in non-coordinating acetone with nucleophilic succinic anhydride (to obtain the chlorido(3-carboxypropanoato) derivative) [20] afforded products not pure enough to proceed with further coupling reactions. Indeed, in both cases HPLC-MS analyses showed traces of the expected products in mixtures of unreacted **1**, hydrolyzed **1** and a Pt(IV) complex containing two equatorial **L**, together with other not identified species. Therefore, such a strategy was abandoned.

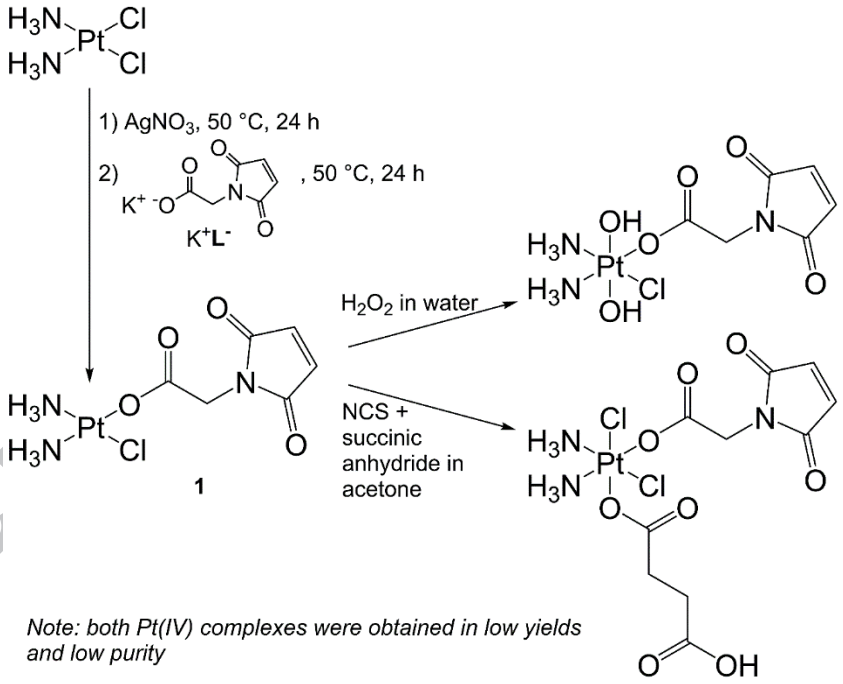

Figure 1. Reaction scheme for the synthesis of Pt(IV) complexes containing equatorial maleimide; NCS = *N*-chlorosuccinimide.

The second strategy involved the introduction of axial **L** into two different kinds of cisplatin-based Pt(IV) complexes (Figure 2). In this case, cisplatin was oxidized with hydrogen peroxide in ethanol or acetic acid to get the corresponding monohydroxido derivatives (**2** and **3**, respectively) according to previously published procedures [19, 38, 39]. The reaction of these intermediates with the activated form of ligand **HL** (by using *N*,*N*'-dicyclohexylcarbodiimide, DCC) [16] resulted in the syntheses of maleimide-containing Pt(IV) complexes **4** and **5** useful for further reaction with furan derivatives.

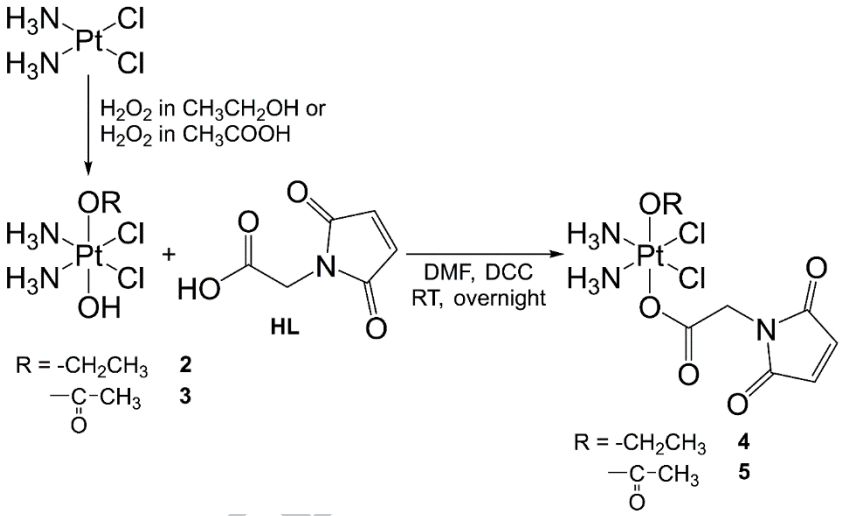

Figure 2. Reaction scheme for the synthesis of Pt(IV) complexes containing axial maleimide. DCC = *N*,*N*'-dicyclohexylcarbodiimide.

2.2. Diels-Alder reaction of the Pt(IV) complexes with furan

The Diels-Alder reaction is a [4 + 2] cycloaddition between an alkene and a conjugated diene to form a cyclic molecule by rearrangement of their π electrons and the formation of two new σ bonds. In this framework, the maleimide-containing complexes **4** and **5** (the dienophile) can be loaded onto nanoparticles decorated with furan (the conjugated diene) for DTD purposes. To set up the Diels-Alder

reaction conditions, the reaction between **4** or **5** and furan as a model was studied (Figure 3) by monitoring it with ^{1}H NMR.

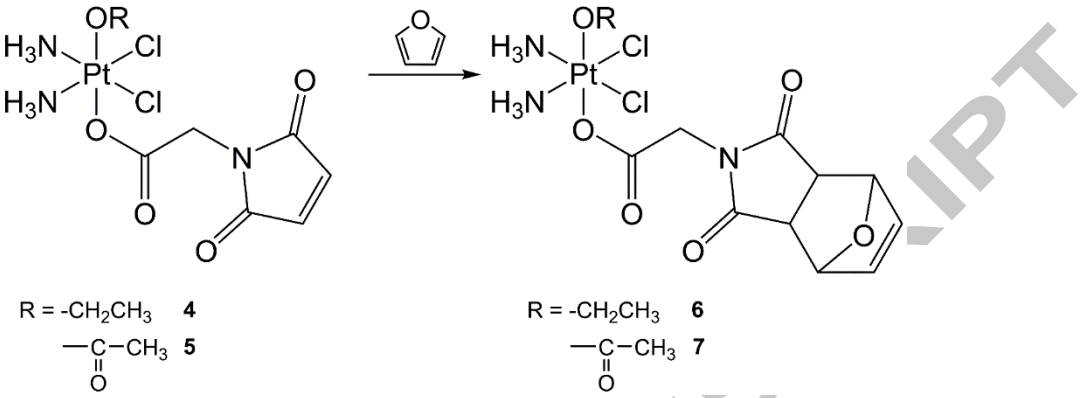

Figure 3. Scheme of the Diels-Alder reaction between furan and complexes **4** and **5**.

First, complex **4** and furan were dissolved in D_2O at room temperature (in 1:1 molar ratio) and an initial ^{1}H-NMR spectrum was acquired. The mixture was then warmed to 40 °C and the reaction was monitored by ^{1}H-NMR for 5 d recording the spectra almost every 2 h (see Supplementary data) [40]. It was observed that the -(*H*C=C*H*)- signals of maleimide and furan gradually decreased, whereas, in the range between 5.4 and 5.6 ppm, the characteristic signals of the bound furan were progressively formed, together with those of bound maleimide in the δ = 3-4 ppm region [40]. However, several new signals due to degradation were also observed. Accordingly, the solution color changed from yellow (almost unchanged within 24 h) to very dark orange after 5 d. For this reason, the reaction was carried out at 25 °C to avoid or limit such a degradation process.

Also in this case, both **4** and furan were dissolved in D_2O, and the reaction was followed for 5 d recording the ^{1}H-NMR spectrum almost every 2 h. Once again, the signals of free furan gradually decreased, while increasing the characteristic signals of the bonded furan. At the same time, the signal of the original -(*H*C=C*H*)- maleimide decreased in favor of bound maleimide. However, some new signals also appeared, even though less quickly than in the previous case, and the -(*H*C=C*H*)-

maleimide- signal did not completely disappear. The main changes were observed within 24 h (see Figure 4 and Supplementary data). In this case, the solution color (yellow) remained unchanged during the whole reaction period (5 d).

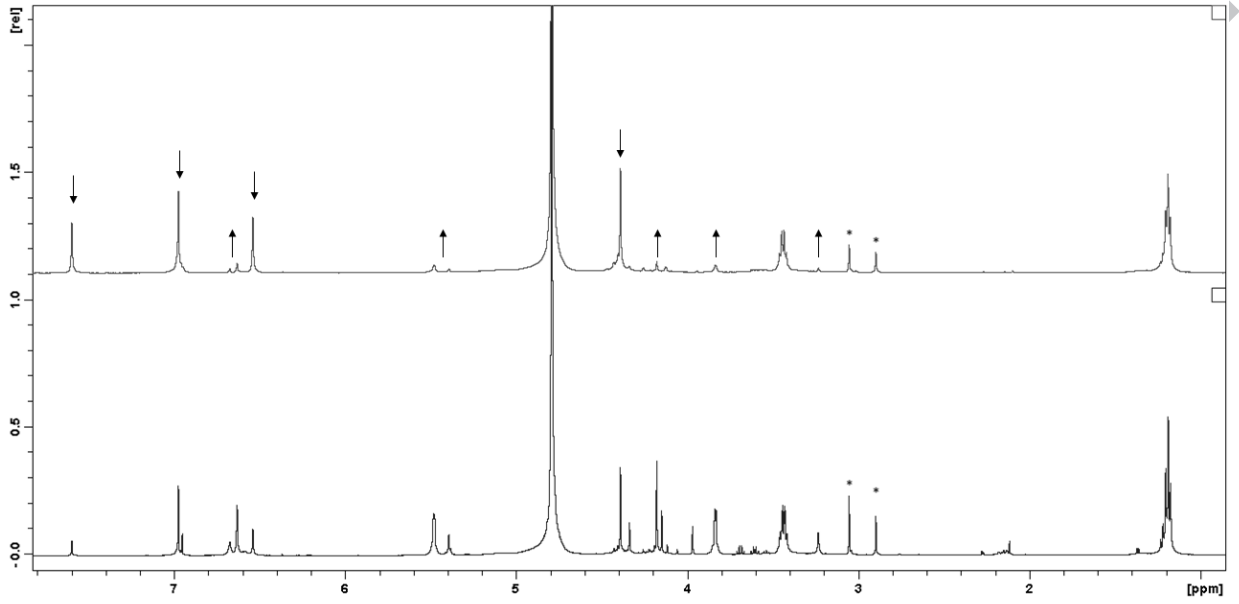

Figure 4. ^{1}H-NMR spectra of the Diels-Alder reaction of **4** with furan (1:1 ratio) in D_2O at 25 °C at reaction time = 0 h (upper spectrum) and 17 h (lower spectrum), respectively. Arrows point out decreasing (**L** and furan) and increasing (**L** modified after cycloaddition) signals. Residual DMF is also present (*).

The Diels-Alder product **6** showed a ^{195}Pt NMR signal in D_2O at 906 ppm ($^{1}J_{Pt\text{-}N}$ = 197.7 Hz), consistent with a "$N_2O_2Cl_2$" coordination sphere around the Pt(IV) core. Moreover, using Heteronuclear Single Quantum Correlation (HSQC) NMR technique also ^{1}H and ^{13}C signals of complex **6** after 50 h reaction were identified (see Supplementary data). It is important to note that a double series of signals was observed and this is justified by the formation of exo/endo products, the attribution of which was carried out according to the literature [41].

The reaction with furan at 25°C was carried out also with complex **5**, with similar results. The reaction was almost complete in 18 h and the ^{1}H NMR signals of complex **7** were assigned based on the comparison with compound **6**. Interestingly, fewer by-products were observed with respect to the formation of complex **6**. Finally, when using furan in 2:1 ratio, instead of 1:1, the maleimide was almost completely consumed in the same reaction time (18 h) (See Supplementary data).

2.3. Diels-Alder reaction of the Pt(IV) complexes with furan-decorated silica nanoparticles

Based on the previous results, nonporous silica nanoparticles (SNPs) decorated with primary amino groups were chosen as model DTD vectors for the Pt(IV) antitumor prodrug upon Diels-Alder reaction. Indeed, SNPs have acceptable biocompatibility, low cost large-scale synthesis and easy control of surface properties and can be modified in the external shell with chemical functionalities useful to link drugs. SNPs containing the 3-aminopropyl arm were synthesised according to literature methods [38, 42, 43] and further coated with a shell of 3-(furan-2-yl)propionate (after removal of its ethyl protecting group and subsequent activation of its carboxylic group with 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate, HATU, as a coupling agent to form amide bonds). Complexes **4** and **5** were loaded via Diels-Alder reaction (overnight, 25 °C), and the Pt content was evaluated by inductively coupled plasma - mass spectrometry (ICP-MS) (see Experimental Section). The results indicated a similar loading for the two complexes (mean values 3.7×10^{-2} and 3.5×10^{-2} mmol Pt g^{-1} SNPs for **4** and **5**, respectively), albeit lower with respect to those obtained with the same SNPs used to load directly the two cisplatin-like (*OC*-6-44)-diamminedichloridoethoxidosuccinatoplatinum(IV) and (*OC*-6-44)-acetatodiamminedichloridosuccinatoplatinum(IV) complexes through the formation of an amide bond

between the axial succinato ligand and the amino groups on the SNPs (7.9×10^{-2} and 5.8×10^{-2} mmol Pt g^{-1} SNPs, respectively) [42].

This quick model test demonstrated that the maleimide linker can be successfully used to link Pt prodrugs to furan-modified vectors through a reaction that can be performed under mild conditions, in particular when the reaction times are not a significant factor.

To verify the stability of the conjugates, they were suspended in 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH = 7.4) and the Pt release was checked by means of inductively coupled plasma - mass spectrometry (ICP-MS). The released Pt reached 13% at 4 h and this behavior, in agreement with that observed for similar conjugates, is justified by the detachment of the 3-aminopropylsilane arm from the silica core by hydrolysis of a Si–O–Si bond [38, 42-47].

3. Conclusions

In this study the maleimide-containing ligand 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetic acid, **HL**, was employed for the synthesis of Pt(IV) complexes having this carboxylate in equatorial or axial position. The best compromise between yield and purity was obtained for complexes **4** and **5** containing **L** as axial ligand. Such complexes proved to be able to undergo Diels-Alder reaction with furan, used as a model to set up the reaction conditions. The reaction at 40 °C was faster than at 25 °C, but the amount of by-products rapidly increased. For this reason, a reaction time no longer than 10-12 h is recommended.

On the contrary, at 25 °C, the reaction could be extended to 50 h, with limited decomposition, even though 18 h can represent a good compromise. It is important to consider that, even though the Diels-Alder reaction is not complete, and/or some traces of by-products are formed, undesired small molecules can be easily removed by dialysis or washing out when the cycloaddition is performed on

furan-functionalized macromolecules or nanoparticles, respectively, for passive DTD purpose. As a proof of concept, the loading of the Pt(IV) complexes **4** and **5** on furan-functionalized silica nanoparticles shows the feasibility of the Diels-Alder reaction for DTD and may represent an alternative to other click reactions, less compatible with the Pt(IV) chemistry. The evaluation of the potentiality of such conjugates needs *in vivo* tests, where the EPR effect is operating.

4. Experimental Section

4.1. General procedures

All the chemicals (Sigma Aldrich or Alfa Aesar-Thermo Fisher Scientific) were used as received and without further purification. Complexes **2** and **3** were prepared according to previously published procedures [19, 38, 39, 42]. The purity of all the synthesized compounds was assessed by analytical RP-HPLC and elemental analysis. Chromatographic analyses were carried out using a C18 Phenomenex Phenosphere-NEXT (5-μm, 250×4.6 mm ID) column on a Waters HPLC-MS instrument (equipped with Alliance 2695 separations module, 2487 dual lambda absorbance detector, and 3100 mass detector). The UV-visible detector was set at 210 nm, the eluent was a 70/30 mixture of 15 mM formic acid/CH_3OH and the flow rate was 0.5 mL min^{-1}. Mass spectra were recorded using source and desolvation temperatures set to 150 and 250 °C, respectively, with nitrogen used as both drying and nebulizing gas. The cone and the capillary voltages were usually +30 V (positive ion mode) or -30 V (negative ion mode) and 2.70 kV, respectively. The quasi-molecular ion peak $[M+H]^+$ or $[M-H]^-$ of the synthesized complexes were assigned on the basis of the *m/z* values and of the simulated isotope distribution patterns. Elemental analyses were carried out for complexes **1**, **4** and **5** by means of a EA3000 CHN Elemental Analyzer (EuroVector, Milano, Italy). The NMR spectra were measured on a

NMR-Bruker Avance III spectrometer operating at 500 MHz (^{1}H), 125.7 MHz (^{13}C) and 107.2 MHz (^{195}Pt), respectively, or on a JEOL Eclipse Plus spectrometer operating at 400 MHz (^{1}H), 100.6 MHz (^{13}C) and 86.0 MHz (^{195}Pt), respectively. ^{1}H and ^{13}C NMR chemical shifts were reported in parts per million (ppm) referenced to solvent resonances. ^{195}Pt NMR spectra were recorded using a solution of $K_2[PtCl]_4$ in saturated aqueous KCl as the external reference. The shift for $K_2[PtCl]_4$ was adjusted to −1628 ppm from Na_2PtCl_6 (δ = 0 ppm).

*4.2. Synthesis of 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetic acid, **HL***

The maleimide containing ligand **HL** was synthesized according to a previously published two-steps procedure, as briefly reported below [33, 34].

4.2.1. Synthesis of the (Z)-4-((carboxymethyl)amino)-4-oxobut-2-enoic acid

To a solution of maleic anhydride (1.08 g, 11.1 mmol) in glacial acetic acid (14 mL), glycine (0.830 g, 11.1 mmol) was added. The suspension was magnetically stirred for 5 h at room temperature. Then, the product was separated by centrifugation, washed with cold ultrapure water and methanol, and dried *in vacuo*. Yield: 1.76 g (92 %). ^{1}H-NMR (DMSO-d_6) δ: 3.91 (d, 2H, -C*H*$_2$, ^{3}J = 5.85 Hz), 6.31 (d, 1H, -C*H*CO$_2$H, ^{3}J = 12.4 Hz), 6.43 (d, 1H, -C*H*CONH, ^{3}J = 12.4 Hz), 9.19 (t, 1H, -N*H*, ^{3}J = 5.35 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 41.0 (-*C*H$_2$), 130.0 (-*C*HCONH), 133.0 (-*C*HCO$_2$H), 165.2 (-CH*C*O$_2$H), 166.0 (-CH*C*ONH), 170.4 (-CH$_2$*C*O$_2$H) ppm. ESI-MS (positive ion mode): 174 *m/z* $[M+H]^+$, calcd for $C_6H_8NO_5$ $[M+H]^+$ 174 *m/z*.

4.2.2. Synthesis of 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetic acid, ***HL***

The above synthesized product (500 mg, 2.89 mmol) was suspended in 75 mL of toluene. Then, after the addition of 1.4 mL of triethylamine, the suspension was left under vigorous stirring at 80 °C in an oil bath until the reagent was completely dissolved (about 2 h). The resulting clear solution was separated from the brownish oil on the flask bottom by centrifugation. Toluene was removed under reduced pressure and the resulting colorless residue was dissolved in ultrapure water (about 50 mL). The pH of this solution was then adjusted to 2.0±0.1 with few drops of concentrated HCl. Two extractions with ethyl acetate were carried out and the organic phase was made anhydrous with anhydrous sodium sulfate and evaporated to dryness by a rotary evaporator. The hygroscopic product was collected and dried *in vacuo*. Yield: 322 mg (72 %) .^{1}H-NMR (DMSO-d_6) δ: 4.14 (s, 2H, -OCOC*H*$_2$), 7.13 (s, 1H, -NCOC*H*) ppm; ^{13}C-NMR (DMSO-d_6) δ: 38.5 (-OCO*C*H$_2$), 134.9 (-NCO*C*H=*C*H), 168.9 (-O*C*OCH$_2$), 170.4 (-N*C*OCH=CH) ppm. ESI-MS (negative ion mode): 154 *m/z* [M-H]$^-$; calcd for $C_6H_4NO_4$ [M-H]$^-$ 154 *m/z*.

4.3. Synthesis of (SP-4-3)-diamminechlorido(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetate)platinum(II), ***1***

To a suspension of cisplatin (100 mg, 0.333 mmol) in 5 mL of anhydrous DMF, a solution of $AgNO_3$ in DMF (54 mg, 0.317 mmol, in about 5 mL) was added dropwise and the mixture was magnetically stirred at room temperature for 24 h in the dark. The suspension was then filtered (PTFE filter with a porosity of 0.45 μm) in order to remove AgCl (precipitated during the displacement of the chloride ligand). The solution was added to a solution of potassium 2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetate (K**L**) in anhydrous DMF [previously prepared from **HL** (57 mg, 0.369 mmol) and

KOH (21 mg, 0.369 mmol) in ultrapure water adjusting pH to 5-6, removing water under reduced pressure and suspending salt in anhydrous DMF]. The reaction was carried out at 50 °C for 24 h in the dark. The mixture was then filtered (0.45 μm porosity) and DMF was removed under reduced pressure. The yellow product was precipitated by adding ethanol/diethyl ether, washed with ethanol and methanol and finally dried *in vacuo*. Yield: 59 mg (42 %). ^{1}H-NMR (DMF-d_7) δ: 3.97 (s, 2H, -OCOCH_2), 4.16 (m, 3H, -NH_3 in *trans* to the carboxylate ligand), 4.70 (m, 3H, -NH_3 in *trans* to the chloride ligand), 7.02 (s, 2H, -C*H*=C*H*CON) ppm; ^{13}C-NMR (DMF-d_7) δ: 40.1 (-OCO*C*H_2), 135.1 (-NCO*C*H=*C*H), 171.2 (-N*C*OCH=CH), 174.1 (-O*C*OCH$_2$) ppm; ^{195}Pt-NMR (DMF-d_7) δ: -1749 ppm. ESI-MS (positive ion mode): 420 *m/z*, $[M+H]^+$, calcd for $C_6H_{11}ClN_3O_4Pt$ $[M+H]^+$ 420 *m/z*. Elem. Anal. found: C 17.53; H 2.62; N 9.88 %; calcd. for $C_6H_{10}ClN_3O_4Pt$: C 17.21; H 2.41; N 10.04 %

4.4. Synthesis of (OC-6-44)-diamminedichlorido(ethanolato)(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetato)platinum(IV), ***4***

The carboxylic acid of ligand **HL** was activated according to a published procedure [16]. Briefly, a solution of **HL** (48 mg, 0.309 mmol) and DCC (64 mg, 0.309 mmol), dissolved in 1 mL of anhydrous DMF, was sonicated in an ultrasonic bath for 15 minutes at room temperature. The colorless solution became dark red and a white solid (dicyclohexylurea, DCU) precipitated. Then the precipitate was separated by centrifugation and the supernatant was added very slowly (dropwise for 30 minutes) to a suspension of (*OC*-6-44)-diamminedichloridohydroxido(2-hydroxyethanolato)platinum(IV), **2**, (100 mg, 0.276 mmol) in 5 mL of anhydrous DMF. The reaction was carried out overnight at room temperature. The mixture was then cooled to -20 °C for about 30 min and the precipitate was eliminated by filtration (PTFE filter with a porosity 0.45 μm). The solvent was evaporated by a rotary evaporator and the yellow oil obtained was dissolved in ultrapure water, in order to allow the

precipitation of the remained DCU. The yellow solution was filtered (0.45 μm porosity), the water was removed under reduced pressure and the reaction product was precipitated with acetone/diethyl ether and then dried *in vacuo*. Yield: 83 mg (60 %). ^{1}H-NMR (D_2O) δ: 1.19 (t, 3H, -OCH$_2$C*H*$_3$, ^{3}J = 6.95 Hz), 3.43 (q, 2H, -OC*H*$_2$CH$_3$, ^{3}J = 6.95 Hz), 4.39 (s, 2H, -OCOC*H*$_2$), 6.97 (s, 2H, -NCOC*H*=C*H*) ppm; ^{13}C-NMR (D_2O) δ: 16.1 (-OCH$_2$*C*H$_3$), 40.0 (-OCO*C*H$_2$), 67.7 (-O*C*H$_2$CH$_3$), 134.7 (-NCO*C*H=*C*H), 172.4 (-N*C*OCH=CH), 176.3 (-O*C*OCH$_2$) ppm; ^{195}Pt-NMR (D_2O) δ: 904 ppm (multiplet of five lines, $^{1}J_{Pt\text{-}N}$ = 197.4 Hz). ESI-MS (positive ion mode): 500 *m/z* $[M+H]^+$, calcd for $C_8H_{16}Cl_2N_3O_5Pt$ $[M+H]^+$ 500 *m/z*. Elem. Anal. found: C 19.58; H 3.24; N 8.13 %.; calcd. for $C_8H_{15}Cl_2N_3O_5Pt$: C 19.25; H 3.03; N 8.42 %.

4.5. Synthesis of (OC-6-44)-acetatodiamminedichlorido(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetato)platinum(IV), ***5***

Complex (*OC*-6-44)-acetatodiamminedichloridohydroxidoplatinum(IV), **3**, (56 mg, 0.15 mmol) suspended in 1 mL of DMF was added to a solution of activated ligand **HL** (0.74 mmol) in 1 mL of DMF (see above). The mixture reacted for 24 h at room temperature. Then, the precipitate was discarded by centrifugation and the solvent removed by means of a rotary evaporator. The residue was dissolved in 200 μL of methanol and precipitated by adding 25 mL of diethyl ether. The crystalline solid is washed with diethyl ether. Yield: 43 mg (56 %). ^{1}H-NMR (DMSO-d_6) δ: 1.91 (s, 3 H, CH_3), 4.17 (s, 2 H, CH_2), 6.49 (m, 6 H, NH_3), 7.09 (s, 2 H, CH) ppm; ^{13}C-NMR (DMSO-d_6) δ: 22.5 (CH_3), 47.5 (CH_2), 134.8 (CH), 170.4 (NC(O)), 174.4 (OC(O)CH_2), 178.0 (OC(O)CH_3) ppm; ^{195}Pt-NMR (DMSO-d_6) δ: 1230 ppm. ESI-MS (positive ion mode): 514 *m/z* $[M+H]^+$, calcd for $C_8H_{14}Cl_2N_3O_6Pt$

$[M+H]^+$ 514 *m/z*. Elem. Anal. found: C 18.96; H 2.78; N 7.93 %; calcd. for $C_8H_{13}Cl_2N_3O_6Pt$: C 18.72; H 2.55; N 8.19 %.

4.6. Diels-Alder reaction with furan

In a NMR tube, furan and the Pt(IV) complexes **4** or **5**, in 1:1 or 2:1 ratios, were dissolved in 0.6 mL of D_2O (final Pt concentrations = 40 mM). The temperature of the mixture was then set to 40 °C or 25 °C and the reaction was followed by ^{1}H-NMR spectroscopy for 5 d recording the spectra every 2 h.

*4.7. Loading of Pt(IV) complexes **4** and **5** on furan-functionalized SNPs*

Ethyl 3-(furan-2-yl)propionate (1.4 µL, 8.8 mmol) was deprotected with a LiOH solution. In particular, the ethyl 3-(furan-2-yl)propionate was dissolved in 25 mL of tetrahydrofuran (THF) and cooled in an ice bath. A solution of LiOH (0.560 g, 13.3 mmol in 13 mL of ultrapure water) was added slowly, followed by 5 mL of isopropanol obtaining an orange solution. The reaction mixture was stirred at room temperature overnight. The solvent was removed by reduced pressure and the residue was dissolved in 10 mL of ultrapure water and extracted twice with diethyl ether (7.5 mL). After acidification with 1 M HCl, the aqueous layer was extracted four times with ethyl acetate (10 mL). The joint organic phases were dried with magnesium sulfate and filtered. The solvent was then removed by reduced pressure to obtain the product as a light orange solid (yield 76% 0.953 g). The resulting 3-(furan-2-yl)propionic acid was activated by reaction with 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) as a coupling agent. In particular, 6 mg of HATU (0.0158 mmol) and 2 mg of 3-(furan-2-yl)propionic acid (0.0129 mmol) were introduced

into a 10 mL round bottom flask with a suspension of SNPs (100 mg, 0.0259 mmol NH_2) in 1.5 mL of anhydrous dimethylformamide (DMF); then 4 μL of N,N'-diisopropylethylenediamine (DIPEA, 0.023 mmol) were added obtaining a yellow suspension. The reaction was carried at room temperature overnight in dark condition. The suspension was washed twice with ultrapure water and thrice with ethanol. The nanoparticles were collected by centrifugation and resuspended in ethanol. Furan-functionalised nanoparticles (50 mg) were suspended in 1.5 mL of anhydrous DMF. Complex **4** or **5** (58 mg, 0.116 mmol for **4** and 60 mg, 0.116 mmol for **5**) was dissolved in 500 μL of DMF and added to the suspension. The yellow mixture was stirred for overnight at 25 °C in the dark. The conjugate was then washed several times with ultrapure water and ethanol to remove unreacted species and DMF. Finally, the Pt-furan-SNPs conjugate was suspended in ethanol.

4.8. Characterization of nanoparticles and conjugates

Dynamic light scattering (DLS) and ζ potential analyses were performed in 10 mM KNO_3 (after pre-treatment of the SNPs with HCl 0.5 M and following centrifugation) at 37 °C with a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at a fixed scattering angle of 173°, using a He–Ne laser and DLS software for Windows (version 6.11, Malvern, UK). Each value is the average of three independent measurements. The furan-SNPs showed DLS diameter = 230 ± 10 nm and ζ potential = 48 ± 4 mV. After loading with Pt complexes, an increase in DLS diameters and a decrease in ζ potential was observed (DLS diameter = 294 ± 15 nm, ζ potential = 23 ± 1 mV for **4**; DLS diameter = 255 ± 13 nm, ζ potential = 21 ± 1 mV for **5**).

For the quantification of the Pt content, an aliquot of the ethanol suspension of the conjugate was centrifuged to remove the solvent and the conjugate (2-3 mg) was dried before its acid digestion in quartz cuvettes with 800 μL of HNO_3 69% w/w and 200 μL of hydrogen peroxide 30% w/w in water.

The cuvettes were then introduced into a closed vessel in the microwave oven (Milestone Start D, Milestone Srl, Sorisole, Italy), where a temperature-controlled heating was applied for 45 min (microwave power = 1200 W, temperature = 200 °C). The vessel was cooled to room temperature and the content was transferred into a marked flask using 1% v/v HNO_3 and sonicated for 1 h at 60 °C in an ultrasonic bath. The solution was diluted with 1% v/v HNO_3 and its Pt content was quantified by inductively coupled plasma – mass spectrometry (ICP-MS, Thermo Optek X Series 2). A platinum standard stock solution of 1000 mg L^{-1} was diluted in 1% v/v HNO_3 to prepare calibration standards. Instrumental settings were optimized to yield the maximum sensitivity for platinum and the most abundant isotopes of platinum and indium (used as an internal standard) were measured at *m/z* 195 and 115, respectively, to quantify the Pt content of the samples. The results indicated: $(3.7\pm0.3)\times10^{-2}$ mmol Pt g^{-1} SNPs for **4** and $(3.5\pm0.4)\times10^{-2}$ mmol Pt g^{-1} SNPs for **5**.

4.9. Release of platinum from conjugates

The conjugates were magnetically stirred in HEPES buffer (1 mM, pH 7.4) for 4 h (the time interval usually employed for cellular accumulation experiments)[38, 42], then the suspension was centrifuged (10 min, 10,000 rpm) to separate the nanoparticles. The amount of Pt released was measured in the supernatant fraction by means of ICP-MS (see above).

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Appendix A. Supplementary data

Supplementary data to this article can be found on line at…

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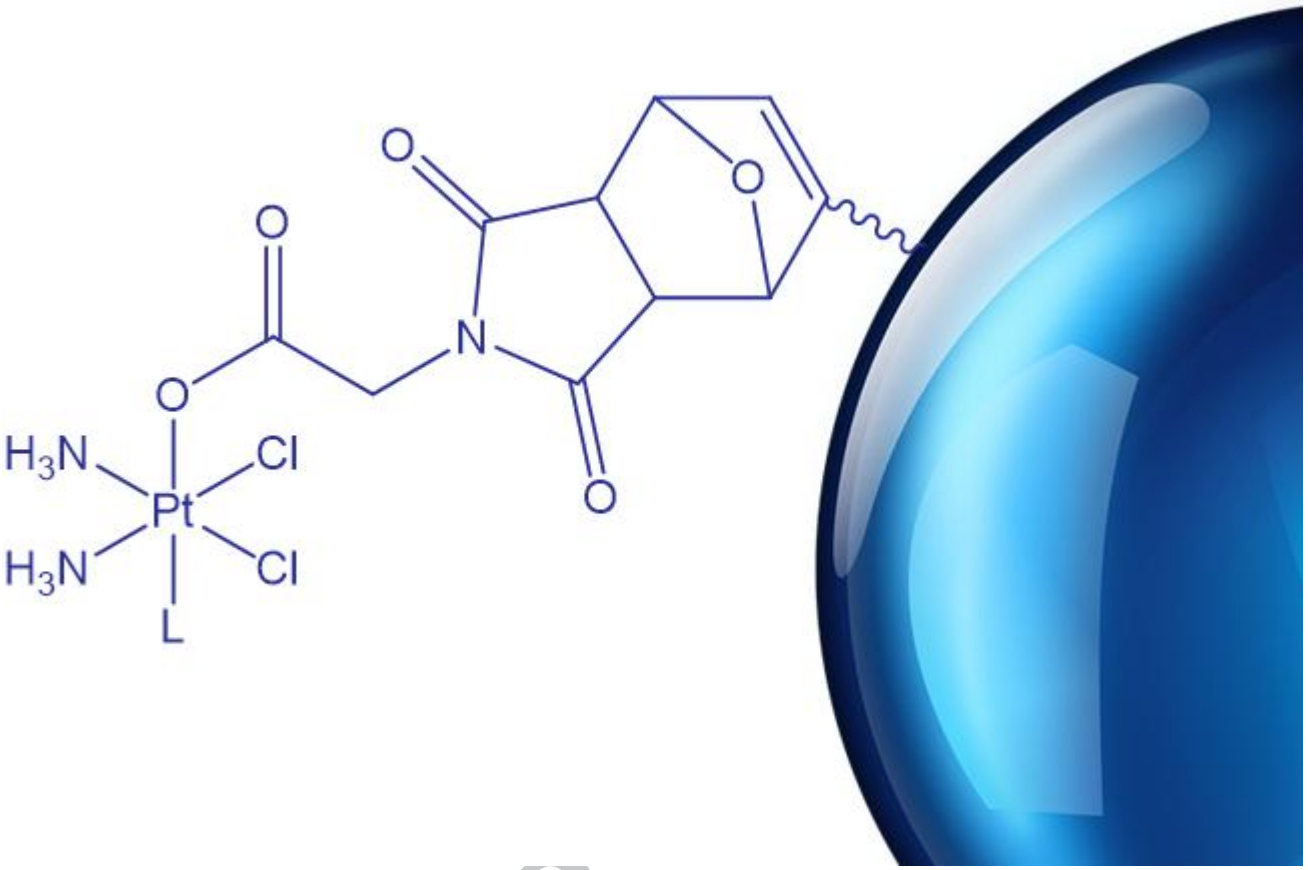

- Diels–Alder reaction is a powerful tool to design drug delivery systems
- Two Pt(IV) complexes containing 2-maleimido acetic acid as axial ligand are proposed
- Clickability of the maleimide-containing Pt(IV) complexes was demonstrated with furan
- As a proof-of-concept, the Pt(IV) conjugates were appended to furan-modified SiO_2 nanoparticles