Abstract: Metal-mediated intracellular reactions are becoming invaluable tools in chemical and cell biology, and hold promise for strongly impacting the field of biomedicine. Most of the reactions reported so far involve either uncaging or redox processes. Demonstrated here for the first time is the viability of performing multicomponent alkyne cycloaromatizations inside live mammalian cells using ruthenium catalysts. Both fully intramolecular and intermolecular cycloadditions of dyynes with alkynes are feasible, the latter providing an intracellular synthesis of appealing anthraquinones. The power of the approach is further demonstrated by generating anthraquinone AIEgens (AIE = aggregation induced emission) that otherwise do not go inside cells, and by modifying the intracellular distribution of the products by simply varying the type of ruthenium complex.

Introduction

Cells can be viewed as complex, tiny factories performing thousands of simultaneous chemical reactions. As chemists, we are not bound to reproduce natural processes, and can therefore aspire at conducting non-coded catalytic reactions within living environments. While native enzymatic transformations are unbeatable with regard to rate and selectivity, abiotic catalytic processes might be advantageous in terms of versatility and substrate scope. Furthermore, introducing non-natural reactivity in cellular contexts can provide unprecedented opportunities for cellular and metabolic intervention. However, performing exogenous catalytic reactions within living cells is far from trivial, as it requires to maneuver among multitude of components and functional groups, and in an aqueous, crowded and compartmentalized environment.

For many years, it was considered that organometallic catalysis was incompatible with aqueous and biological environments; however, the last decade has witnessed an increasing number of reports on bioorthogonal, and even intracellular transformations, mediated by transition metals. However, the repertoire of reactions is yet scarce, and mainly limited to uncaging processes; albeit other reactions, like reductions, isomerizations and cyclizations, have also been recently introduced. Therefore, there is a great interest in increasing the repertoire of metal-mediated reactions that can be performed in live settings, as this will contribute to further establish this field, and expand its biotech and biomedical potential. A particularly attractive type of transformations are cycloaddition reactions, owing to their synthetic power, and the lack natural counterparts, albeit there have been claims on the existence of Diels–Alderases. Intracellular cycloadditions promoted by transition metals have been limited to sporadic applications of the renowned copper-mediated alkyne-azide cycloaddition (CuAAC) (Fig. 1, i), a key reaction for the establishment of the field of bioorthogonal chemistry. Unfortunately, its efficiency in intracellular settings is compromised by the toxicity and oxidation lability of copper. We recently developed a ruthenium-promoted azide-alkyne cycloaddition (MAAC) (Fig. 1, ii), a key reaction for the establishment of the field of bioorthogonal chemistry. Unfortunately, its efficiency in intracellular settings is compromised by the toxicity and oxidation lability of copper. We recently developed a ruthenium-promoted azide-alkyne cycloaddition (MAAC) (Fig. 1, ii).
nium-mediated alternative that involves thioalkynes as azide partners (Figure 1, ii). However, it couldn’t be implemented inside living cells.[12]

At some point of our research we envisioned that moving from alkynes to appropriately tethered diynes, there might be better chances for bioorthogonal “fishing” of transition metal reagents owing to the bidentate coordination ability of the substrate (A, Figure 1).[33] An ensuing oxidative cyclometalation would give a metallacalycy (B/C, Figure 1) that could be trapped by another alkyne to provide aromatic adducts formally resulting from a (2+2+2) cycloaddition (D, Figure 1, iii). This type of cycloaddition of alkynes is well-known in the realm of synthetic chemistry,[14] and has also been even accomplished in aqueous media, using Co, Rh, or Ru catalysts,[15,16] but never in a cellular context.

In this manuscript we demonstrate the viability of this process, by reporting the first examples of a multicomponent cycloaddition carried out in the interior of live, unfixed mammalian cells. The reaction, which is mediated by nontoxic ruthenium complexes, allows to make three C–C bonds, and at least two new cycles, in a single step, therefore enabling a remarkable increase in structural complexity.

We demonstrate that the cyclotrimerization is not only viable in fully intramolecular cases, but also when using isolated alkynes as external reaction partners (Figure 1, iv and v). Remarkably, this latter reaction allows to generate “in cellular” anthraquinones, secondary metabolites which cannot be produced by mammalian cells. We also demonstrate that whereas anthraquinone derivatives with AIE (aggregation induced emission) characteristics do not internalize, they can be indirectly “introduced” inside cells using our reaction.

We can even go one step further and promote the preferential generation of cycloadducts in different intracellular locations.

**Results and Discussion**

Our initial experiments were planned considering previous work of Cadierno and co-workers,[15b] and of Teply,[16] demonstrating the viability of achieving cyclotrimerizations of alkynes under different aqueous conditions, using [RuCp∗Cl(COD)] (Ru1) as catalyst. However, these reactions were carried out using relatively high concentration of reactants, and required long reaction times, which raised doubts on their exportability to biological environments. We first tested the cyclotrimerization of triyne 1, because it is an intramolecular process, and the resulting pentacyclic product is fluorescent (Figure 2a), thereby providing for tracking the reaction using microscopy. After some optimization, we managed to accomplish the reaction in complex biological media such as phosphate-buffered saline solution (PBS), cell culture media (DMEM) and HeLa cell lysates, even using micromolar concentrations of the precursor (Figure 2a).

We then moved to a real biological scenario, a mammalian living cell. Therefore, HeLa cells were incubated with Ru1 and washed twice with DMEM to remove the excess of the complex. The presence of ruthenium inside cells was confirmed by ICP-MS analysis (Figure S17). Then, cells were mixed with substrate 1 for 1 h. Panel A in Figure 2b displays the cells after treatment only with substrate 1, which show no fluorescence. In contrast, we did observe some fluorescence in cells that had been pretreated with Ru1 (panel B), in agreement with the formation of the product 2. Remarkably, using the complex Ru2, which we knew from previous studies that presents a good cellular uptake and tolerance,[54] we observed a more intense intracellular emission (Figure 2b, panel C), consisting with the generation of a larger amount of product. Indeed, we calculated a three-fold higher fluorescence intensity when compared with that resulting from the use of Ru1 (Figure 2c). The ruthenium (IV) complex Ru2 is likely reduced in situ to an active Ru3 species,[54] and its better performance in cells seems in part associated to an improved uptake with respect to Ru1 (Section S12). The processes didn’t produce observable changes in the morphology of the cells or affect the survival at the operating times (Figure S8).

The above results are consistent with the generation of product 2 inside cells, through a formal (2+2+2) multicomponent annulation. Albeit the intramolecularity of the process might favor the annulation, we envisioned that once ruthenacycle intermediates like the ones in Figure 1b are formed, they could be trapped with external alkynes (Figure 1, v). Gratifyingly, treatment of diyne 3 with terminal alkyn 4a...
The yield increases up to 94% when the reaction is carried out in DMEM and in presence of HeLa cell lysates (95% and 89% yields, respectively, Figure 3a), and can be promoted with other complexes like [RuCp-

The AQ skeleton could be assembled in a single step by means of a Ru-promoted annulation between bispropargylic ketones of type 6 and alkynes (Figure 3a, bottom). In nature, the main polycyclic core of AQs is biosynthesized from acetylCoA and malonylCoA, in a multistep process governed by the multi-domain enzyme polyketide synthase (PKs) present in some plants, fungi, insects and bacteria (Figure 3b). However, mammalian cells do not code for the biosynthesis of AQs, which added further interest to explore the possibility of building these products inside these cells.

Gratifyingly, the bispropargylic ketone 6a reacted with commercially available alkyne 4a (4 equiv) in presence of Ru1 (50%) to give the expected tricycle 7a in 79% yield after 2 h at rt, in water (Figure 3a, bottom). The catalyst loading can be reduced to 25%, albeit there is a decrease in the yield (59%). Importantly, the concentration of 6a could be decreased to 500 μM (53% yield). The reaction can be also accomplished in PBS even at 25% of Ru1 (65% yield). In cell culture media, such as DMEM, or in HeLa cell lysates the reaction presents almost no turnover (37 and 28% respectively, using 25% of Ru1). The cycloaddition is also feasible using an internal 1,6-diyne such as 6b, albeit it was somewhat slower (45% yield, using 50% of Ru1 in water). In this case, we found that the use of an organic co-solvent (H2O/MeCN 8:2), allowed a slightly better yield (59%). Other terminal alkynes (4) also participate in the cycloaddition (Table S3).

Substrates 6a and 4a are not fluorescent, however the anthraquinone product 7a presents bright green and blue fluorescence emission, which facilitates monitoring in cellular settings. For cellular experiments we preferred to use Ru2, owing to its better uptake[5e] (although catalyst Ru1 was also able to elicit the reaction in HeLa cells, Figure S10). These experiments were carried out by incubating the cells with Ru2, followed by treatment with alkyne 4a and diyne 6a (Figure 4a and S9). After 4 h, we were glad to observe an intense fluorescence build-up inside the cells, consistent with the formation of anthraquinone product 7a (Figure 4a, panels A, B).

Control experiments confirmed that addition of anthraquinone product 7a to HeLa cells elicits a clear intracellular fluorescence (panel D, Figure 4a). In contrast, cells co-incubated with substrates 6a and 4a were nearly non-fluorescent (Figure 4a, panel C). As expected, addition of each component alone, either 6a or 4a, to cells that had been previously treated with the ruthenium reagent, led only to residual intracellular fluorescence (arising from the substrates, Figure S10).

The intracellular formation of AQ products was further confirmed by carrying out the reaction with the alkyne derivative 4P, which features a phosphonium tail (Figure 4b). This group facilitates the identification of the product owing to its good performance in ESI-MS spectrometry. Therefore, MS analysis (extracted ion chromatogram) of methanolic extracts of cells treated with 4P, and either 3 or 6a, under the standard reaction conditions, revealed the presence of the expected cycloadducts (Figure 4b, Section S14). These results suggest that this type of molecular labels (phosphonium cations or similar), could allow to overcome the current limitation of detection tactics to fluorescent products.

To further expand the scope and potential of the technology, we envisioned the synthesis of products with tetraphenylethylene skeletons like 9a, using alkyne 8a as reaction partner (Figure 5a).
This type of structures, known to present aggregation-induced emission properties (AIE), are very attractive from a photophysical perspective. Unfortunately, while the alkyne 8a displays an excellent emission, the corresponding cycloadduct 9a presents a strong decrease in its AIEgen properties, perhaps because of an increased molecular rotation (Figure S11). However, AQ 9b, obtained from alkyne 8b, behaves as an excellent AIEgen (Figure 5a, Figure S12).

Importantly, the product (9b) can be generated inside live mammalian cells using the cyclotrimerization reaction. Therefore, the addition of substrates 6a and 8b to HeLa cells previously treated with the ruthenium complex Ru2, leads to an intense intracellular red staining (Figure 5b, panel D), enlarged images in E, F). This result is especially significant because the anthraquinone 9b is unable to travel inside cells. Indeed, incubation of cells with the anthraquinone 9b leads to the formation of aggregates in the extracellular media (Figure 5b, panel G, and enlarged images in H, I). This product can be internalized only after permeabilization of the
membrane with digitonin (Figure S16). Therefore, our approach allows to indirectly introduce in cells a product (anthraquinone 9b) that otherwise aggregates in the extracellular space. Importantly, these results further corroborate the intracellular character of the reaction.

An additional asset of the strategy stems from the possibility of controlling the intracellular distribution of the AQ product 9b depending on the ruthenium reagent. Therefore, by using complex Ru1, the cellular fluorescence shows a cytosolic profile (slightly less intense than with Ru2, Figure 6, panels A–C). However, using complex Ru3, which is known to preferentially accumulate in mitochondria owing to its lipophilic cationic character,[54] the fluorescence is mainly located in these organelles (Figure 6, panels D–F, Figure S13).

Finally, it is important to note that in all the above experiments neither substrates nor products compromised the cell viability at the observation times, inducing only a slight decrease after 12 h of incubation at high concentrations (Figure S8).[56]

Conclusion

In conclusion, our results demonstrate that transition metals can promote challenging (2+2+2) multicomponent cycloadditions in live mammalian cells. The use of abiotic reactants with bidentate metal coordination abilities (diyne) seems instrumental for the success of the process. In addition to provide a cutting-edge addition to the palette of cell-compatible metal-mediated transformations, our work demonstrates the viability of synthesizing complex, biorelevant polycycles inside cells from simple precursors, and therefore goes beyond the more common uncaging or ligation reactions. Our intracellular synthetic strategy allows to generate products that otherwise cannot be delivered to the cell, as well as controlling their spatial distribution by using suitable ruthenium reagents. Indeed, the possibility of generating the desired products in specific subcellular locations, just by changing the targeting characteristics of the reagents, is exciting. While further work to increase the efficiency of the reactions is needed, our discoveries should further foster this young field of intracellular metal catalysis, and trigger important applications in chemical and synthetic biology, and in biomedicine.

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Conflict of interest

The authors declare no conflict of interest.

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