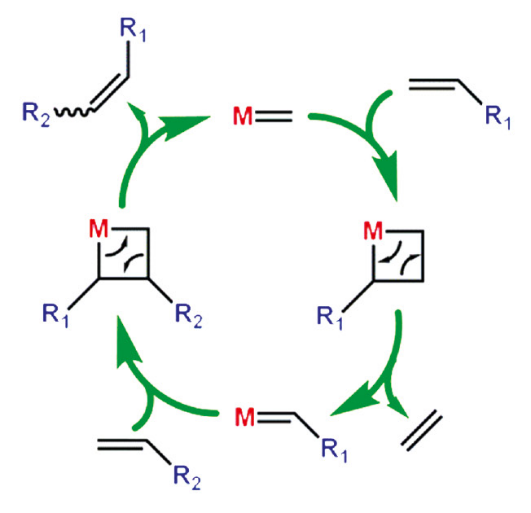
**In Vivo Olefin Metathesis – Challenges, Approaches and Applications**

# Introduction

1. Olefin metathesis

Formation of new carbon-carbon bonds is one of the major objectives of modern organic chemistry. Alongside mechanisms like the Wittig reaction and palladium-catalyzed coupling, olefin metathesis is an important tool for achieving this goal.

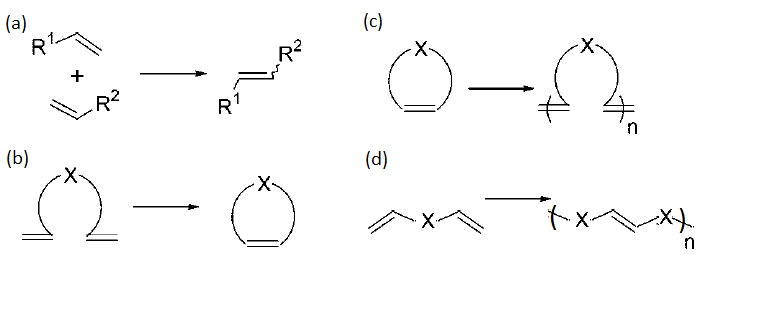
Olefin metathesis usually involves the exchange of partners between two double bonds, though the same concept has also been applied to reactions in enynes (between the double and triple bond), in which a diene is formed.

The mechanism of the catalytic cycle was proposed by Yves Chauvin in 1971.1 It involves initiation of the catalyst by a [2+2]-cycloaddition to create a metallacyclobutane intermediate that immediately undergoes cycloreversion to form a species with the metal atom of the catalyst bonded to the carbon atom of the first alkene. In the propagation step, another cycloaddition and cycloreversion cycle with the second alkene creates the metathesis product (figure 1). The release of small alkenes, like ethylene, can make the reaction entropically favorable.

**Figure 1** Schematic catalytic cycle of olefin metathesis

according to the Chauvin mechanism2

OM reactions are grouped by the type of the reactants and products (figure 2) – intermolecular cross-metathesis (2a) involves the exchange of double-bond partners between two separate molecules. Ring-closing metathesis (RCM; 2b) is an intramolecular reaction that can be driven by the relative stability of five- and six- membered rings. Polymerization reactions include ring-opening metathesis polymerization (ROMP; 2c) and acyclic diene metathesis (ADMET; 2d), which competes with RCM in some cases. In general, most metathesis reactions are not highly selective – in terms of product distribution and stereoselectivity – unless reactants with steric bulk or differing reactivity are used.3



**Figure 2** Different olefin metathesis reactions4,5

The evolution of metathesis catalysts includes two major progressions – choice of the central transition metal involved and its ligands. Early reactions utilized heterogenous catalysts with Ti, Nb and Al salts. Later, after the cycloaddition-cycloreversion mechanism was proposed, tungsten (W) and molybdenum complexes were introduced and enabled – especially in Mo complexes – superior catalytic activity. However, their low functional group tolerance and sensitivity to moisture and oxygen were significant drawbacks for many reactions.4,6 In 1992, Grubbs et al. introduced the first ruthenium alkylidene complex for olefin metathesis, exhibiting lower activity but a significant improvement in stability in oxygen- and water-containing environments and for various functional groups.7

The second factor determining the catalyst's characteristics – its ligands – has also progressed significantly. Most Ru catalysts contain the carbene ligand responsible for the initiation – alkylidene or benzylidene – two anionic ligands like chloride and two neutral ligands. In early catalysts these neutral ligands were phosphines, such as PCy3, but they introduced an inherent tradeoff – because the active species of the catalyst is the one most prone to decomposition, so either the reactivity or the stability of the catalyst can be optimized. The development of carbene ligands resolves this issue, since they are strong σ donors and enhance dissociation of the other neutral ligand (i.e. activation) and stabilize the resulting electron-deficient active species.6 A plethora of N-heterocyclic carbene (NHC), cyclic alkylamino carbene (CAAC) ligands have been developed, which employ various modification for increased efficient, stability of functional group tolerance.4

1. Principles of bioorthogonal chemistry

Bioorthogonal chemistry describes a set of reactions that can be used in a biological context and include reactants or catalysts that are not commonly found in nature. Thus, their reactivity with endogenous biological molecules like proteins, sugars and nucleic acids can be limited. Other key characteristics of bioorthogonal reactions are high yields and reactions rate, water tolerance and generally small reaction partners, which minimize perturbance to the biological system.8 These requirements enable highly selective and efficient modification of molecules in biological environments.

Multiple bioorthogonal reactions have been reported, including native chemical ligation to create amide bonds in protein synthesis, Copper-catalyzed azide-alkyne cycloaddition (CuAAC) that forms triazoles, tetrazine ligation that forms bicyclic compounds and several types of photoinducible reactions, in which light activates stable reactants.8

Bioorthogonal reactions are often modified forms of well-known reactions that were optimized for the strict requirements of biological systems. For example, the Staudinger reaction – between a phosphine and an azide – was described in 1919 but was not useful for the creation of an amide bond in an aqueous environment because of spontaneous hydrolysis. Changing the phosphine to include an "electrophilic trap" that enabled rearrangement to a stable structure prevented this and uncovered a highly selective and biocompatible tool, nowadays called the Staudinger ligation.9

Bioorthogonal techniques complement strategies like genetic engineering and protein tags for many applications – drug development and delivery, expansion of the genetic code by incorporation of unnatural amino acids, targeted protein degradation and cell imaging by tagging different kinds of target molecules.

In this work, I shall summarize the recent advances in olefin metathesis in the context of bioorthogonal chemistry and focus on the techniques and catalysts used to progress from reactions in aqueous media to metathesis in living cells (in-vivo).

# Body

## Use cases for in-vivo metathesis

Although bioorthogonal chemistry enabled significant progress in research and was even used in certain medical applications,10 the reactions mentioned in the section above have a limited scope of reactants and products – most additions involve azides, nitrogen heterocycles or alkynes and the formation of amide or similar heteroatom-containing bonds.8 These can be useful in biological contexts, but there is still a need for reactions that create a new carbon backbone.

Olefin metathesis can be a valuable tool in this endeavor – its versatility enables synthesis of many biological and biologically reactive molecules, such as amino acids, peptides, proteins, sugars, lipids nucleic acids and drugs. A couple of the researched and proposed usages are hereby presented.

Synthesis and modification of proteins is one of the best studied applications of bioorthogonal olefin metathesis. Not only can metathesis catalyze the formation of a protein similar to one found in nature, but through a careful choice of the reacting residues, the synthesized protein can be improved in terms of stability and ligand affinity in comparison to the "natural" form. For example, when an ethylene bridge replaces the disulfide bond in protein this can result in greater conformational rigidity and stability. A study comparing the activity and half-lives of oxytocin agonists and antagonists with these "dicarba analogues", that were synthesized through RCM, found that the substitution increases the half-life while retaining the biological activity of this peptide hormone.11

Post-translational modifications can alter proteins' stability, lifecycle and characteristics, and are vital to their function. Therefore, changing them through metathesis is another effective way to modulate biological activity. Histone acetylation is a well-known modification that regulates gene expression.12 This modification was successfully mimicked by cross-metathesis on an alkene-bearing histone and even recognized by an antibody that naturally binds to this epigenetic marker. Next, the acetyl group was removed by elimination to restore the alkene. This unique "write-read-erase" cycle, though harder to perform in a living cell, is a prime example of the flexibility and switch mechanism metathesis reactions enable.13

Another DNA-related metathesis was reported for the formation of building blocks for DNA libraries – a technology for the identification of small molecules. A ruthenium NHC catalyst used in excess managed to catalyze both CM and RCM on substrates bound to an oligonucleotide backbone.14 Catalysis on nucleic acids themselves was demonstrated for the nucleoside uridine, which was successfully dimerized using an anionic CAAC catalyst.15 The ability to modify DNA and DNA-bonding molecules can be used to regulate genes, identify mutations and detect gene expression.

Ring-closing metathesis was also applied to RNA dinucleotides, in order to increase the steric bulk of the RNA and control its secondary structure.16 The purpose of the study utilizing this reaction was to create a conformationally restricted oligonucleotide, but a similar reaction design may also be used to enhance three-dimensional folding or control the specific structure of the RNA, to change its affinity and reactivity.

Metathesis in-vivo can be a valuable tool in drug delivery, as the bond forming or breaking process can liberate bioactive molecules from a cage in the required tissue or microenvironment. A study of virus-like particles as carrier molecules for peptide drugs involved the use of ROMP as the uncaging process. The particles were functionalized with NHS-activated norbornene, and so a ROMP reaction that created a bond between two norbornene molecules drove the disassembly of the vesicle and release of the cargo peptide .17 This generic mechanism may be used for other types of drugs and for other vesicles, as long as they can bind metathesis substrates.

A diagram of a molecule

Description automatically generated**Figure 3** Particle disassembly driven by ROMP reaction for drug delivery.17

Instead of protecting the bioactive compound of a drug in a cage, drugs can be administered in their inactive form – prodrug – and then converted to the bioactive form inside the body, in the target tissue. This approach can both minimize side effects, because the active drug will not reach unnecessary tissues, and increase the activity, because the drug will only act in the target tissue or cells. Olefin metathesis is especially suitable for such syntheses because it changes the carbon backbone of a molecule, which can lead to different affinity and binding.

Prodrug activation was investigated for the potential antitumor drug combretastatin-A4. An artificial metalloenzyme (ArM) was synthesized, containing a Hoveyda-Grubbs second-generation catalyst anchored to human serum albumin protein. Localization to tumors was achieved by incorporation of the pentapeptide cGRD, which interacts with integrin, a protein that's overexpressed in cancer cells. cGRD was bound to the surface of the ArM so metathesis only proceeded near tumors. The metalloenzyme managed to catalyze the formation of the drug from its precursor through RCM in a solution containing blood, with moderate yield (39%). This technique was next tested in vivo, in mice with implanted tumors. The prodrug administered with the metalloenzyme proved effective in halting tumor growth and achieved better results than direct administration of the drug itself, suggesting that increased specificity does enhance the effectiveness, at least for this drug.18

Bioorthogonal olefin metathases can also be used in basic research, since it's ideal for creating artificial metabolic or signaling pathways. A study investigating abiogenesis and the development of modern cells employed this strategy in protocells consisting of only DNA. These cells were loaded with ArMs – Hoveyda-Grubbs catalysts conjugated to streptavidin through biotin. The ArM catalyzed an RCM reaction that resulted in the release of fluorescent – and thus self-reporting – molecules, and molecules that intercalate into double-stranded DNA and lead to cell swelling by dynamization of the DNA membrane. More cellular effects – cell growth, fusion and functional adaptation – were mimicked without any "natively biological" reactions, illustrating the way abiotic reactions can be adopted by pre-cellular systems that could have evolved into modern cells.19

In all the examples above, olefin metathesis was utilized in cells for the products to affect the organisms. But even when synthesizing simpler molecules, cells may provide a good environment for the reaction to take place. Photoautotrophic organisms, like unicellular microalgae, are attractive candidates because they naturally produce unsaturated fatty acids, which can react in olefin metathesis. A recent study utilized CAAC and NHC ruthenium catalysts to catalyze cross-metathesis of these unsaturated acids to alkenes or diesters. Since these substrates are stored in lipid bodies inside the cells the catalysts were conjugated to a lipophilic fluorescent BODIPY moiety that localized them to these organelles. The conversion rate inside the cells was high – 72-79% – suggesting that sustainable and efficient polymer synthesis may be achieved using this type of cellular factories.20

## Challenges and requirements

A major challenge in utilizing olefin metathesis reactions in biological systems stems from nature of their biorthogonality – unconjugated alkenes, and especially terminal ones, are the common reactant in metathesis and may not be found in the cells where the desired metathesis should occur.21 Thus, in most cases the reactants should be administered with the catalyst or synthesized in-situ from a naturally occurring compound. Those olefins should be stable in the cellular environment and not cause harm to the cell.

Two ways to increase the yield and rate of olefin metathesis reactions are lowering the pH and increasing the temperature.22-24 These settings, although easily controlled in a lab solution, usually cannot be modified in cell culture or inside an organism – biologically-regulated homeostasis will work to return the acidity and temperature to their original state and more importantly, such a change is likely to harm the cell by causing protein denaturation or dynamizing lipid membranes, for example.

Another important factor that influences reactivity is the solvent. For most metatheses with ruthenium complexes, organic solvents like toluene were found to provide the best results.21,23 Cell cytoplasm, however, is an aqueous solution and cannot be modified.

Optimizing catalyst reactivity in aqueous conditions often involves addition of a cosolvent – DMSO, tBuOH or PEG compounds. 24-26 The cosolvent increases the solubility of the catalyst and prevents aggregation.24 However, just like for the solvent, in living systems a cosolvent cannot be added without serious damage to the cell.

Even without challenging substrates and biological condition, olefin metathesis can lead to undesired side reactions, the most common of which is isomerization – migration of the product's double bond as a result of beta-hydride elimination. Isomerization occurs with most common catalysts – though with varying degrees – and not only lowers the yield of the desired product but can also lead to the formation of harmful compounds.27

### Substrate-specific challenges

Not all olefins react with the same rate and yield, and catalysts that seem successful in simple lab reactions may fail to convert biological compounds. This challenge has two main causes – steric congestion and electronic properties.

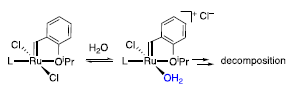
The steric demand of metathesis is simple – the mechanism in fig. 1 demonstrates how coordination of the alkene to the metal atom is required for each step in the conversion. If either of the reactants or the catalyst contain bulky groups which impede this coordination, the reaction will not proceed or achieve lower yields. This effect is prominent for protein-conjugated catalysts and hindered sugar derivatives like GlcNAc, which either require longer reaction times or fail to catalyze at all in comparison to equivalent small reactants.28

Electron-withdrawing groups like ammonium also reduce metathesis efficiency.28 The catalytic cycle explains this as well – an electron-deficient moiety prevents coordination with the electron deficient catalyst.

Another disruptive factor is a tendency for self-metathesis – since most useful reactions include conjugation of two different groups, the alkene cross-linking with itself will lead to undesired side products, especially if this product is stable and does not re-enter the catalytic cycle. This effect was observed for bulky allyl sulfides.28 The metathesis reactant must therefore be reactive, but not too reactive in order to form the desired product.

### Water-related challenges

As mentioned before, the reaction solvent highly affects the rate, selectivity and yield of the reaction. In-vivo reactions must proceed in aqueous environments, which are far less-than-ideal for olefin metathesis.

The main effect of water is catalyst decomposition – the anionic ligands of the metathesis catalyst interact with the polar groups of hydrogen-bonding water molecules to form a hydrous species that's inactive in metathesis, or binuclear ruthenium complexes.15,22

**Figure 4** Formation of a Ru aqua-complex

leading to catalyst decomposition.15

Ruthenium hydroxide and hydride species – the result of exchange of the halide for hydroxide – do not catalyze metathesis but cause double bond migration and isomerization, leading to unexpected side products.29,30

This pathway can lower yield significantly even when water is only a contaminant or cosolvent, but is most prominent when it's the main solvent. Fast-initiating catalysts are more susceptible to decomposition because the active form is more likely to interact with water.29 Therefore, the design of catalysts that can achieve efficient TONs while being soluble and water tolerant is a major challenge of aqueous olefin metathesis.

Water can also lower metathesis yield by changing the reactant's conformation – hydrogen bonding to water molecules changes the free energy of the alkene. For some RCMs tested, interaction with water destabilized the pre-cyclic conformation by disrupting π-orbital stacking arrangement so that the free energy of the state increased by several kcal/mol. This makes the reaction less thermodynamically favorable and lowers the yield.29

### Biology-related constraints

Lessons learned from other bioorthogonal reactions provide further constraints for olefin metathesis – since the reaction must proceed in both low substrate and low catalyst concentration (to minimize harm to the cell) and interact with biological molecules, which often undergo very rapid enzymatic changes, the reaction rate must be maximized as well. The decomposition of metathesis catalysts in water exacerbates this demand, because the limited lifetime of every catalyst molecule requires that it catalyzes as many cycles as possible before being inactivated.

It's nearly impossible to set a guideline for the desired reaction rate in TON, TOF or catalytic units like M/s, since every use case and reaction has different constraints, and most metathesis catalysts don't follow Michelis Menten kinetics used for comparison with enzymes.23 We do know that most metathesis reactions suffer a significant rate decrease when organic solvents are exchanged for water,29 and should therefore be optimized to achieve effective yields in-vivo.

Low substrate and catalyst loading in the cellular environment mean that the catalyst must be very specific and not interact with different molecules in the cell, which may be in a higher concentration. Since olefin metathesis is a bioorthogonal reaction not many cell-native terminal alkenes are expected to compete with the substrates, but ruthenium complexes demonstrate an ability to bind to DNA, which can inactivate them.15

Specificity of the metathesis reaction does not mean only that the metathesis should proceed for desired reactants, but also in a specific cell or tissue, and in some cases, a specific cellular compartment. Localization, or selective activation of the catalyst, may be crucial for a successful synthesis that maximizes the effect of the synthesized product, provides a good metathesis micro-environment (e.g. an organelle with a lower pH) or prevents metathesis in sensitive tissues. To achieve this, the catalyst must contain a localizing moiety but it should not change its reactivity.

A unique biological challenge is the peptide glutathione (GSH). GSH is present in high concentrations (1-2 mM) in most cells and servers as an important antioxidant that aids in the removal of peroxides.31 This reduction tendency makes it a potent inhibitor for metathesis complexes – it coordinates to the metal center and deactivates it. The amino acid histidine has similar properties and damages yield in a comparable manner.32 Since removal of these two vital molecules will damage the cell, overcoming the deactivation should be achieved by preventing interaction between the catalyst and GSH.

Removal of the catalyst from the reaction site is a challenge for every reaction, but in this case the set of available tools is much smaller – most techniques employ additives like phosphines, DMSO, supercritical fluid etc. or tagging the catalyst with a group that binds it to another material (e.g. solid support) followed by washing.33 Obviously, none of these can be utilized if the cellular environment should remain living and functioning. An alternative should be found to scavenge the catalyst while keeping the cell intact, or to use such small loads that the cumulative effect of the transition metal complexes on the cell would be minimal. Since ruthenium complexes are known to have toxic and mutagenic effects, special care should be taken to avoid its aggregation.34

## Solutions

Most of the challenges and limitations presented above can be resolved or minimized by careful design of reaction components and conditions. Since limited data on in-vivo metathesis was reported to date, I will also include results regarding aqueous media that may apply to living systems.

### Catalysts

* + 1. Hoveyda-Grubbs catalysts

Choosing the catalyst is one of the most important decisions when planning a synthesis, and in the case of in-vivo olefin metathesis it must fit the strict requirements of the reaction – rapid and efficient catalysis in low concentration, specificity for reactants and selectivity for products, being biologically inert and non-toxic and localization to the relevant tissues and cellular components.

Ru complexes are established as the most stable and functional group- and water- tolerant olefin metathesis catalysts,21 and most recent research in the field of aqueous olefin metathesis involved different ruthenium compounds. Their early versions, with phosphine ligands, were very air sensitive and could catalyze a limited range of metathesis reactions,35 even when equipped with cationic groups to increase solubility.21 Replacing one phosphine ligand with NHC and the other with an isopropoxy group bound to the benzylidene creates the Hoveyda-Grubbs second generation catalyst (HGII), a group of common, efficient and tolerant catalysts.36

Despite these advantages, HGII catalysts have drawbacks – the cellular toxicity of ruthenium, poor solubility in aqueous solutions and rapid decomposition of the catalyst.15 The basic structure of the HGII should be optimized to fit the conditions inside cells.

* + 1. Charged catalysts

Some downsides of neutral, hydrophobic catalysts can be resolved by attaching ionic moieties. AquaMet, for example, is an HGII catalyst in which a quaternary ammonium group is attached to the N-heterocyclic carbine, improves the solubility in water and weakens the coordination of the isopropoxy group to the ruthenium, resulting in faster initiation30,37. It was successfully employed in physiological conditionsand is moderately cytotoxic, significantly impacting HeLa cells viability in concentrations above 0.2 mg/mL but not harming 1MEA cells in similar concentrations.17

However, cationic catalysts like AquaMet have drawbacks as well – they may acidify the water around the ruthenium complex, accelerating their decomposition, and fail to catalyze substrates on nucleic acids because of the attraction between the cationic group and the negatively-charged phosphate backbone.15

AqueMet's reactivity for specific RCM reactions can be improved by replacing one of the chloride ligands with nitrate. This new catalyst – AM-NO3 – decomposes in a similar rate but offers "kinetic protection" – because its initiation is faster more product is formed before the catalyst is inactivated and the total yield is greater.32

Anionic tags provide an attractive alternative to cationic ligands – cyclic alkylaminocarbene (CAAC) ligands functionalized with a sulfonate tag showed greatly improved stability in water – the AquaMet aqua species, which is formed immediately in water, decayed over 2 hours whereas the anionic catalyst remained over 24 hours.15 They also managed to achieve usable TONs (70-1520, depending on the reaction) for RCM and CM of various challenging substrates like the sugar galactopyranoside and the nucleoside uridine. Another significant improvement in comparison to AquaMet is the suppression of isomerization, enabling selective metathesis with little side-products. However, the reactions for these reported TONs proceeded in 70˚C and in the presence of NaCl and should therefore be tested in more realistic biological conditions.

* + 1. Metalloproteins

Preserving the structure and reactivity of the catalyst can be achieved by anchoring it to a protein. Metalloproteins or artificials metalloenzymes (ArMs) offer several advantages – minimizing the cytotoxic effect of free ruthenium species while protecting the catalyst from decomposition and side reactions and directing it to the relevant cell and site.

An appropriate anchoring protein and conjugation strategy of the catalyst to the amino residues are vital to the success of the metalloprotein. β-barrel proteins are good candidates because of the rigidity of the β-sheet motif and compact barrel structure that can protect the catalyst from degradation. More specifically, membrane-spanning β-barrel proteins are relatively large and can fit bulky catalysts in their pores or binding sites.38

A good example for this type of protein is ferric hydroxamate uptake protein component A (FhuA) - a transmembrane β-barrel protein isolated from E. coli. After attaching a maleimide linking unit and a cysteine residue to the NHC ligand of a HGII catalyst, the catalyst was successfully anchored to the protein and catalyzed a ROMP reaction in aqueous conditions of oxanorbornene with high TONs (300-500) but without cis/trans selectivity. It's worth noting that for such a large protein the conjugation required genetically engineering a protease cleavage site, denaturing and digesting the protein and renaturing it afterwards to achieve refolding to the original secondary structure.39

Streptavidin is a small bacterial β-barrel protein with very high affinity to biotin. This enables non-covalent conjugation of a biotinylated HGII catalyst to the protein. A metalloenzyme created this way outperformed both classic HGII catalyst, AquaMet and the free biotin-Ru species for RCM reactions inside E. coli cells.25

Exploitation of existing binding-site affinity was also used for synthesis of human carbonic anhydrase II (hCAII) ArM. Since this enzyme natively contains a Zn ion, installing an arylsulfonamide anchor on a HGII catalyst promoted dative binding of the catalyst to the protein and catalyzed RCM in aqueous conditions with TON of 28 in physiological conditions.40

Regardless of the type of protein or reaction, the length of the spacer between the catalyst and the protein's peptide is significant to the reactivity and selectivity of the reaction as it affects the second ligand sphere – the amino acids that the catalyst contacts and the substrate's steric constraints for contact with the catalyst. The study on FhuA protein compared multiple lengths of the chain linking the catalyst to the FuhA protein. It was found that the shortest spacer led to the highest TONs and a slightly higher cis/trans ratio of the product – it may be concluded that in this case a longer spacer enables coordination with hydrophilic amino acid residues on the anchoring protein that compete with the metathesis reaction.39 However, when the same catalyst was incorporated to the smaller β-barrel protein nitrobindin, a longer spacer was required both for successful synthesis of the metalloenzyme – to accommodate the bulky NHC ligand – and achieved the highest TONs in ROMP reaction.41 Therefore the design of a metalloenzyme must take into account the balance between minimizing contact with nearby amino acids residues and enabling interaction with the substrate, a balance that depends on the protein, the catalyst and the catalyzed reaction.

The stability and reactivity of the metalloenzyme may be affected by the anionic ligand bound to the ruthenium. An experiment comparing three different halides – chloride, iodide and bromide – in an HGII catalyst conjugated to nitrobindin discovered that iodide complexes had the highest TONs for several RCM reactions in aqueous environment. Chloride complexes were the second best, with bromide metalloenzymes having the lowest TONs and failing to catalyze in some conditions. The improvement in reactivity for iodide-substituted metalloenzymes is attributed to the hydrophobicity of this ligand in comparison to chloride. Since it doesn't form hydrogen bonds in the aqueous environment, it's less susceptible to halide-hydroxide ligand exchange the leads to the decomposition of the catalyst. The higher stability over a long reaction time enables higher TONs.22 The same effect – both for catalyst stability and RCM yield – was observed for a different metalloenzyme containing albumin, suggesting that iodide substitution will be helpful for any ArM in which the catalyst is exposed to the aqueous environment.18

The synthesis of the FhuA ArM required mutagenesis to insert a cleavage site for the incorporation of the HGII catalyst;39 experiments using the smaller protein nitrobindin introduced mutations to construct a hydrophobic cavity for catalyst anchoring.41 The most striking usage of mutagenesis for ArM optimization was exhibited in the directed evolution of a streptavidin (SAV)-based metathase. In contrast to the previous examples, the SAV research employed multiple consecutive rounds of mutagenesis that improved the cell-specific activity of the ArM for a specific reaction. The mutations included substitutions of amino acids close to the catalysis site to increase the flexibility of the tertiary structure, reduce steric hindrance or increase the entropy of reaction.25 Different mutants of the protein performed best for different metathesis reactions, suggesting that a global "ideal metathase" for all reactions may not be developed, but each use-case can benefit from engineering of a customized mutant through directed evolution.

Another promising technology for catalyst uptake is through a polymersome – an artificial organelle composed of a polymer and the catalyst. In a recent study, a polyethylene glycol (PEG) derivative was conjugated – either covalently or through hydrophobic interactions – to an HGII catalyst to form polymersomes. These were successfully internalized by HeLa cells, localized in the lysosome and catalyzed RCM formation of umbelliferone inside the cell. The combined effect of encapsulation and localization inside an intracellular organelle both protects the cell from the cytotoxicity of free HGII catalysts and prevents decomposition of the catalyst by cellular compounds like GSH. It should be noted that the conversion rate of this reaction was quite low, perhaps due to said encapsulation isolating the catalyst from the substrate.42 Further optimization is necessary to adapt this interesting technique to other metathesis reactions and on a wider scale.

### Substrates and reaction partners

Metatheses in which one of the substrates is a peptide or a protein require the incorporation of alkene-containing amino acids, which are unnatural amino acids (UAAs). This can be achieved chemically – by modifying an existing residue – or genetically.

Chemical modification can be achieved by direct allylation with allyl halides. This method was used to convert cysteine to S-allylcysteine (Sac), and achieved high conversion rates with mild conditions and without denaturing the protein. Since allyl halides may also react with other amino acids – such as lysine and histidine – a more specific allylation protocol was developed for cysteine in which the amino acid was first converted to Se-allyl selenenylsulfide using allyl selenocyanate and then underwent rearrangement to form Sac. Both methods provided an active protein that also reacted in olefin metathesis with allyl alcohol.43

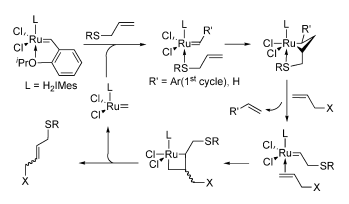
Genetic incorporation of UAAs exploits their similarity to natural amino acids which allows them to occupy the binding site in aminoacyl-tRNA synthetase. A study testing the incorporation of S and Se methionine analogues to E. Coli methionyl-tRNA synthetase revealed that the turnover was the highest for S-allylhomocysteine (Ahc). Ahc was also incorporated successfully to multiple proteins in-vivo while keeping their original secondary structure and function. Cross-metathesis by HGII catalyst with allyl alcohol or the fluorescent tracer fluorescein was performed with moderate to high yields (55%-95%), depending on the protein and the reaction partner. The same UAA was also installed into the Fc region of immunoglobulin G in a human cell line and reacted with olefin-biotin, illustrating the potential use for biotin tagging.26

Derivatives of sulfur-containing amino acids, like cysteine, are frequently used in protein olefin metathesis and achieve better yields than other amino acids.28 This can be explained, at least in part, by the allylic chalcogen effect. The same qualities that make the S-containing GSH a potent deactivator of ruthenium complexes enhance the rate of reactions of allyl sulfides – the sulfur atom tends to coordinate to the central ruthenium in the catalyst, since it's a softer nucleophile than the oxygen in the isopropoxy group. Because the sulfur is bound to the rest of the alkene, this increases the effective concentration of the active species in the metathesis cycle and thus the reaction rate (figure 5). Because metathesis catalysts are prone to decomposition in aqueous conditions, the higher rate leads to higher final yield.28

Since selenium is a larger, softer base than sulfur and with similar electronegativity, it can also be incorporated to UAAs in the allylic position. Se-allylselenocysteine (Seac) was indeed found to be more reactive than Sac for challenging reactions and in some cases, to achieve higher yields.28

**Figure 5** A mechanistic description of the allylic chalcogen

effect in olefin metathesis28

Another factor affecting reaction rate and yield is the position of the UAA on the protein. To decrease the steric strain of the coordination of the alkene to the catalyst, a protein containing cysteine was modified to include acrylamide conjugated to Sac, instead of Sac bound directly to the protein. This modified reactant manage to achieve higher yields and faster rates than Sac for some cross-metathesis reactions.28 Similarly to catalyst design, the length of the linker chain of the reactant is significant to accommodate the bulk of the metathesis reactants and catalyst (though in the cited article, less significant than the effect of selenium substitution).

### Additional reagents

As was mentioned before, in-vivo olefin metathesis is limited by deactivation and decomposition of the catalyst, in some instances by dimerization or chelate formation of the ruthenium complexes.32 Addition of MgCl2 to the solution in aqueous olefin metathesis improves yield in multiple reactions,19,26 in part because the Mg+2, a soft lewis acid, disrupts chelate formation.30 The chloride plays a role as well, as NaCl decreases catalyst decomposition too – it's assumed that a higher chloride concentration shifts the equilibrium constant of the halide exchange decomposition path to favor the dichloro form of the catalyst.44

### Organism and biological microenvironment

Although metathesis inside cells poses many challenges, careful choice of the environment can minimize them. For example, to eliminate catalyst decomposition by GSH reduction, olefin metathesis in E. coli was localized to the cell's periplasm – the space between the two bacterial membranes – where glutathione is present in its harmless oxidized form.25

The cytotoxicity of metathesis catalysts is established, for multiple cell types,17,20,42 but is not equal for every cell. As a dose-dependent comparison of AquaMet effect on viability has shown, the cell lines HeLa and 1MEA show drastically different survival rates.17 The difference can result from the different source organism – human vs mouse, source tissue – cervical vs liver cancer – or another factor. This variance proves that some cells are less susceptible to damage than others and so can be targeted for metathesis with minimal effects.

Another encouraging result is the high cell viability achieved when the catalyst is delivered by polymersomes. The study testing this technique measured HeLa viability as well, and achieved about 80% viability in polymersome concentrations as high as 1 mg/mL, whereas with unprotected AquaMet viability dropped to 50% for concentration of 0.46 mg/mL.17,42 Therefore, localizing the reaction in an intracellular compartment or organelle may protect the cell.

# Discussion

The scope, both for reactions and application, for in vivo olefin metathesis is wide and the challenges are significant – the few reports of metathesis in living systems demonstrate this. We cannot depend on one new discovery or breakthrough that would suddenly turn this difficult goal possible and enable efficient, selective and harmless reaction for any reaction in any cell. However, the research on aqueous olefin metathesis provides some useful guidelines that should be considered when planning a reaction.

The most significant and easily controlled factor is the catalyst. As common industry-level HGII catalysts are not water tolerant and biologically safe, one of two alternative paths may be taken – optimizing small ruthenium complexes for solubility and cell viability (for example, by adding anionic groups or changing the halides), or synthesizing artificial metalloenzymes. While the former option is much simpler and more economical, there's a limit to the efficiency and biocompatibility of unprotected ruthenium complexes in aqueous environments. Therefore, they may be used in the first steps of planning a synthesis or when selectivity and viability are not highly prioritized. The costly and time-consuming process of designing a protein and conjugating a catalyst, which should involve site-specific mutations for further optimization, will probably provide better results which are necessary for products with a strong effect (e.g. drugs) or experiments in living animals.

Although the theoretic range of metathesis reactions is very wide – every CM, RCM, ROMP and more – the number of truly possible reactions is lower. Olefins with bulky or electron-deficient groups – which are frequent in biological setting – often convert slowly, with low yields or fail to react at all. When possible, at least one of the reacting alkenes should be designed to maximize the length of the link to the hindering molecule and to contain an allyl sulfide or allyl selenide as the reacting group. Additionally, most of the reviewed articles featured RCM reactions as the ones with best yield and rate, probably because of the increased stability of 5- and 6-membered rings, so they are recommended for testing new catalysts or reaction methods.

Aqueous olefin metathesis experiments showed that some reagents prevent catalyst decomposition and increase yield, the most prominent being MgCl2. However, cell cytoplasm can't be modified easily so we shouldn't rely on such modification, unless the reaction is localized to a compartment with distinct ion content (like the sarcoplasmic reticulum contains high Ca+2 concentration). Instead, catalyst delivery techniques like polymersomes can be used to minimize contact with deactivating agents.

Some usages for olefin metathesis in living cells were proposed and even less experimentally tested, most of them concerning protein modification. The results so far seem promising, especially for histone modification, which was demonstrated in a reversible manner and could be utilized for "genetic switches". Another interesting modification can be ubiquitylation, which can be used to control protein lifecycle. Further research on other important proteins is needed, and on potential metabolic utilization of fatty acids metathesis, which proceeded successfully in microalgae cells. Sugar modification was achieved for monomers and its usage in multicellular systems can alter metabolic and cell signaling processes.

These promising prospects are limited by the difficulties of delivering the reactants and catalyst to the cell while causing minimal harm, and not less importantly, eliminating the remaining catalyst and by-products after the reaction. So far, few suggestions for bio-compatible scavenging methods have been published and this remains an obstacle.

This obstacle can be at least partially bypassed by using cells which should be destroyed, like cancer cells. Thus, the decreased viability caused by ruthenium-carbene complexes becomes an advantage and because of the lowered extracellular pH in tumor microenvironments,45 olefin metathesis can be favored there.

# Conclusion

In this work I attempted to review the existing knowledge about in vivo olefin metathesis and the ways to realize it. I discovered that although metathesis reactions are becoming more and more common for a multitude of applications, most current catalysts and techniques do not fit the constraints needed to operate in or near living cells, which are demonstrated in other bioorthogonal reactions – efficiency, specificity, selectivity, high rate and harmlessness. To my disappointment, I found few accounts of metathesis in living organisms.

However, the enormous potential of this versatile reaction is too tempting to abandon the idea altogether – the quickly-improving field of aqueous olefin metathesis shows that the reaction can be optimized by various modifications, the most prominent of which is catalyst design. More specifically, genetic engineering of both catalysts and substrates can significantly improve yields and rates.

As a new member of the bioorthogonal toolkit, the most rewarding or challenging applications of olefin metathesis were not determined yet – existing work includes "proof-of-concept" setups, involving mostly modifications of arbitrary reporter molecules or proteins. I believe that once the reaction is relatively easy and performant (and current discoveries are getting us closer to that state) it will enable great progress in the fields of biochemistry, cellular biology and pharmacology.

# References

1. Jean-Louis Hérisson, P.; Chauvin, Y. Die Makromolekulare Chemie 1971, 141 (1), 161–176. https://doi.org/10.1002/macp.1971.021410112.
2. Reany, O.; Gabriel Lemcoff, N. Light Guided Chemoselective Olefin Metathesis Reactions. Pure and Applied Chemistry 2017, 89 (6), 829–840. https://doi.org/10.1515/pac-2016-1221.
3. ‌Chatterjee, A. K.; Choi, T.-L.; Sanders, D. P.; Grubbs, R. H. A General Model for Selectivity in Olefin Cross Metathesis. Journal of the American Chemical Society 2003, 125 (37), 11360–11370. https://doi.org/10.1021/ja0214882.
4. ‌ Vougioukalakis, G. C.; Grubbs, R. H. Ruthenium-Based Heterocyclic Carbene-Coordinated Olefin Metathesis Catalysts†. Chemical Reviews 2010, 110 (3), 1746–1787. https://doi.org/10.1021/cr9002424.
5. Caire da Silva, L.; Rojas, G.; Schulz, M. D.; Wagener, K. B. Acyclic Diene Metathesis Polymerization: History, Methods and Applications. Progress in Polymer Science 2017, 69, 79–107. https://doi.org/10.1016/j.progpolymsci.2016.12.001.
6. ‌ Trnka, T. M.; Grubbs, R. H. The Development of L2X2RuCHR Olefin Metathesis Catalysts: An Organometallic Success Story. Accounts of Chemical Research 2001, 34 (1), 18–29. https://doi.org/10.1021/ar000114f.
7. ‌ Nguyen, S. T.; Johnson, L. K.; Grubbs, R. H.; Ziller, J. W. Ring-Opening Metathesis Polymerization (ROMP) of Norbornene by a Group VIII Carbene Complex in Protic Media. Journal of the American Chemical Society 1992, 114 (10), 3974–3975. https://doi.org/10.1021/ja00036a053.
8. ‌ Scinto, S. L.; Bilodeau, D. A.; Hincapie, R.; Lee, W.; Nguyen, S. S.; Xu, M.; am Ende, C. W.; Finn, M. G.; Lang, K.; Lin, Q.; Pezacki, J. P.; Prescher, J. A.; Robillard, M. S.; Fox, J. M. Bioorthogonal Chemistry. Nature Reviews Methods Primers 2021, 1 (1), 1–23. https://doi.org/10.1038/s43586-021-00028-z.
9. ‌ Saxon, E. Cell Surface Engineering by a Modified Staudinger Reaction. Science 2000, 287 (5460), 2007–2010. https://doi.org/10.1126/science.287.5460.2007.
10. ‌ Bird, R. E.; Lemmel, S. A.; Yu, X.; Zhou, Q. A. Bioorthogonal Chemistry and Its Applications. Bioconjugate Chemistry 2021, 32 (12), 2457–2479. https://doi.org/10.1021/acs.bioconjchem.1c00461.
11. ‌ Stymiest, J. L.; Mitchell, B. F.; Wong, S. J.; Vederas, J. C. Synthesis of Oxytocin Analogues with Replacement of Sulfur by Carbon Gives Potent Antagonists with Increased Stability. 2005, 70 (20), 7799–7809. https://doi.org/10.1021/jo050539l.
12. ‌ Shvedunova, M.; Akhtar, A. Modulation of Cellular Processes by Histone and Non-Histone Protein Acetylation. Nature Reviews Molecular Cell Biology 2022. https://doi.org/10.1038/s41580-021-00441-y.
13. ‌ Lin, Y. A.; Boutureira, O.; Lercher, L.; Bhushan, B.; Paton, R. S.; Davis, B. G. Rapid Cross-Metathesis for Reversible Protein Modifications via Chemical Access to Se-Allyl-Selenocysteine in Proteins. Journal of the American Chemical Society 2013, 135 (33), 12156–12159. https://doi.org/10.1021/ja403191g.
14. ‌ Lu, X.; Fan, L.; Phelps, C. B.; Davie, C. P.; Donahue, C. P. Ruthenium Promoted On-DNA Ring-Closing Metathesis and Cross-Metathesis. Bioconjugate Chemistry 2017, 28 (6), 1625–1629. https://doi.org/10.1021/acs.bioconjchem.7b00292.
15. ‌ Blanco, C. O.; Castellanos, R. R.; Fogg, D. E. Anionic Olefin Metathesis Catalysts Enable Modification of Unprotected Biomolecules in Water. ACS Catalysis 2024, 14 (15), 11147–11152. https://doi.org/10.1021/acscatal.4c02811.
16. ‌ Steffansen, S. I.; Christensen, M. S.; Børsting, P.; Nielsen, P. Synthesis of Conformationally Restricted Nucleic Acid Fragments Using Ring-Closing Alkene and Enyne Metathesis Reactions. Nucleosides, nucleotides & nucleic acids 2005, 24 (5-7), 1015–1018. <https://doi.org/10.1081/ncn-200060347>.
17. Kelly, M. P.; Napolitano, T.; Anand, P.; Justin; Jabeen, S.; Kuppan, J.; Sujoy Manir; Holford, M. Induced Disassembly of a Virus-like Particle under Physiological Conditions for Venom Peptide Delivery. Bioconjugate Chemistry 2020, 32 (1), 111–120. https://doi.org/10.1021/acs.bioconjchem.0c00494.
18. ‌ Igor Nasibullin; Yoshioka, H.; Akari Mukaimine; Nakamura, A.; Kusakari, Y.; Chang, T.-C.; Tanaka, K. Catalytic Olefin Metathesis in Blood. Chemical science 2023, 14 (40), 11033–11039. https://doi.org/10.1039/d3sc03785a.
19. ‌Samanta, A.; Sabatino, V.; Ward, T. R.; Walther, A. Functional and Morphological Adaptation in DNA Protocells via Signal Processing Prompted by Artificial Metalloenzymes. *Nature Nanotechnology* **2020**, *15* (11), 914–921. <https://doi.org/10.1038/s41565-020-0761-y>.
20. Schunck, N. S.; Mecking, S. In Vivo Olefin Metathesis in Microalgae Upgrades Lipids to Building Blocks for Polymers and Chemicals. Angewandte Chemie International Edition 2022, 61 (44). https://doi.org/10.1002/anie.202211285.
21. ‌ Binder, J. B.; Raines, R. T. Olefin Metathesis for Chemical Biology. Current Opinion in Chemical Biology 2008, 12 (6), 767–773. https://doi.org/10.1016/j.cbpa.2008.09.022.
22. ‌ Ingram, A. A.; Wang, D.; Ulrich Schwaneberg; Okuda, J. Grubbs-Hoveyda Catalysts Conjugated to a β-Barrel Protein: Effect of Halide Substitution on Aqueous Olefin Metathesis Activity. Journal of Inorganic Biochemistry 2024, 258, 112616–112616. https://doi.org/10.1016/j.jinorgbio.2024.112616.
23. ‌ Bieniek, M.; Michrowska, A.; Usanov, Dmitry L.; Grela, K. In an Attempt to Provide a User’s Guide to the Galaxy of Benzylidene, Alkoxybenzylidene, and Indenylidene Ruthenium Olefin Metathesis Catalysts. Chemistry - A European Journal 2008, 14 (3), 806–818. https://doi.org/10.1002/chem.200701340.
24. ‌ Sabatino, V.; Ward, T. R. Aqueous Olefin Metathesis: Recent Developments and Applications. Beilstein Journal of Organic Chemistry 2019, 15, 445–468. https://doi.org/10.3762/bjoc.15.39.
25. ‌ Jeschek, M.; Reuter, R.; Heinisch, T.; Trindler, C.; Klehr, J.; Panke, S.; Ward, T. R. Directed Evolution of Artificial Metalloenzymes for in Vivo Metathesis. Nature 2016, 537 (7622), 661–665. https://doi.org/10.1038/nature19114.
26. ‌ Bhushan, B.; Lin, Y. A.; Bak, M.; Anuchit Phanumartwiwath; Yang, N.; Bilyard, M. K.; Tanaka, T.; Hudson, K. L.; Lercher, L.; Stegmann, M.; Mohammed, S.; Davis, B. G. Genetic Incorporation of Olefin Cross-Metathesis Reaction Tags for Protein Modification. Journal of the American Chemical Society 2018, 140 (44), 14599–14603. https://doi.org/10.1021/jacs.8b09433.
27. Lehman, S. E.; Schwendeman, J. E.; O’Donnell, P. M.; Wagener, K. B. Olefin Isomerization Promoted by Olefin Metathesis Catalysts. Inorganica Chimica Acta 2003, 345, 190–198. <https://doi.org/10.1016/s0020-1693(02)01307-5>.
28. Lin, Y. A.; Chalker, J. M.; Davis, B. G. Olefin Cross-Metathesis on Proteins: Investigation of Allylic Chalcogen Effects and Guiding Principles in Metathesis Partner Selection. Journal of the American Chemical Society 2010, 132 (47), 16805–16811. https://doi.org/10.1021/ja104994d.
29. ‌ Blanco, C. O.; Sims, J.; Nascimento, D. L.; Goudreault, A. Y.; Steinmann, S. N.; Michel, C.; Fogg, D. E. The Impact of Water on Ru-Catalyzed Olefin Metathesis: Potent Deactivating Effects Even at Low Water Concentrations. ACS Catalysis 2021, 11 (2), 893–899. https://doi.org/10.1021/acscatal.0c04279.
30. ‌Lin, Y. A.; Chalker, J. M.; Davis, B. G. Olefin Metathesis for Site-Selective Protein Modification. ChemBioChem 2009, 10 (6), 959–969. <https://doi.org/10.1002/cbic.200900002>.
31. Forman, H. J.; Zhang, H.; Rinna, A. Glutathione: Overview of Its Protective Roles, Measurement, and Biosynthesis. Molecular aspects of medicine 2009, 30 (1-2), 1–12. <https://doi.org/10.1016/j.mam.2008.08.006>.
32. James, C. C.; Petrus; Bruin, B. de; Joost. Kinetic Protection of a Water‐Soluble Olefin Metathesis Catalyst for Potential Use under Biological Conditions. ChemCatChem 2023, 15 (7). <https://doi.org/10.1002/cctc.202201272>.
33. Kotha, S.; Mirtunjay Kumar Dipak. Strategies and Tactics in Olefin Metathesis. *Tetrahedron* **2012**, *68* (2), 397–421. <https://doi.org/10.1016/j.tet.2011.10.018>.
34. Yasbin, R. E.; Matthews, C. R.; Clarke, M. J. Mutagenic and Toxic Effects of Ruthenium. *Chemico-Biological Interactions* **1980**, *31* (3), 355–365. <https://doi.org/10.1016/0009-2797(80)90023-x>.
35. Messina, M. S.; Maynard, H. D. Modification of Proteins Using Olefin Metathesis. Materials Chemistry Frontiers 2020, 4 (4), 1040–1051. <https://doi.org/10.1039/c9qm00494g>.
36. Garber, S. B.; Kingsbury, J. S.; Gray, B. L.; Hoveyda, A. H. Efficient and Recyclable Monomeric and Dendritic Ru-Based Metathesis Catalysts. Journal of the American Chemical Society 2000, 122 (34), 8168–8179. <https://doi.org/10.1021/ja001179g>.
37. Jordan, J.; Grubbs, R. Small‐Molecule N‐Heterocyclic‐Carbene‐Containing Olefin‐Metathesis Catalysts for Use in Water. Angewandte Chemie International Edition 2007, 46 (27), 5152–5155. <https://doi.org/10.1002/anie.200701258>.
38. Sauer, D. F.; Schiffels, J.; Hayashi, T.; Schwaneberg, U.; Okuda, J. Olefin Metathesis Catalysts Embedded in β-Barrel Proteins: Creating Artificial Metalloproteins for Olefin Metathesis. Beilstein Journal of Organic Chemistry 2018, 14 (14), 2861–2871. <https://doi.org/10.3762/bjoc.14.265>.
39. Sauer, D. F.; Bocola, M.; Broglia, C.; Arlt, M.; Zhu, L.; Brocker, M.; Ulrich Schwaneberg; Okuda, J. Hybrid Ruthenium ROMP Catalysts Based on an Engineered Variant of β‐Barrel Protein FhuA ΔCVFtev: Effect of Spacer Length. Chemistry - An Asian Journal 2014, 10 (1), 177–182. <https://doi.org/10.1002/asia.201403005>.
40. Zhao, J.; Kajetanowicz, A.; Ward, T. R. Carbonic Anhydrase II as Host Protein for the Creation of a Biocompatible Artificial Metathesase. Organic & Biomolecular Chemistry 2015, 13 (20), 5652–5655. <https://doi.org/10.1039/c5ob00428d>.
41. Sauer, D. F.; Tomoki Himiyama; Kengo Tachikawa; Fukumoto, K.; Onoda, A.; Eiichi Mizohata; Inoue, T.; Bocola, M.; Ulrich Schwaneberg; Hayashi, T.; Okuda, J. A Highly Active Biohybrid Catalyst for Olefin Metathesis in Water: Impact of a Hydrophobic Cavity in a β-Barrel Protein. ACS Catalysis 2015, 5 (12), 7519–7522. <https://doi.org/10.1021/acscatal.5b01792>.
42. Roy; Shao, J.; Marleen; Wu, H.; Tania Patiño Padial; Loai K. E. A. Abdelmohsen; Jan. Biodegradable Grubbs-Loaded Artificial Organelles for Endosomal Ring-Closing Metathesis. Biomacromolecules 2023, 24 (9), 4148–4155. <https://doi.org/10.1021/acs.biomac.3c00487>.
43. Chalker, J. M.; Lin, Y. A.; Boutureira, O.; Davis, B. G. Enabling Olefin Metathesis on Proteins: Chemical Methods for Installation of S-Allyl Cysteine. Chemical Communications 2009, No. 25, 3714–3714. <https://doi.org/10.1039/b908004j>.
44. Church, D. C.; Takiguchi, L.; Pokorski, J. K. Optimization of Ring-Opening Metathesis Polymerization (ROMP) under Physiologically Relevant Conditions. Polymer Chemistry 2020, 11 (27), 4492–4499. <https://doi.org/10.1039/d0py00716a>.
45. Ward, C.; Meehan, J.; Gray, M. E.; Murray, A. F.; Argyle, D. J.; Kunkler, I. H.; Langdon, S. P. The Impact of Tumour PH on Cancer Progression: Strategies for Clinical Intervention. Exploration of Targeted Anti-tumor Therapy 2020, 1 (2), 71–100. https://doi.org/10.37349/etat.2020.00005.