Palladium in biological media: Can the synthetic chemist's most versatile transition metal become a powerful biological tool?

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Richard C. Brewster[†], Eva Klemencic[†], Amanda G. Jarvis*
EaStCHEM School of Chemistry, Joseph Black Building, David Brewster Rd, University of Edinburgh, Edinburgh, EH9 3FJ.*Corresponding author: amanda.jarvis@ed.ac.uk

Highlights

- Palladium reactions as new tools for selective protein modification.
- Bioorthogonal and biocompatible palladium complexes and their reactions.
- Enzymes plus palladium – use in cascade reactions and as biohybrid catalysts.
- Toxicity and non-specific metal binding reduce utility of Pd *in vivo.*
- Judicious use of ligands and buffer maximise the use of Pd reactions in biological media.

Abstract

Palladium catalysed reactions are ubiquitous in synthetic organic chemistry in both organic solvents and aqueous buffers. The broad reactivity of palladium catalysis has drawn interest as a means to conduct orthogonal transformations in biological settings. Successful examples have been shown for protein modification, *in vivo* drug decaging and as palladium-protein biohybrid catalysts for selective catalysis. Biological media represents a challenging environment for palladium chemistry due to the presence of a multitude of chelators, catalyst poisons and a requirement for milder reaction conditions e.g. lower temperatures. This review looks to identify successful examples of palladium-catalysed reactions in the presence of proteins or cells and analyse solutions to help to overcome the challenges of working in biological systems.

1. Introduction

The versatility of palladium as a catalyst is widely recognized, especially for cross-coupling chemistry [1] which was awarded the Nobel prize in 2010 [2], but also in Pd-catalysed oxidations and hydrogenations [1]. To accommodate a shift to more sustainable chemistry and to expand the biocompatible applications of Pd, extensive work has been done on Pd chemistry in water [3], [4] and, where reactants are insoluble in aqueous media, efforts have been made to develop "on water" chemistry [5]–[7].

The advantages of Pd in comparison to other transition metal systems is that, while there needs to be some caution when setting up reactions, Pd can withstand the presence of oxygen and moisture, and in some forms is less toxic than some other transition metals, such as Cu [1], [8]. Pd-catalysed reactions are biorthogonal to reactions found in nature and they have found use in labelling studies, decaging reactions for potential medical applications and for controlling protein targets [9]. Although Pd is not found in natural biological systems, Pd-protein hybrid systems have been designed where the incorporation of a new reactive metal centre allows abiotic enzymatic reactions.

Despite the prevalence of Pd catalysis in synthetic chemistry, hybrid Pd-protein catalysts are relatively rare. While Pd offers advantages in terms of reactivity, there are also disadvantages when proteins are present: (i) Pd can form non-specific interactions with proteins as the amino acids have moderate binding affinity for metal ions [10]–[13]; (ii) Pd(II) complexes can act as synthetic peptidases and

[†] RB and EK contributed equally to this work.

catalyse the hydrolysis of amides [14]; (iii) some Pd reactions, e.g. C-H activation, need higher temperatures and prefer organic solvents limiting the proteins that can be used for these reactions to those that tolerate higher temperatures and organic solvents; (iv) lastly, it has been observed that Pd species can inhibit enzymatic reactions [15]. For *in vivo* systems, difficulties include the uptake of Pd, toxicity of Pd and inactivation of Pd in the presence of thiols, proteins and other cell components [16].

In this review we focus on how the field of Pd catalysis in the biological context has progressed, describing i) protein modifications, ii) *in vivo* Pd chemistry, iii) one-pot Pd/enzyme reactions and iv) the building of biohybrid catalysts to highlight the challenges and opportunities of Pd-protein chemistry. We have limited the review to the use of Pd *in vitro* in the presence of proteins and examples where Pd has been used *in vivo.*

2. Protein Modification

Protein modification is an essential technique that has been used to study the structure and function of proteins. The key to successful protein modification is selective modification of one amino acid residue over all the others. Cysteine (Cys) is the most commonly targeted residue as it is the most nucleophilic amino acid and also one of the least abundant, although lysine modification is also extremely widely used, especially for the synthesis of drug-antibody conjugates. Non-canonical amino acid incorporation has allowed a variety of bioorthogonal chemistries to be used to selectively modify proteins both *in vitro* and *in vivo*.

Protein labelling as a field has been focused on selectivity (through enhanced relative rate) rather than reactivity and the goal is to achieve maximum conversion and shorter reaction time. It is therefore common to add super stoichiometric amounts of Pd, even for catalytic transformations. The main challenges the field faces are the insolubility of reagents/substrates, the need to use protein concentrations in the nM or µM range, and lastly proteins acting like ligands for the metals, leading to poisoning or degradation [17].

2.1 Protein modification *via* cross-coupling reactions

Cross-coupling chemistry is a broad range of reactions that describe the joining of two species by a transition metal catalyst, creating a carbon-carbon or carbon-heteroatom bond. These reactions have become a mainstay in organic synthesis since their discovery in the 1970s. Although other transition metals such a Cu and Ni are used for cross-coupling chemistry, palladium is by far the most common metal used. Major advantages of cross-coupling reactions are that they are biocompatible as the reagents are not typically found in nature (aryl halides, boronic acids, alkenes and alkynes) and examples such as the Suzuki-Miyaura, Heck and Sonogashira reactions can be performed in water.

2.1.1. Suzuki-Miyaura

The Suzuki-Miyaura (SM) reaction, which describes the coupling of an aryl halide with an aryl/alkyl boronic acid was investigated on a 4 kDa aryl-iodide containing peptide substrate by Ojida *et al.* using stochiometric amounts of the Pd precursor, K_2PdCl_4 and excess of the boronic acid [18]. They found the reaction was enhanced by the addition of glycerol as a co-solvent (10%) and increasing temperature to 40 °C, however in this ligand free example, optimised the yields only to 60%.

A major breakthrough for SM reactions on proteins was made by Chalker *et al.* using 4,6-dihydroxypyrimidine (ADHP) to make the water soluble $Pd(ADHP)_2{\cdot}(OAc)_2$ complex, **1** [19]. The authors highlight the benefits of using nitrogen-based ligands over phosphine ligands, as the catalyst is stable in solution and reactions can be performed in air without risk of oxidation that occurs with phosphines. A single Cys residue was introduced into the protein subtilisin from *Bacillus lentus* (SBL) and was alkylated to give an aryl iodide containing subtilisin which was used as a model protein. The group

demonstrated SM coupling with 7 different arylboronic acids and two alkenylboronic acids, giving >95% conversion at 37 °C in 30-60 minutes. The reaction was run at pH 8 in phosphate buffer using 50 eq. of **1** and 500 eq. of boronic acid to speed up the reaction (Figure 1). ADHP and ligands bearing similar structural motifs have since been used in a variety of protein SM reactions including ligation of fluorophores [20], PEG2k chains[21], ^{18}F labelling [22], cell surface labelling [23] and to probe the mechanism of glyconeogenesis [24].

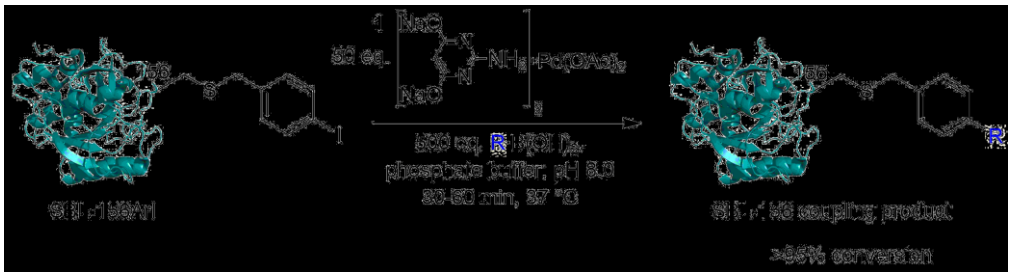

Figure 1: SM coupling of an aryl iodide modified Cys in subtilisin, SBL, using the water soluble complex, $Pd(ADHP)_2{\cdot}(OAc)_2$ (**1**) [19]**.**

N-heterocyclic carbene (NHC) ligands are classic ligands for Pd as they give rise to highly stable and catalytically active metal complexes due to their strong sigma-donor properties. Ma *et al.* screened five different water soluble NHC-Pd complexes for SM coupling of *N-tert*-butoxycarbonyl-4-iodo-*L*-phenylalanine with 3-(hydroxymethyl)phenylboronic acid in phosphate buffer, pH 8 at 37 °C [25]. The best catalysts proved to be the least bulky monodentate carbenes **2**, achieving >95% conversion in 4 h with a rate of 1.1×10^{-2} $M^{-1}s^{-1}$, comparable to Staudinger ligation ($k = 2.5 \times 10^{-3}$ $M^{-1}s^{-1}$). For the coupling of a biotin or fluorophore arylboronic acid to aryl iodide labelled bovine serum albumin (BSA), conversion increased with increasing Pd loading, up to 43 eq. Pd and 85 eq. boronic acid (Figure 2). Finally, the group labelled Lys residues on the surface of HeLa cells with an aryl iodide which they then biotinylated by a SM coupling in 1h, retaining >85% cell viability at concentrations up to 200 μM Pd-NHC.

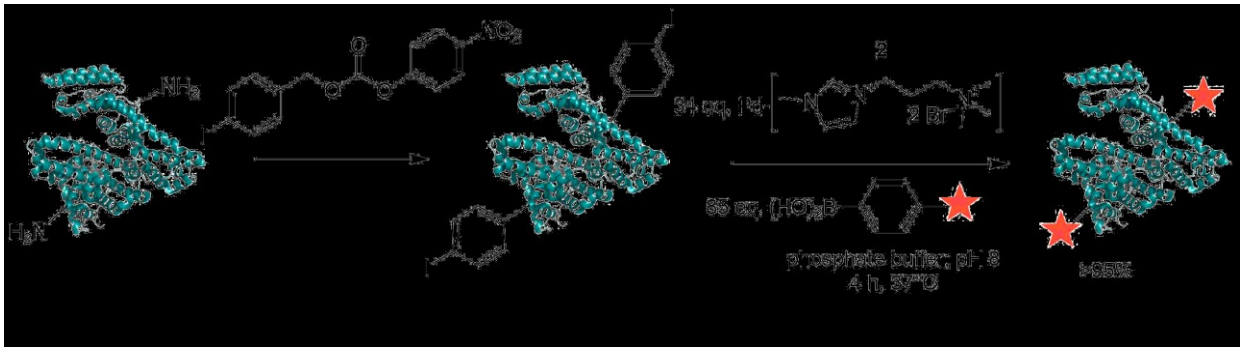

Figure 2: SM coupling of a aryl iodide modified Lys containing protein using a water soluble Pd-NHC complex [26].

Pd-protein interactions are perhaps a drawback of the complex **1**, which coordinates Pd through nitrogen. Protein surfaces contain a large number of nitrogen atoms which presumably compete for Pd binding with the ligand. NHCs coordinate Pd more strongly which should reduce the possibility of protein-Pd interactions. However, the rate of reaction with NHCs appears to be slower than the ADHP ligand.

Genetic incorporation of the unnatural amino acid *p*-iodophenylalanine (*p*iodoPhe) gives a much more attractive single step modification method than the two-step method first used by the Davis group. However while investigating SM coupling on a maltose binding protein containing *p*iodoPhe they encountered a dramatic loss in MS signal in the presence of Pd [27]. Further investigation revealed the same effect upon mixing **1** with other commonly used proteins such a myoglobin. Surprisingly, protein bound to multiple Pd ions was observed using LC-MS analysis where proteins are desalted and denatured on the column before they reach the MS source. This suggests strong non-specific Pd-protein interactions gave rise to a heterogenous mixture with multiple ionisation states thus reducing the signal intensity. The group were able to overcome this issue by scavenging excess Pd before LC-MS analysis using 3-mercaptopropionic acid. Recently Zhao *et al.* reported the SM coupling of a range of boronic acids with proteins incorporating the unnatural amino acid fluorosulfate-*L*-tyrosine (a *pseudohalide* for *p*iodoPhe) both *in vitro* and *in vivo* using **1**[28].

The only example of a SM coupling on a protein modified with an aryl boronic acid was by Brustad *et al.* who evolved a tRNA/aminoacyl-tRNA synthetase pair for the genetic incorporation of *p*-boronophenylalanine (*p*BPhe) [29]. After expression and purification of T4 Lysozyme with *p*BPhe, they were able to achieve a 30% yield of modified protein with a boron-dipyrromethene (BODIPY) iodide using $Pd(dba)_2$ in 20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS), pH 8.5 at 70 °C. The authors hypothesised that employing a more water soluble Pd source might allow for lower temperatures and higher conversion. Boronic acids are known to coordinate to serine residues so an alternate hypothesis for the observed reduced reactivity is thought to be due to the boronic acid coordinating to amino acid residues in the protein, slowing transmetallation.

2.1.2. Mizoroki-Heck (MH) reaction

Kodama *et al.* were the first to show a Heck coupling on a protein. Using a cell free expression system, they were able to genetically incorporate *p*iodoPhe into His_6-fused Ras protein [30]. Optimised conditions for labelling with a vinyl biotin reagent used DMSO (12 %) combined with $MgCl_2$ (80 mM), pH 8 tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS) buffer and Pd-triphenylphosphine-3,3′,3′′-trisulfonic acid trisodium salt (TPPTS) for 50 h at 50 °C under Ar, giving about 2% conversion (Figure 3). They proposed that DMSO opens the protein structure, giving increased access to the *p*iodoPhe residue and that >80 mM $MgCl_2$ supressed Cys alkylation, possibly by decreasing electrostatic repulsion of the negatively charged protein and catalyst. A major side reaction was the dehalogenation of Ras-32pIPhe-His, ~24%.

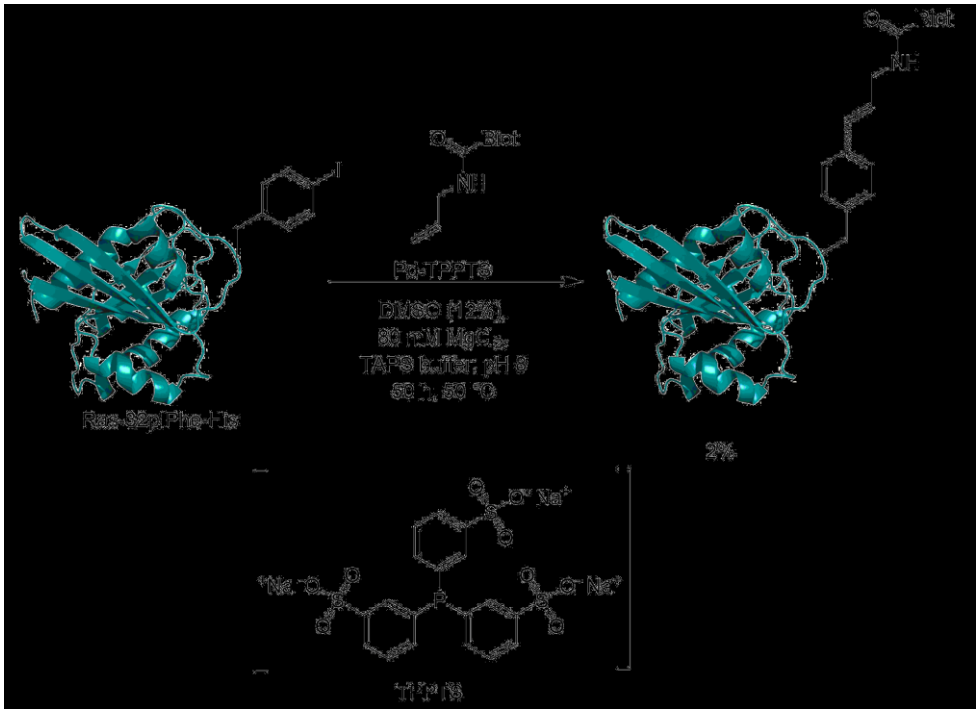

Figure 3: Biotinylation of the Ras-32pl protein containing a *p*iodoPhe residue *via* a MH coupling [30].

The group returned the following year having done a considerable amount of reaction optimisation on a short peptide in the presence of BSA [31]. Essentially this did not improve the yield or dehalogenation of the protein substrate, but they found $MgCl_2$ was essential in stopping protein precipitation in the presence of Pd, especially under aerobic conditions. It is possible that the alkene they were coupling, an allyl, was not reactive enough to achieve efficient coupling and showed more success with the Sonogashira reaction (see section 2.1.3.).

Simmons *et al.* used a pre-formed Ar-Pd-CO_2CF_3 complex, **3**, that contained the fluorophore Cy5 attached to the aryl group, to perform a MH-type coupling on lysozyme labelled with a styrene [32]. Coupling **3** to the protein in *tris*(hydroxymethyl)aminomethane (Tris) buffer at pH 8 gave 75% conversion in 4 h (Figure 4). No fluorescence was observed for unmodified proteins which showed the excellent selectivity of this reagent.

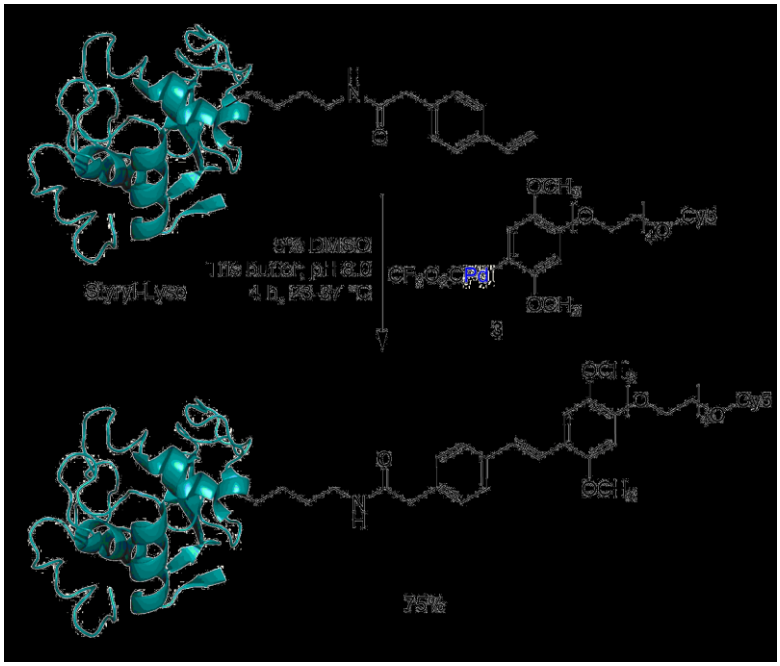

Figure 4: MH type coupling of styrene labelled Lysozyme using **3** [32].

2.1.3 Sonogashira reaction

Following on from their work on the MH reaction, Kodama *et al.* investigated the Sonogashira coupling of their *p*iodoPhe labelled His6-Ras protein [31]. Once again, conditions for the Sonogashira coupling were screened using a short peptide in the presence of BSA and Pd-TPPTS to propargyl biotin. The mild reducing agent ascorbic acid enhanced the reaction, whereas dithiothreitol completely inhibited product formation and tris(2-carboxyethyl)phosphine (TCEP) had a mild inhibitory effect. Optimized conditions show complex reaction mixtures, with the best overall yield being 25% biotinylated product and 13% dehalogenation (Figure 5).

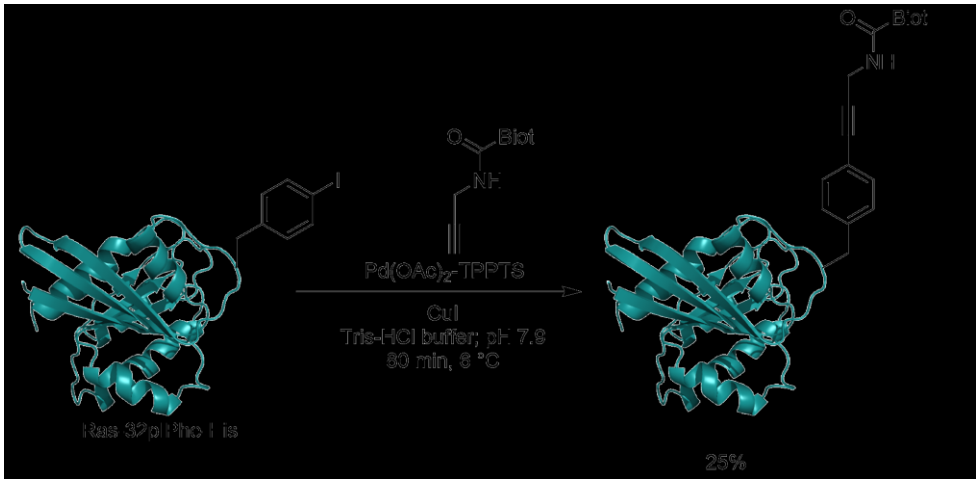

Figure 5: Sonogashira coupling of *p*IodoPhe incorporated Ras protein with a biotin alkyne [31].

Inspired by the activity of ADHP as a ligand in SM coupling on proteins, Li *et al.* tried to use it in a Sonogashira coupling of fluorescein iodide and an homopropargyl glycine (HPG) containing peptide, but no product was detected [33]. A screen of other pyrimidine containing ligands showed the 2-*N*-dimethylamino-4,6-dihydroxypyrimidine (DMADHP), gave 91% conversion in 40 minutes using 30 mol% catalyst. The reaction was achieved *in vivo*, simply by addition of the Pd complex, **4**, and fluorescein iodide to *E. coli* cells expressing HPG-ubiquitin (Figure 6). Hauke *et al.* were able to use

similar conditions to demonstrate coupling to an aryl iodide-labelled lipoic acid ligase [34] and Li *et al.* demonstrated coupling on the cell surface of mammalian cells [35].

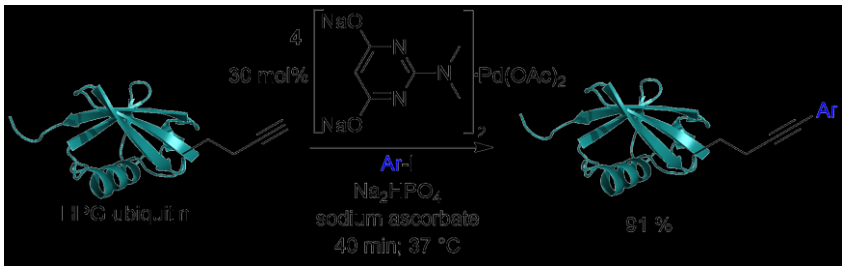

Figure 6: *In vitro* and *in vivo* copper free Sonogashira coupling of alkyne labelled ubiquitin with Aryl-iodides using **4** [33].

Further *in vivo* studies were reported by Li *et al*. including the intracellular Pd-mediated Sonogashira coupling in *E.coli* and compared to CuAAC chemistry [36]. The group screened a range of Pd sources and ligands for the *in vitro* coupling between an alkyne labelled green fluorescent protein (GFP) and *p*iodoPhe tagged fluorophore, in phosphate buffered saline (PBS) with sodium ascorbate in air. Surprisingly ligand-free reactions with only $Pd(NO_3)_2$ or Na_2PdCl_4 yielded the highest conversions, 95% and 90% respectively in 40 min using 10 eq. Pd, a similar rate to that of comparable CuAAC reaction (Figure 7). Reactions where the protein was labelled with the alkyne proceeded faster than where proteins were labelled with an aryl iodide. The authors also report that the inclusion of a short PEG linker between the aryl iodide and dye increased the rate of reaction, suggesting the PEG linker stabilises the Pd reducing its aggregation to form Pd-black. Interestingly the Davis group also noticed a marked increase in reactivity of Pd for the Suzuki coupling in the presence of PEG [21]. For the Sonogashira coupling, it is proposed that the addition of Pd(II) salts with sodium ascorbate causes the formation of Pd(0) nanoparticles which are the catalytically active species. Cellular Pd uptake was much greater on the addition of $Pd(NO_3)_2$ compared to using the negatively charged Pd-$[DMADHP]_2$ complexes [36].

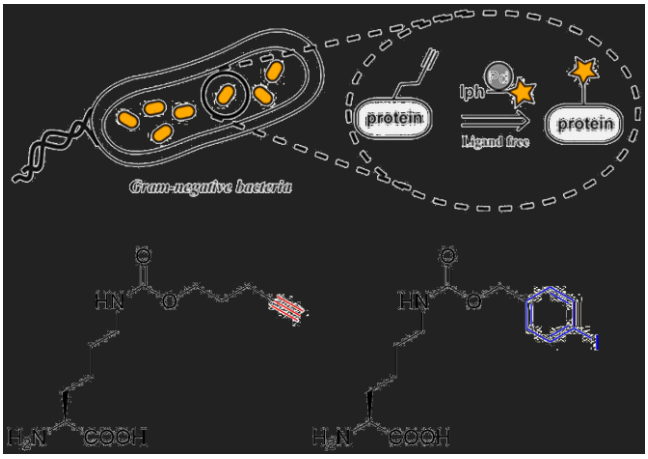

Figure 7: Ligand free i*n vivo* Pd catalysed Sonogashira coupling. Adapted from ref. [36].

The stability of a labelled GFP protein was also monitored under CuAAC or Sonogashira coupling conditions by measuring fluorescence (denatured GFP has less fluorescence) [37]. Under the conditions Cu(I) was found to cause a much greater loss of fluorescence than $Pd(NO_3)_2$. Moreover, 200 µM $Pd(NO_3)_2$ caused no change in cell viability or proliferation in *E.coli* or *Shigella* in contrast to a marked decrease using Cu(I). Finally, they were able to fluorescently label a type-III Secretion (T3S) toxin-OspF protein in *Shigella* cells using a Sonogashira coupling.

As seen with the MH reaction, preformed palladacyles can also be used to promote the Sonogashira coupling of homopropargyl glycine (HPG) labelled ubiquitin. Using the amide form **5a,** where the carbonyl of the amide acts as a directing group to Pd, Cheng *et al.* reported almost complete conversion was observed in 30 minutes using 25 eq. of Pd in PBS at 37 °C [38] (Figure 8). Other substituted heterocycles were slower to react (up to 4 h). By changing the amide to carbamate **5b**, reactions proceeded in 3 minutes using only 4 eq. of reagent giving an astonishing rate constant of 19 700 $M^{-1}s^{-1}$ (on a small molecule substrate) [39] comparable to tetrazine-transcyclocteneene coupling [40].

Figure 8: Extremely fast Sonogashira coupling to an alkyne labelled ubiquitin using a pre-formed palladacycle carbamate **5** [38][39].

2.1.4. Thioetherification (C-S Buchwald-Hartwig coupling)

Another coupling reaction that has been used in bioconjugation chemistry is thioetherification, and has recently been extensively reviewed [41].

Cysteine is a common target for protein modification reactions, due to its nucleophilicity and rare natural occurrence. Building on the work of Simmons [32] and Cheng [38], the Pentelute and Buchwald groups used preformed aryl-Pd(RuPhos) **6** complexes to perform Cys arylation reactions [42]. The reaction is very specific for Cys and occurs in 5-30 minutes using 10 eq. Pd at room temperature over a wide pH range using a variety of aryl and heteroaryl substituents. The requirement of 5% organic co-solvent (for solubility of the Pd complex) was negated by the use of the water soluble phosphine sSPhos [43]. A wide variety of Cys containing proteins have since been modified using this technique and notably an antibody drug conjugate in the presence of the reducing agent TCEP. Removing excess Pd appears problematic however, 73% of added Pd was removed after simple desalting. The use of buffer exchange and then size exclusion chromatography (SEC) (including thiopropionic acid in the buffer) improved this to remove 94% Pd but reduced the protein yield to 39% (Figure 9).

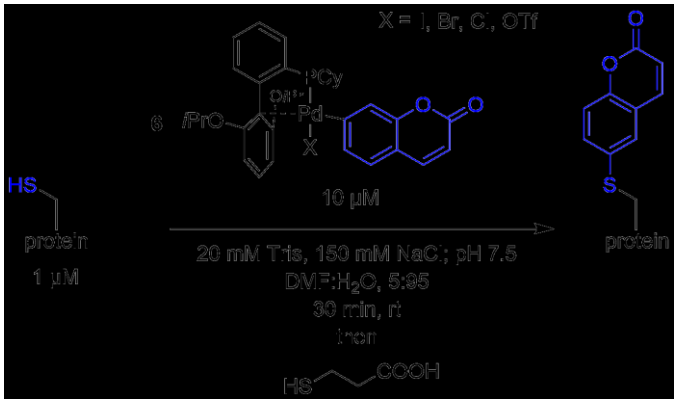

Figure 9: Cys arylation of proteins using pre-formed Pd-RuPhos reagent **6** [43].

2.2. Protein modification *via* Allylation reactions

Alongside cross coupling reactions, Pd-catalysed allylations are a major reaction class that go through a Pd-π-allyl intermediate. Tilley and Francis showed how tyrosine could react selectively with a π-allyl complex to create *O*-allyl modified proteins [44]. Using chymotrypsinogen A as a model protein, they modified a single solvent exposed tyrosine with a rhodamine labelled allylic acetate using $Pd(OAc)_2$ (0.2 eq.), TPPTS (2.4 eq.) in phosphate buffer at pH 8.5 in 45 minutes. The reaction gave about 60% conversion and interestingly did not react with a Cys residue. This was not due to the location of the Cys, which had previously been shown to be reactive with a maleimide. This is one of the few protein modification examples where Pd is used catalytically. This methodology has been used for selective modification of Tyr108 in the protein superoxide dismutase to probe changes in polarity and conformation in response to heat and changes in pH [45] and Cserép *et al*. designed a terminal alkyne labelled allyl-acetate for Tyr-conjugation and subsequent CuAAC modification, demonstrated using the protein bovine serum albumin[46].

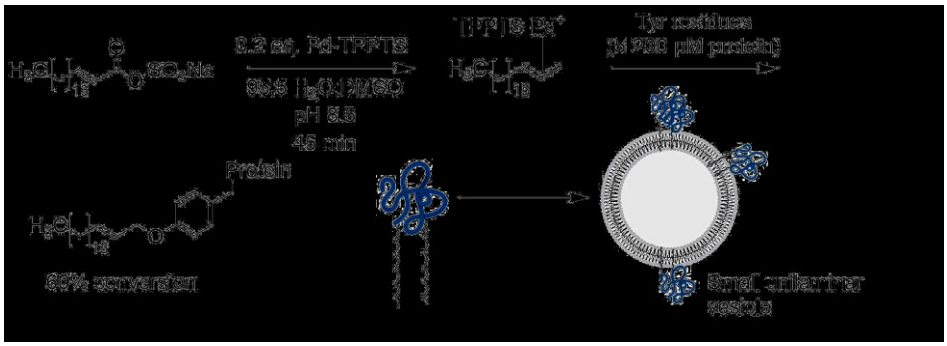

Figure 10: Modification of tyrosine using a Pd-TPPTS catalysed *O*-allylation with a C_{17} chain, forming protein small unilamellar vesicles [44].

In contrast to selective Tyr-allylation using Pd-TPPTS and allyl-acetates, the Breinbauer group found that highly selective Cys-allylation could be achieved by combination of Pd-BIPHEPHOS (BIPHEPHOS = 6,6'-[(3,3'-Di-tert-butyl-5,5'-dimethoxy-1,1'-biphenyl-2,2'-diyl)bis(oxy)]bis(dibenzo[d,f][1,3,2]dioxaphosphepin) and allylic carbamates [47]. They were able to couple a wide range of functional groups onto a peptide model and form a peptide staple between two Cys residues. Furthermore, S-allylation of mono and bis Cys mutants of ubiquitin like protein-**3**, and a Cys mutant of heat shock protein were modified using just 1.2 eq Pd per Cys in 2-4 h. However, reactions required 50% MeCN, presumably to aid solubility of the catalyst and reagents, which would not be tolerated by most proteins.

3. Palladium reactions for medical applications

Transition-metal catalysts can catalyse unique and chemoselective transformations *in vivo*, giving rise to the area of bioorthogonal organometallic (BOOM) reactions [48]. This relatively recent field has mostly focused reactions that have medical applications, e.g. decaging, which enables selective activation of drugs, dyes or proteins inside cells. In 2006 Streu and Meggers described the first biocompatible ruthenium catalysed allylcarbamate deprotection of a pro-fluorophore inside living human cells [49] and since examples have utilised other metals such as Au, Cu, Ir, Pt and Os [50][51].

Pd is an attractive metal to use however due to its broad reactivity. Heterogenous Pd(0) has perhaps shown the most success, being biologically inert and the least toxic amongst Pd species, however, successful homogeneous Pd approaches are starting to emerge.

3.1. Imaging and decaging

The first example of *in vivo* Pd-catalysed decaging was the deprotection of the pro-fluorophore, m-pro-FlCl [52][53]. Five-day-old zebrafish were incubated in E3 embryo media containing m-pro-FlCl (20 μM) for 30 min at 28 °C. After m-pro-FICl had been removed from the media, zebrafish were incubated with increasing concentrations of $PdCl_2$ for 30 min. Strong fluorescence due to Pd-catalysed depropargylation was observed. After dissection, they discovered that Pd accumulates in the brain, eyes, fin and to a smaller extent in heart and liver.

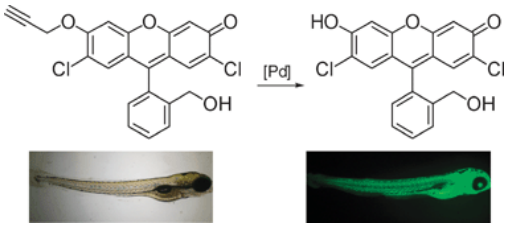

Figure 11: Decaging of m-pro-FlCl in a Zebrafish model using Pd. Reproduced from Ref. [53] with permission from The Royal Society of Chemistry.

The Bradley group were the first to use a Pd(0) microsphere *in vivo*. Pd(0) microspheres were built from amino-functionalized polystyrene (Figure 12) and improved Pd stability and cellular uptake compared to simple Pd salts and stopped them accumulating to form less reactive Pd particles [48], [54]. After 24 h, 75% of HeLa cells had taken up one or more of the Pd(0)-microspheres and showed >91% viability after 48 h. Cells treated with the Pd(0) microspheres were able to catalyse decaging of bis-N,N'-allyloxycarbonyl ($Alloc_2$) rhodamine 110 (R110), which could be observed by fluorescence of free R110. Interestingly, in the presence of glutathione (5 mM) the catalytic activity of Pd(0) microspheres was higher, with a turn over number (TON) up to 30 when both glutathione and cell extract were present. Moreover, TONs of Pd(0)-microspheres were the same in DMF, PBS, and cell lysate.

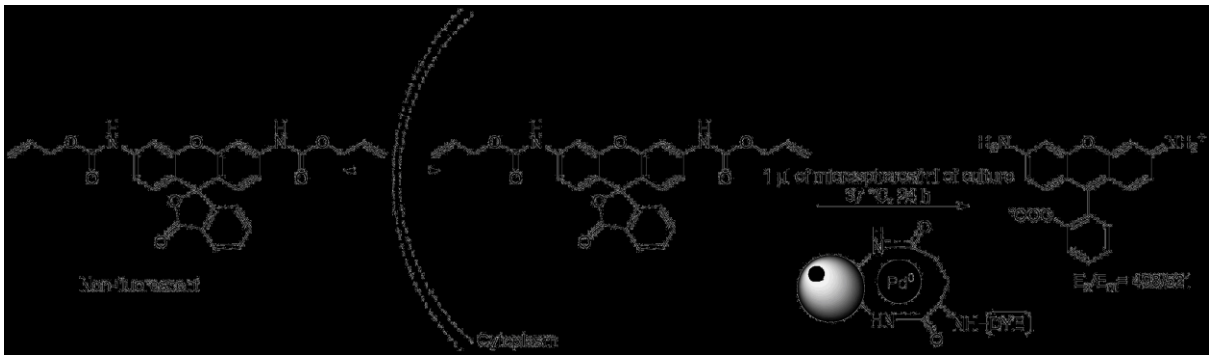

Figure 12: Synthesis of Pd(0) functionalised polystyrene microspheres and their use in intracellular de-allylation reaction chemistry [47].

Functionalisation of a Pd(0)-microsphere catalyst with the addition of a cyclic-Arg-Gly-Asp(RGD) cancer-cell targeting peptide, induced specific uptake by U87-MG glioblastoma cells was observed. This catalyst was used for *in vivo* decaging of prodrug 5-fluoro-1-propargyl-uracil (Pro-5FU) (Figure 13a)[55].

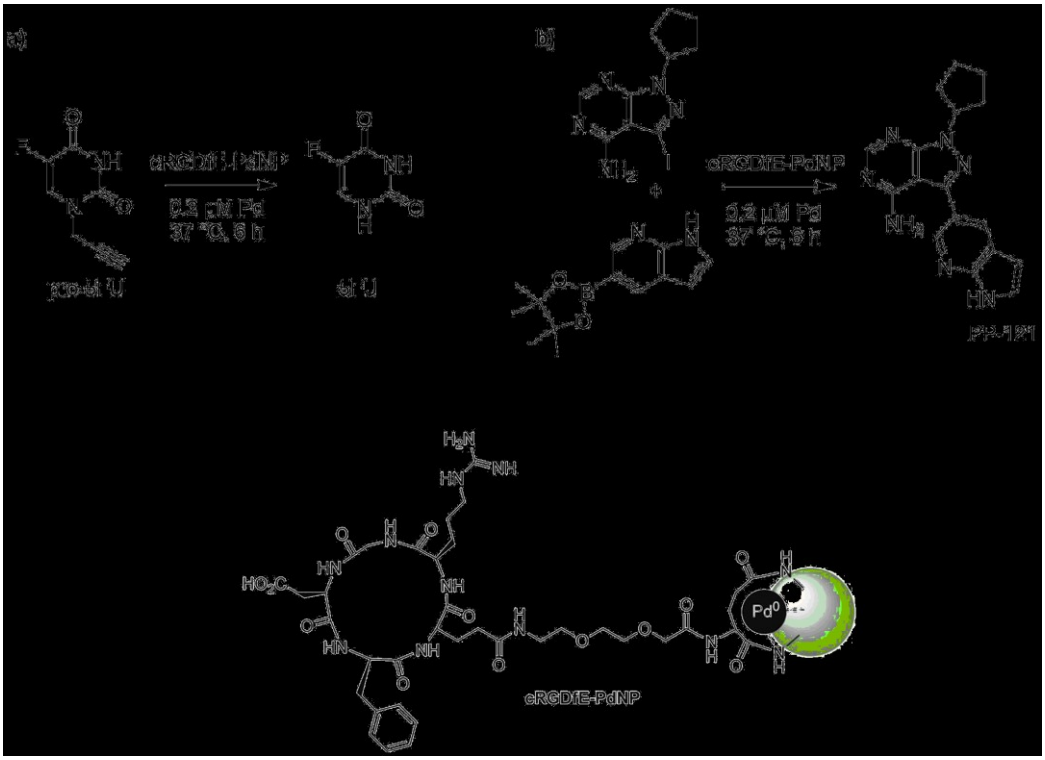

Figure 13: Pd(0)-microsphere catalyst with a cyclic-RGD cancer-cell targeting peptide (cRGDfE-PdNP) was used for; a) *in vivo* decaging of prodrug 5-fluoro-1-propargyl-uracil (Pro-5FU) and b) a SM cross-coupling reaction to form a cytotoxic drug PP-121. Reproduced from Ref. [55].

An alternative extracellular approach to molecular decaging by Unciti-Broceta and co-workers [56] used polyethylene glycol (PEG)-polystyrene resin as a Pd(0) support to give a Pd(0) resin (Pd(0)-PEG/PS) that is larger than human cells. Extracellular activation of Pro-5FU allowed 5FU to enter cells and exhibited anti-proliferative activity. The biocompatibility and catalytic abilities of Pd(0)-PEG/PS were tested in zebra fish and showed no toxicity or alteration of phenotype. Furthermore, fish surgically implanted with Pd(0)-PEG/PS that were cultured with N,N'-bis(propargyloxycarbonyl)($Proc_2$) R110 showed fluorescence only localised to the resin [56]. Further investigation by the group showed the Pd(0)-PEG/PS could be surgically implanted into tumours and used to activate a doxorubicin prodrug *in vivo* [57]. Activation of a pro-drug of the anti-tumour agent SN-38 was also possible, where the phenolic OH was protected using a 2,6-bis(propargyloxy)benzylgroup [58].

Whilst surgical insertion of Pd resins is an elegant solution to drug targeting, exosomes represent another strategy that can hijack natural biochemical communication and delivery mechanisms for targeted drug delivery. Exosomes are membrane-enclosed vesicles that are released into the extracellular space by cancer cells and it is believed they play a role in progression, angiogenesis and invasion. Efforts to introduce nanoparticles into exosomes is challenging due to the fragile nature of the vesicle, but the Unciti-Broceta group developed a strategy to form Pd(0) nanostructures inside exosomes using CO as a mild reducing agent, giving Pd-Exosome (Pd-Exo) (Figure 14). The Pd-Exo structures were able to deprotect the propargyl group of **7** and a prodrug form of Panobinostat **9**. Furthermore the Pd-Exo derived from A549 cells, were up-taken by A549 cells and indicated a preferential tropism for their progenitor cells [59].

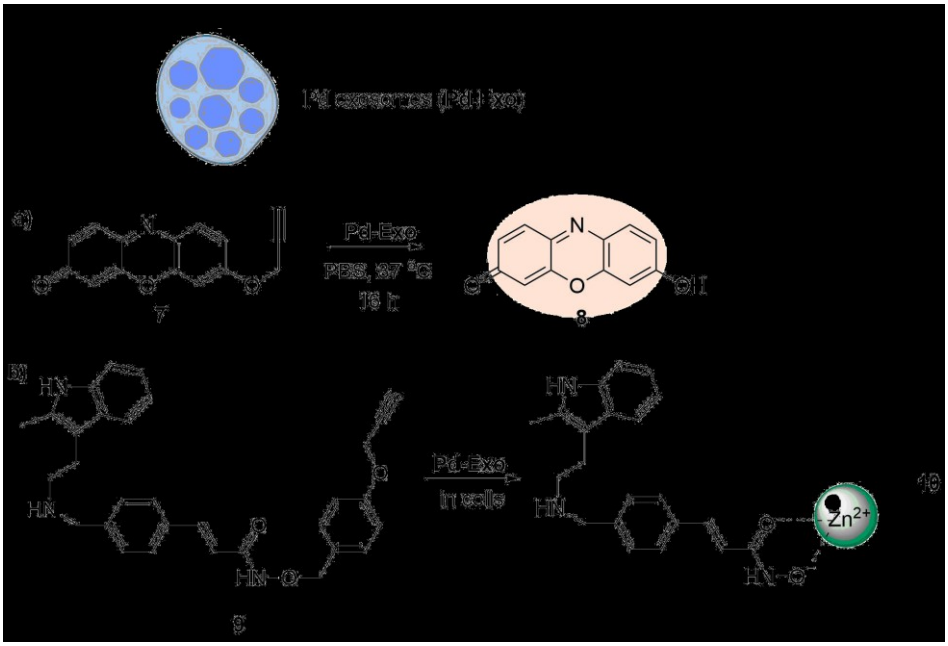

Figure 14: Formation of Pd(0) exosomes by reduction of Pd(II) with CO. Pd(0)-exosomes were active for the depropargylation of fluorophore **7** and prodrug **9** [59].

The first steps towards homogeneous Pd decaging in PBS and HeLa cells were made by the Chen group in 2014 [9]. They described a Pd-mediated deprotection reaction to activate a protein with caged lysine residue *in vivo*. The group used simple and single component palladium salts that could enter bacterial cells more easily and were less toxic than complexes containing phosphine ligands [37]. A number of classic air-stable Pd salts were screened in PBS for depropargylation of $Proc_2$-R110 and Proc-lysine [9]. All the Pd salts tested catalysed the conversion, with $Pd(dba)_2$ and $[Pd(allyl)Cl]_2$ being the most successful. Interestingly, water-soluble ligands TPPTS, ADHP and DMADHP decreased the reactivity for Pd both salts. $Pd(dba)_2$ and $[Pd(allyl)Cl]_2$ at 10 µM exhibited almost no toxicity in HeLa cells and when protein OspF-K134-ProcLys was expressed in HeLa cells, Pd triggered decaging was estimated to 28 % conversion.

In 2017 the Weissleder group observed that classic Pd precursors show low activity for decaging of $Alloc_2$-R110 in cell culture media [60]. The use of the electron-poor phosphine ligand, tri(2-furyl)phosphine (TFP), improved activity compared to the electron rich phosphine TPPTS in cell culture medium, but activity in fetal bovine serum or whole tumour homogenate was much lower. Encapsulation of $PdCl_2(TFP)_2$ in poly(lactic-co-glycolic acid) (PLGA) and PLGA-PEG nanoparticles improved activity by 16-fold compared to unencapsulated $PdCl_2(TFP)_2$ in cell lysates. The encapsulated Pd catalyst was safely delivered into mice, where it activated an alloc-Doxorubicin prodrug through allylcarbamate cleavage. Using the same nanoparticles the group showed an alternative approach to decaging for *in vivo* imaging by using MH coupling to synthesise a coumarin fluorophore inside the cell.

The Mascareñas group investigated a range of Pd complexes (**11**-**16**) that varied in charge and solubility in depropargylation reactions and demonstrates how homogenous Pd complexes that are most active in water or buffer are not suitable for *in vivo* reactions (Figure 15) [61]. Complexes with phosphine ligands (**13**-**16**) performed depropargylation in cell lysates in contrast to **11**, **12**, due to phosphine ligands giving more stable Pd complexes, resistant to ligand competition in the cellular environment. Interestingly, the use of additives such as sodium ascorbate and glutathione improved the yields obtained with **14** and **16** in cell lysates. Complexes **13-16** showed better cell permeability

than **11**, **12** and are essentially non-toxic below 50 µM in HeLa cells. The catalytic abilities *in vivo* were tested inside HeLa cells using R110; **11**, **12** raised only very low intracellular fluorescence, whilst with **13-15** strong intracellular green fluorescence was observed. The estimated TON values (based on intracellular Pd concentration by inductively coupled plasma mass spectrometry) were for **14**, an average TON of 10, whereas for **15** TON >5. Phosphine hydrophobic ligands act as targeting groups for mitochondria and the complex **16** had higher mitochondrial accumulation than other Pd complexes.

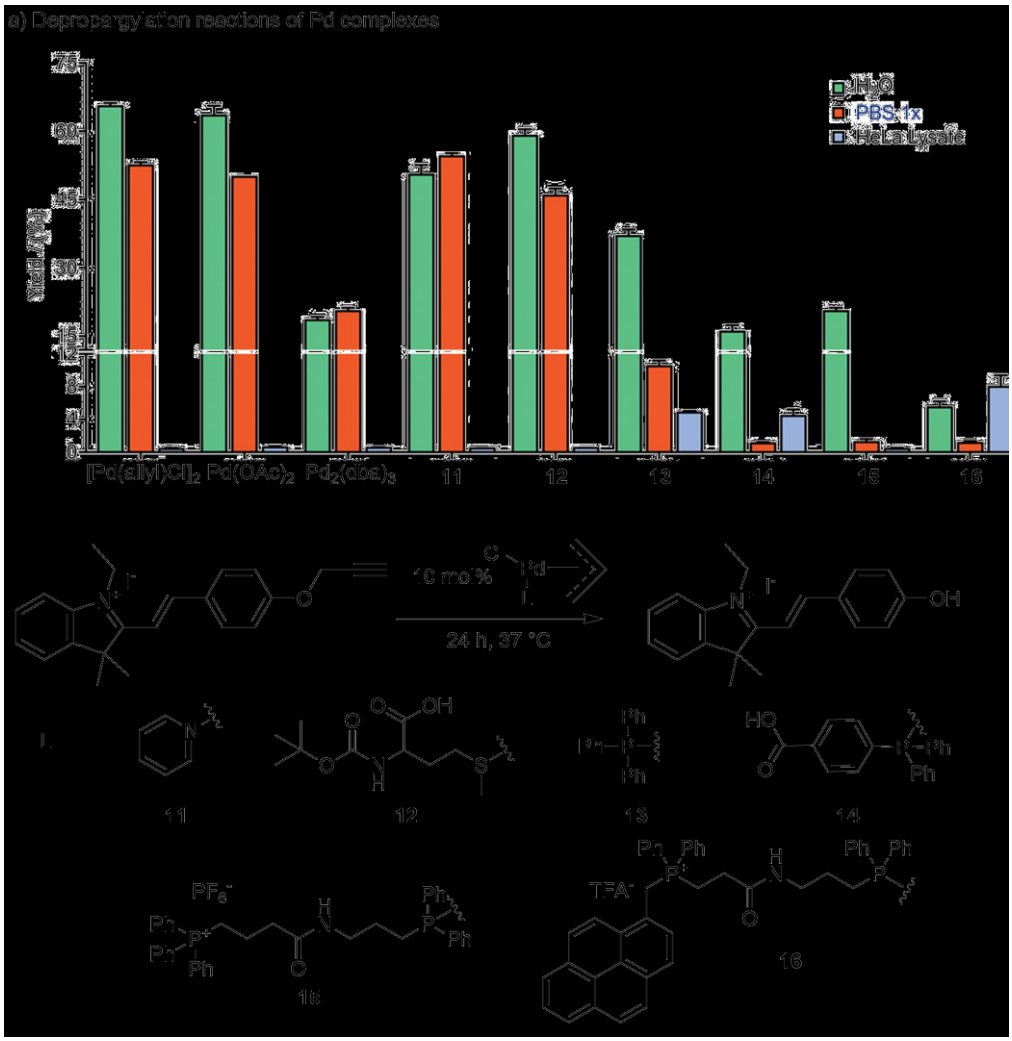

Figure 15: Depropargylation of a pro-fluorophore using homogenous Pd catalysts. a) Comparison of depropargylation in different media using different Pd complexes, H_2O (green, left), PBS (orange, middle) and HeLa cell lysate (blue, right); b) Pd localisation to mitochondria using phosphines and phosphonium salts. Adapted from Ref. [61] https://pubs.acs.org/doi/10.1021/acscatal.8b01606.

NHC ligands are another commonly used ligand for Pd. The Bradley group used a homogeneous NHC Pd catalyst coupled to a peptide for the catalytic decaging of R110 *in vivo* (Figure 16) [62]. By combining the NHC with cationic tri-lysine peptides **17**, Pd-complexes with cell permeability were obtained. The complexes displayed high biocompatibility, with no cytotoxicity observed even at 200 µM. Cells incubated with the peptide complex and R110 showed localised fluorescence in the cytoplasm and nucleus, indicating the cellular uptake of the complex.

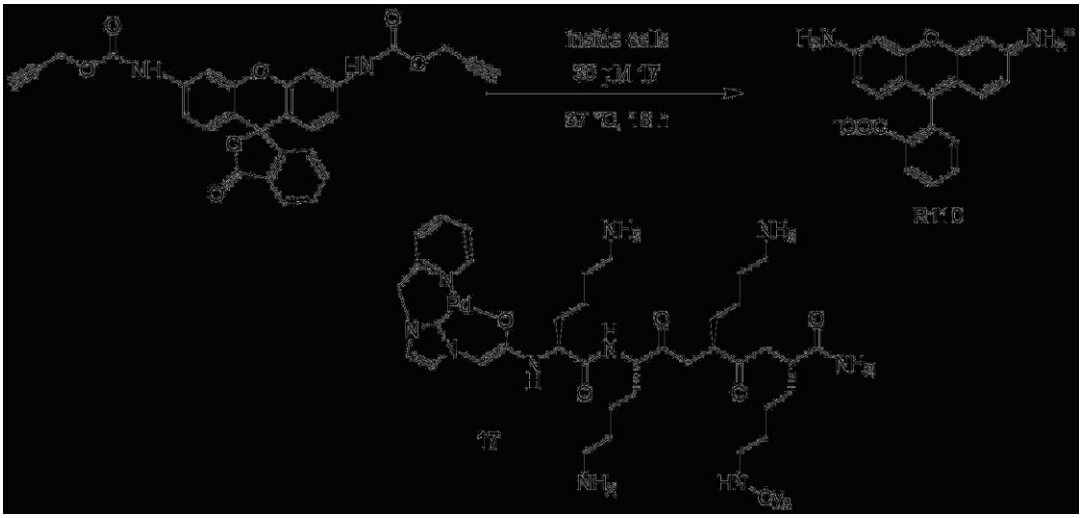

Figure 16: The use of a Pd-NHC peptide **17** for intracellular deprotection of (proc$_2$)R110 [62].

A peptide fragment from the DNA binding protein GCN4 was investigated by the Mascareñas group after they discovered that introduction of a bis-histidine i-i+4 mutation and subsequent binding of Pd(en)Cl_2 allowed the stapled peptide to enter HeLa cells and bind into the major groove of DNA [63]. Peptides bound to Pd(COD)Cl_2 and Pd(Bpy)Cl_2 were also found to enter HeLa cells and were screened for reactivity for the deprotection of pro fluorophore HBPTQ (Figure 17) [64]. The peptide complex formed on addition of Pd(COD)Cl_2 showed *in vivo* deprotection of HBPTQ, however, no deprotection was observed on addition of Pd(Bpy)Cl_2 and Pd(en)Cl_2 precursors.

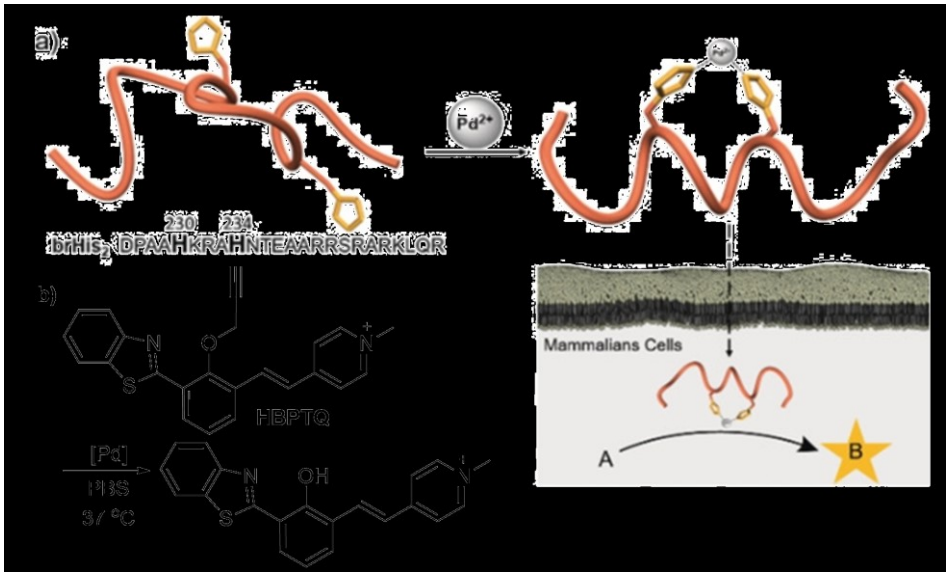

Figure 17: Peptides bound to Pd-COD entered HeLa cells and showed *in vivo* deprotection of HBPTQ. Adapted from Ref. [64].

To this point deprotection strategies have focused on allyl/propargyl ethers and carbamates, however the Bernardes group designed a thioether-directed propargyl carbamate. They demonstrated the linker could be deprotected *in vivo* using Pd(COD)Cl_2 using the same or lower Pd concentrations as required when using Proc-Lys. The group also showed they could modify their thioether to include a Cys reactive group. After modification of a nanobody with their cleavable prodrug linker they were able deprotect it with Pd, suggesting a new targeting strategy [65].

3.2. Pd catalysis *in vivo*: *in situ* drug synthesis

The Bradley group were the first to report a SM coupling inside a mammalian cell [54] using polystyrene Pd(0)microsphere. Cells were incubated with a pro-fluorophore mono-triflate and a mitochondria targeting alkylaminophenylboronate. After fixing and imaging the cells by confocal microscopy, fluorescence was observed in the cell mitochondria confirming SM coupling had occurred. The group later used the same polystyrene support but coupled to a cancer cell targeting peptide cyclic-RGD to perform an intracellular SM coupling to form the cytotoxic agent PP-121 *in situ*. Cell viability was found to decrease only when Pd nanosphere and both coupling partners of the SM coupling were present (Figure 13b) [55].

4. Enzyme-Pd 'one-pot' reactions

Synthetic chemists often look to improving the sustainability and efficiency of transformations by combining multiple steps in one-pot or telescoping reactions together. Therefore, there is an interest in combining chemo- and enzymatic reactions in one pot. This simple idea can be extremely challenging as; i) normally conditions for one reaction do not favour the other; ii) it is difficult to achieve the selectivity between reagents, intermediates and products and; iii) the metal/protein mixture can inhibit one another. This section will aim to highlight some of the methods used to overcome these issues.

To be successful, almost all reactions involving small molecule homogeneous Pd and enzyme transformations that are 'one-pot' are carried out in a two-step process, allowing for changes in temperature, pH and solvent for each step. Burda *et al.* investigated a $Pd(PPh_3)_2Cl_2$ catalysed SM coupling followed by an ketone reduction by an alcohol dehydrogenase (ADH) to give enantiopure biarylethyl alcohols as shown in the general scheme in Figure 18 [66]. Despite both reactions working well independently, it was found that excess PPh_3 and $PhB(OH_2)_2$ caused significant inhibition of the ADH, while $PdCl_2(PPh_3)_2$ had a much smaller inhibitory effect. By removing excess PPh_3 from the reaction and not using excess boronic acid, good yields (>90%) could be obtained for the ADH reduction. In a later paper they find that a combination of $PdCl_2$ and TPPTS is compatible with ADH reductions [67].

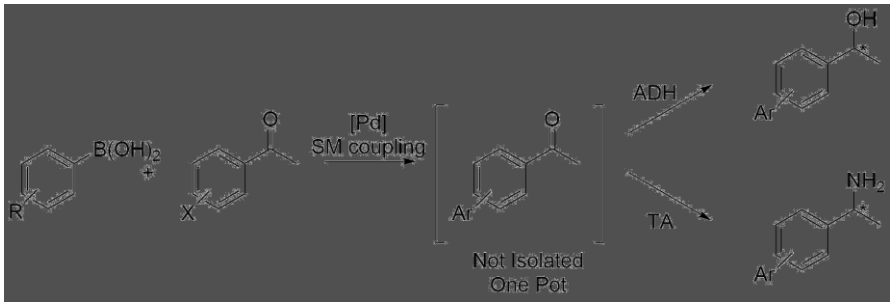

Figure 18: A general scheme for a one pot, 2 step synthesis of chiral biarylethyl alcohols and amines. A SM coupling is followed by ketone reduction using an alcohol dehydrogenase (ADH) or transamination using a transaminase (TA).

Enzymes are an extremely powerful and simple way to introduce chiral amines into organic molecules. The Turner and Flitsch groups used phenylalanine ammonia lyase or D-amino acid dehydrogenase to synthesise both *S* and *R* enantiomers of 4-bromophenyl alanine respectively, which after Boc-protection was coupled with an arylboronic acid using a SM reaction in a one pot, three step process [68]. $PdCl_2MeCN_2$ was found to be more active than complex **1** and use of a microwave reactor gave overall yields of up to 70%.

The use of designer surfactants such as D-α-tocopherol polyethylene glycol succinate (TPGS)-750-M has been shown by the Lipshutz group to enhance rates of synthetic and enzymatic reactions in water using micelles. They have also combined metal catalysed reactions using Pd, Cu, Rh, Fe or Au, with ketone reductions using an ADH in a one-pot, two-step process [69]. The micelles are believed to act as substrate 'reservoirs', reducing interactions/inhibition of catalysts and reagents with enzymes.

The Turner group also demonstrated the benefit of TPGS-750-M for aqueous Buchwald-Hartwig amination as shown in Figure 19. The group used a range of enzymes to synthesise aliphatic chiral amines which were then used as substrates for Buchwald-Hartwig amination [70]. Optimised conditions showed $[PdCl(allyl)]_2$ and the phosphine ligand, tBuXPhos, with TPGS-750-M and 1M NH_4Cl gave >90% conversion with both purified enzyme and filtered cell free extract at 50 °C, although additional NaOH was required for the cross-coupling, meaning this is still a two-step protocol.

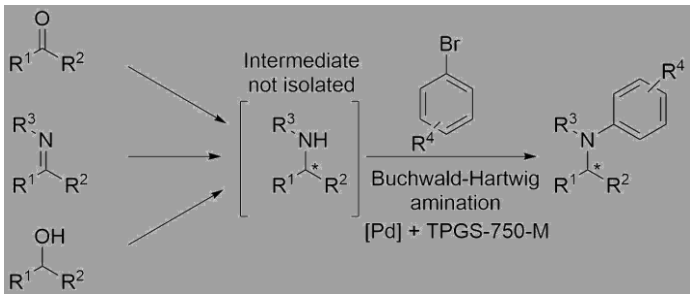

Figure 19: Synthesis of chiral N-aryl amines using TA, imine reductases or hydrogen borrowing enzymes to get chiral amines, substrates for the Buchwald-Hartwig amination [70].

The use of organic solvents is tolerated by some enzymes, such as the engineered transaminase from *Asperguillus fumigatus* employed by the Bornschauer group [71]. Similar to the scheme in Figure 18, taking the crude reaction mixture of a ligand free $PdCl_2$ catalysed SM coupling of 5-bromo-3-acetyl-pyridine to phenylboronic acid in 50% DMF, after adjusting pH and addition of isopropyl amine and pyridoxal phosphate, the solution was passed slowly through a column filled with immobilised transaminase (TA) giving a biaryl chiral amine product in 41% overall yield. Deep eutectic solvents have also been applied to the same transformation by Paris *et al.* to increase the concentration of aqueous reactions [72]. Using a mix of choline chloride and glycerol in phosphate buffer, the SM reaction was performed at 200 mM and transamination at 25 mM.

ADH has also featured in an alternative approach to chiral alcohols using a Wacker-Tsuji oxidation of styrene to give acetophenone which was then reduced by an ADH [15]. Both reactions worked well individually, however addition of enzyme to the Pd reaction mixture gave extremely low enzyme turnover. Curiously, addition of each component of the Pd catalysed reaction to the ADH reduction individually did not significantly reduce conversion. Enzyme activity in the reaction mixture was recovered by addition of metal binding ligands, such as 2,2-bipyridine or ethylenediaminetetraacetic acid before addition of the enzyme, suggesting a Pd species was responsible for enzyme deactivation.

Mekmouche *et al*. described a true coupled oxidation system using a laccase enzyme (LAC3) and Pd(II) catalyst. LAC3 is an oxidative enzyme with four bound Cu ions and was found to oxidise Pd(0) complexes to Pd(II) using O_2 thus turning over the Pd catalyst [73]. The reaction discussed here is the oxidation of a model compound of lignin (veratryl alcohol) into veratryl aldehyde (Figure 20). They obtained reasonable catalytic activity under mild conditions (33 TON, 1.4 h^{-1}), however this decreased when the LAC3 concentration was >5 µM, which the authors attribute to formation of stable protein-Pd interactions. The phenol oxidase activity of LAC3 was not affected by presence of Pd, suggesting loss of oxidative activity is through Pd poisoning.

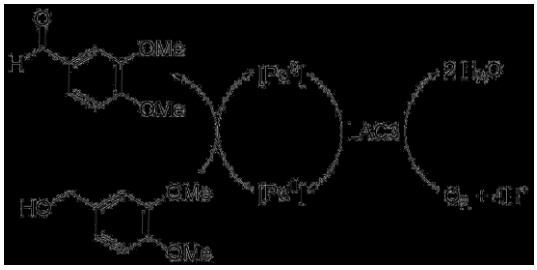

Figure 20: A coupled Pd-enzyme system where Pd^{II} oxidises veratryl alcohol to veratryl aldehyde. The resulting Pd^{0} is reoxidised by the Cu containing LAC3 enzyme, using O_2 as oxidant [73].

Some examples of one-pot, two-step heterogenous Pd catalysis and enzyme catalysis exist [74], however the benefit of this coupled process is much less obvious given that heterogeneous catalysts can be easily removed from solution by centrifugation or filtration before addition of an enzyme. One interesting example, where a clear benefit was shown by having the heterogeneous catalyst in contact with biological media was reported by the Balskus group. They demonstrated alkene hydrogenation using the Pd Royer catalyst [2.44 % palladium on polyethyleneimine (PEI)/silica gel] in the presence of *E. coli* cells genetically engineered to produce hydrogen gas from glucose. 2-Hexenedioic acid and *Z,Z*-muconic acid were converted in 18 h at 37 °C into adipic acid (TON of 12.5), an important industrial chemical (Figure 21). Other heterogeneous Pd/Pt catalysts were screened such as platinum (IV) oxide, platinum on calcium carbonate, Pd on barium sulfate, $Pd(OH)_2$ on carbon, Pd on silica powder, Pd on activated carbon and Pd on alumina, however these catalysts exhibited little to no activity. It is suggested that the superior performance of the Royer catalyst is due to ability of *E. coli* to adsorb onto PEI, which increases catalyst-cell proximity and lower activity was observed when *E.coli* was separated from catalyst in a dialysis bag. The catalyst didn't affect the viability of the bacterial cells and along other data suggests that the reaction takes place outside the cells [75].

Figure 21: An example of a biocompatible chemical reaction, H_2 produced by a modified *E. coli* using glucose is used for the extracellular reduction of alkenes on Pd Royer catalyst [75].

Pd-catalysis has been shown to be a biocompatible chemistry, and used to show that non-enzymatic catalysts can interface with enzymatic pathways [76]. This is an exciting area, however the fact that many examples require two-step processes is still a major limitation. The ideal situation for a coupled Pd/enzyme reaction would be where a product from a reversible enzyme reaction is then reacted by Pd, driving enzymatic turnover in favour of the products.

5. Hybrid Pd catalysts

The previous sections have focused on Pd-catalysed reactions being used in a biological setting or in combination with enzymes. In this section, the focus is on the combination of Pd complexes with bioarchitecture to obtain novel biohybrid catalysts. By reaction class we will cover artificial metalloenzymes and bio-supported nanoparticles, Pd and peptide ligands have been excluded and are covered in previous reviews [77][78]. Binding Pd to proteins can protect the metal from the

environment by the chiral protein scaffold, which could bring enantiomeric excess to the Pd catalysed reactions or facilitate the selectivity of reactions.

5.1 Pd-catalysed cross coupling reactions

5.1.1. Suzuki-Miyaura couplings

The use of proteins as scaffolds for both homogeneous and heterogeneous catalysts is exemplified by studies looking at biohybrid catalysts for SM couplings. Prastaro *et al.* described the formation of Pd nanoparticles encapsulated in an encapsulin *Te*Dps (*Thermosynechoccus elongatus* DNA binding protein from starved cells), a thermostable protein from the ferritin family [79]. The palladium nanoparticles were constructed by mixing K_2PdCl_4 with the protein, followed by reduction with $NaBH_4$. Transmission electron microscopy showed the nanoparticles were approximately 3.5 µm in diameter and a small section of Pd was exposed on the surface of the protein. The group screened the Pd-*Te*Dps for the SM coupling of 4-iodobenzoic acid and 2-tolylboronic acid in a range of buffers and pH at 100 ^{o}C. Tris buffer was found to give higher turnovers than $NaHCO_3$/NaOH or *N*-cyclohexyl-3-aminopropanesulfonic acid buffer, with the best turnovers gained at pH 8.9 and optimum turnover of 22250 over 48 h. When the group attempted to recycle the catalyst, the second use gave 81% conversion, but the third cycle gave only trace amounts. The authors propose the Pd might be leaching from the protein in each run and this may be the catalytically active species, rather than the designed hybrid catalysts.

Abe *et al.* investigated the binding of Pd to apo-Ferritin (apo-Fr), an iron storage protein composed of 24 subunits, with an interior space of 8 nm [80]. Treatment of apo-Fr from horse liver with 100 eq. [Pd(allyl)Cl_2] in Tris/HCl pH 8.0 and acetonitrile (9%) gave a complex containing 96 Pd ions per apo-Fr after purification by SEC. A crystal structure of the protein showed two different binding sites per monomer, both of which coordinate a dinuclear Pd(allyl) complex (Figure 22). Point mutations to the ligands co-ordinating the palladium could be made allowing formation of a trinuclear Pd complex. For the SM coupling of 4-iodoaniline and phenylboronic acid in water and NaCl (0.15 M) at 50 ^{o}C, the wild type (Pd-allyl)-apo-Fr was found to be most active, with TOF of 3500±400 $molh^{-1}$ (TON 42000). The authors do not describe any attempts to optimise conditions.

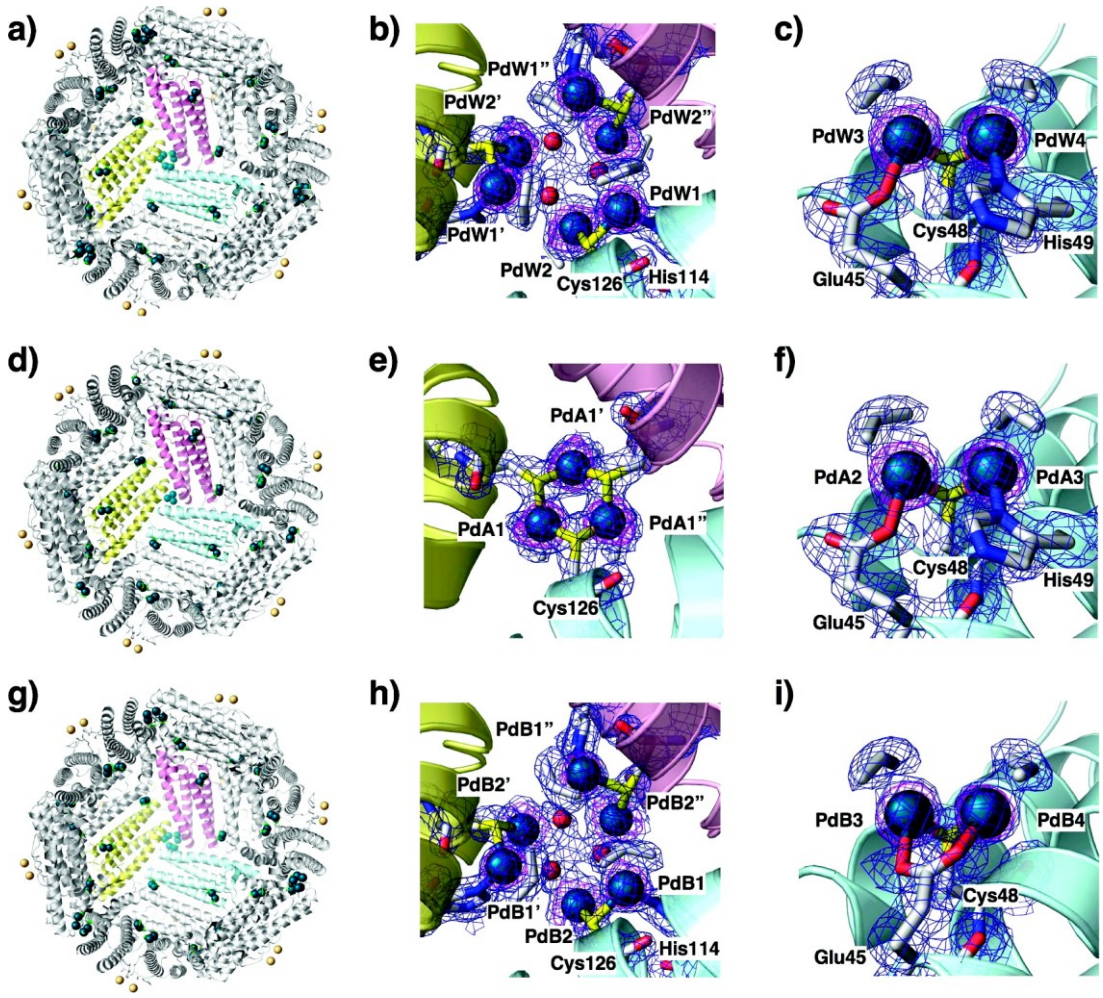

Figure 22: A crystal structure of Pd(allyl)-apo-Fr. The interior structures of a-c) dinuclear Pd(allyl)-apo-Fr, d-e) trinuclear Pd(allyl)-apo-H114AFr, and g-i) dinuclear Pd(allyl)-apo-H49AFr. Adapted from Ref. [80].

An alternative approach to Pd nanoparticle formation was taken using the lipase CalB, which was found by Filice *et al.* to act as a reducing agent for nanoparticle formation, a stabilizing and supporting agent (avoiding nanoparticle aggregation) and a biocatalyst [81]. Addition of $Pd(OAc)_2$ and to CalB with either MeOH or THF co-solvent, caused the protein to aggregate with the formation of 1.3 nm nanoparticles embedded in the protein 'net' giving CalB-PdNP at 23%(w/w). The SM coupling of phenylboronic acid and phenylbromide or iodide using CalB-PdNP in H_2O went to 50 and 55% conversion in the presence of NaOH at 50 ^oC respectively over 24 h [82]. The hydrolytic activity of the solid CalB-PdNP for the hydrolysis of 4-nitrophenolbutyrate was found to be about half that ofsoluble CalB. Whilst the Pd is to some extent reducing the activity of the natural enzymes, it is clear that some activity does remain, suggesting that Pd catalysts and enzymes can be successfully combined for multistep catalysis. Addition of the phase transfer catalyst tetrabutylammonium chloride gave a significant rate enhancement for the aryl bromide, giving 99% conversion in 2.5 h (TON = 3876, TOF = 1550 h^{-1}) and the aggregate could be reused five times without loss of activity. The group reported SM coupling between bromobenzene and phenylboronic acid in another lipase Pd-aggregate, giving 70% conversion over 24 h (TOF = 0.43 h^{-1}, TON = 10.3) [12].

The Ward group described a palladium artificial metalloenzyme, which could perform an enantioselective SM reaction [83]. The group screened a variety of Pd phosphine complexes and a Pd-

NHC complex, all tethered to biotin. Biotin has an extremely high affinity for the protein streptavidin (SAv) and when biotin-Pd compounds are mixed with SAv, biotin binds to SAv, leaving the metal complex in a defined protein cleft. Sodium hydroxide in DMSO:water (1:9) gave a good compromise between conversion and a competing protonolysis side reaction of the boronic acid for the coupling of 1-iodonapthalene and 2-methoxy-1-napthaleneboronic acid (Figure 23). Phosphine ligands (**18-20**) were found to outperform NHC ligands and after screening several point mutations in SAv, product was obtained in 80% enantiomeric excess (ee) with 160 TON. The group then showed that they could expand their substrate scope to include other aromatic substituents, although none improved enantioselectivity or TON.

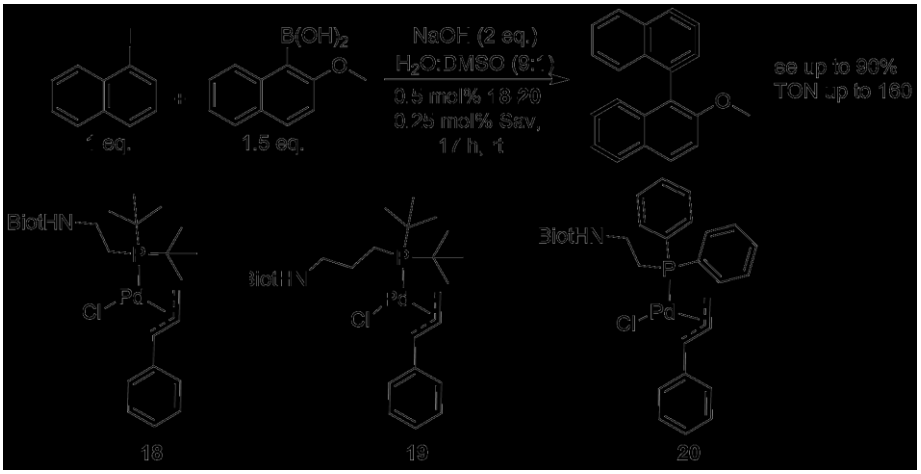

Figure 23: A Palladium SAv based biohybrid catalyst, or 'Suzukiase' enzyme which can perform enantioselective SM reaction of biaryl compounds. Selectivity for *R* or *S* mutants could be selected by mutagenesis of the protein scaffold [83].

5.1.2. Heck reaction

Lipases are a class of enzyme that are known to be remarkably stable in the presence of organic solvent and high temperatures, and have thus provided an attractive scaffold for biohybrid catalysts. The Palomo group reported the preparation of a protein-Pd aggregate using a lipase from *Geobacillus thermocatenulatus* (GTL) [12]. Curiously, the aggregate only formed after genetic mutation to incorporate a Cys (GTL-A193C) and subsequent ligation of Cys with a short phenylalanine containing peptide, giving an aggregate containing 40% (w/w) Pd. The authors suggest that ligation of the short peptide must create a binding site ideal for; a) binding Pd(II) ions; b) reducing Pd(II) to Pd(0) and c) binding Pd(0) NPs. MH coupling of ethyl acrylate and iodobenzene was optimised to give TON of 78.5 and TOF of 3.3 h^{-1} in 9:1 DMF/H_2O at 65 ^oC using 1.5 eq. of NEt_3 as base. Using 3:1 DMF/H_2O they achieved slightly lower TOF/TON, but were able to re-use the catalyst 5 times with 95% activity on the last use.

Filice *et al.* used the active site of lipase from CAL-B immobilised on Sephadex resin, to ligate a Pd-pincer complex **21** through phosphoester hydrolysis [84]. The artificial metalloenzyme showed good activity in 100% DMF at 120 ^oC (TOF = 230 h^{-1}) or in 3:1 DMF:H_2O at 70 ^oC (TOF = 142 h^{-1}) (Figure 24). They were able to further stabilise the enzyme by functionalising the resin with C8 chains after immobilisation of the enzyme. Use of the support gave TOF of 153 h^{-1} at 70 ^oC and could be used for 2 cycles, maintaining 70% activity. They were also able to demonstrate an enantioselective MH reaction between phenyl iodide and 2,3-dihydrofuran giving 97% ee.

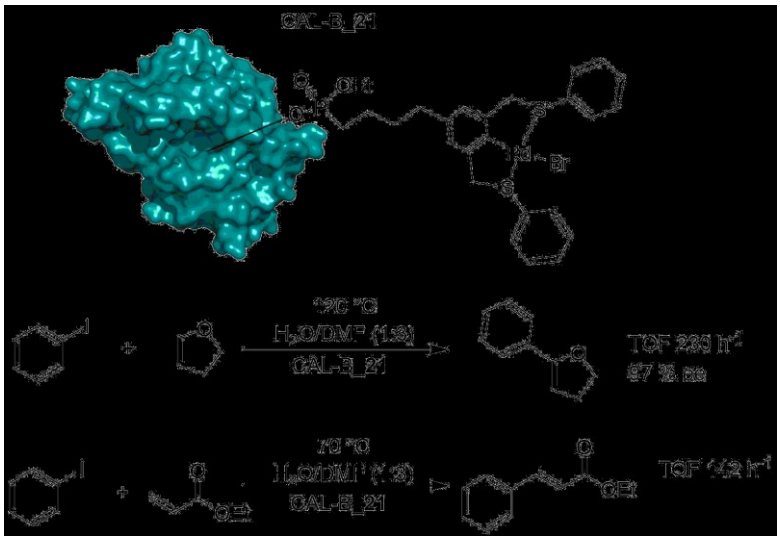

Figure 24: Immobilized CAL-B with a Pd-pincer complex to catalyse enantioselective MH reaction [84].

5.1.3. Sonogashira

Schmidtchen and Dibowski optimised aqueous Sonogashira couplings on unprotected amino acids in the presence of a model protein, RNase A [85]. The authors use a palladium-guanidinoaryl-phosphine ligand **22**, instead of the more commonly employed water-soluble phosphine ligand TPPTS. They reason that using TPPTS, electrostatic repulsion between a negatively charged protein and negatively charged catalyst will have to be overcome to perform a reaction, whereas a positively charged ligand will not. They also state the ligand is more stable against oxidation than TPPTS. The authors showed the coupling of 3-iodotyrosine with propiolic acid gave good yields in short reaction times (2 h) (Figure 25). In the presence of 14 mg/mL RNase A 'the reaction was markedly faster' while the catalytic properties of RNase A were unaffected showing that the reaction can be performed in the presence of proteins.

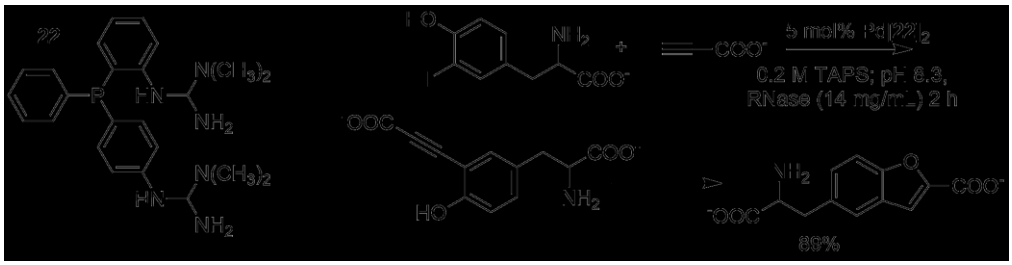

Figure 25: Sonogashira couplings of unprotected amino acids in the presence of RNAse protein [85].

5.1.4. Allylation reactions

Allylation reactions are frequently used as a benchmark reaction for asymmetric catalysts and with biohybrid catalysts this is no exception. The Ward group applied the biotin-(strept)avidin system to create an asymmetric allylic alkylation Pd catalyst [86]. Initial problems with ester hydrolysis of their starting material under the basic, aqueous conditions were solved by the addition of 2 eq. of the surfactant didodecyldimethylammonium bromide (DMB) to the substrate (4 mM) as shown in Figure 26. An initial ligand screen showed only a biotin-bis-phosphine ligand was active. The group then screened a library of biotin-bis-phosphine ligands varying the linker against a site saturation library of SAvS112. The presence of protein was shown to dramatically enhance the rate of reaction compared

to reactions without protein, indicating enzyme like behaviour had been achieved. Changing the SAv mutant used allowed each enantiomer to be accessed in high yields and good enantioselectivities (95%, 90% ee - *R* and 92%, 82% ee - *S*).

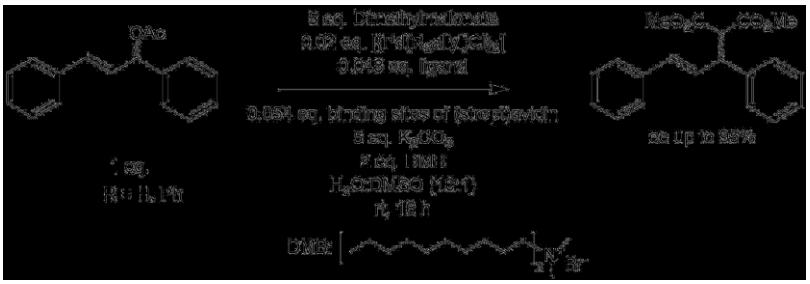

Figure 26: Pd biohybrid catalyst based on SAv to catalyse an enantioselective allylic alkylation reaction [86].

The first Cys conjugated phosphine was reported by the Kamer group [87]. Phosphines and Cys are both nucleophilic, which makes this common alkylation strategy more challenging. It was found that a CDI activated ester was uniquely reactive for the free Cys residue in *apo*-photoactive yellow protein (PYP) at pH 8. Using this strategy, *apo*-PYP was modified with 7 different mono- and bis-phosphines. Creation of an artificial metalloenzyme was achieved by addition of Pd(allyl)Cl_2 to the phosphine activated ester, giving **23,** and subsequent addition to the *apo*-PYP (Figure 27). A pre-coordination strategy was used to minimise the chance of Pd-protein interactions and Pd-protein complexes were observed by MS. The ArM was used for an allylic amination reaction, but conversion could only be achieved using a 1:1 DMF/Bicine buffer mixture at pH 8.5 to give racemic product. Circular dichroism showed the protein started to unfold above 5% DMF, which explained the racemic product.

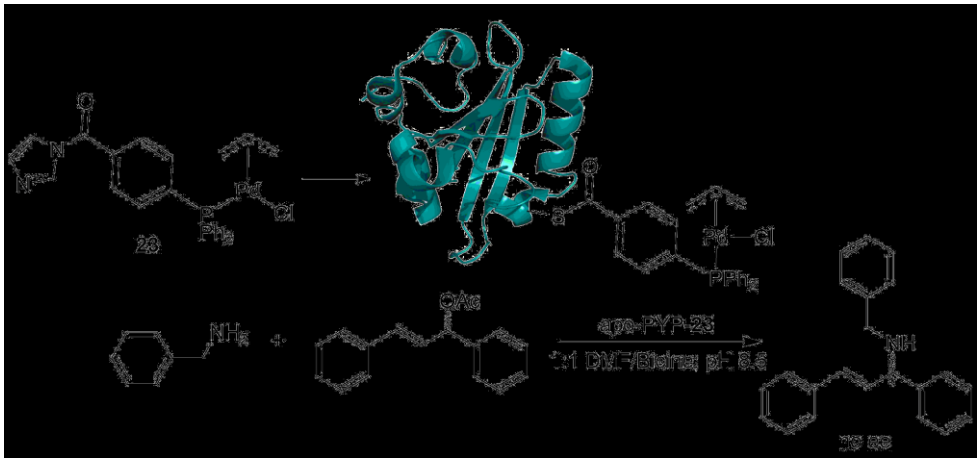

Figure 27: An allylic amination reaction catalysed by a Cys modified Pd-biohybrid catalyst. Although turnover was detected, it is believed the high concentration of DMF required caused denaturation of the protein so no enantioselectivity was observed [87].

More recently Kobayashi *et al.* looked to raise an antibody selective to a bis-triphenylphosphine Pd-allyl complex [88]. The Pd complex **24** used initially was found to be unstable in water, decomposing in an H_2O/DMF mixture in 7 days. The stability of the complex was greatly enhanced by substitution of Pd-allyl for Rh-COD, which after ligation to BSA and immunisation of mice, an IgM antibody was isolated. The antibodies showed K_d = 2.0 or 7.6 × 10^{-5} M for the Pd **24** or Rh complex **25** respectively, affinities for the phosphine ligands alone or benzoic acid were significantly lower. Investigation of an

allylic amination reaction using the Pd-mAb ArM Pd gave a TON = 600 (6% yield) and 98% ee of the (*R*)-enantiomer (Figure 28). Higher loadings of the Pd-antibody complex caused lower conversion and enantioselectivity, possibly due to aggregation and precipitation of the IgM.

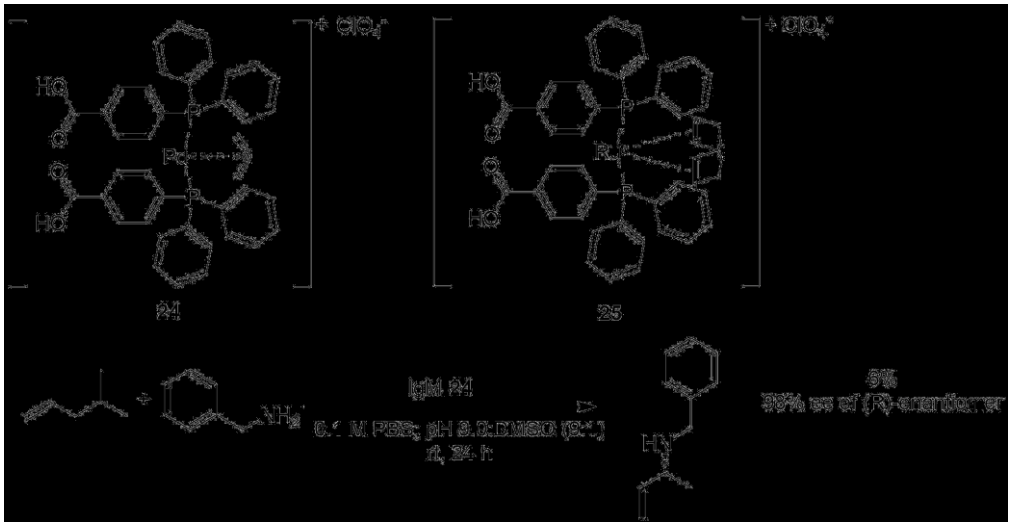

Figure 28: A biohybrid catalyst raised from an IgM antibody, raised against compound **25** for selective binding of Pd complex **24**, showed excellent turnover and ee [88].

5.2 Aldol condensation

The Thorimbert and Salmain groups designed a biohybrid catalyst by binding prochiral Pd(II) NCN-pincer ligands carrying a fatty acid side chain into β-lactoglobulin (β-LG), a protein which has a high affinity for fatty acids. A library of hybrid catalysts, varying by both the fatty acid chain length and N-substituents of the NCN pincer ligand, were screened for activity in Aldol condensation reactions of aldehydes and isocyanoacetates. By performing the Aldol condensation in water, the oxazoline intermediate was hydrolysed to give a formamide β-hydroxyamino acids. The diastereomer obtained was dependent on the ligand. As hypothesised, β-LG was show to effect the stereochemical outcome and thus play a role in determining the diastereoselectivity [89]. This represents a nice example of how alternative supramolecular binding approaches to the S(Av)-biotin system can be used to generate biohybrid catalysts.

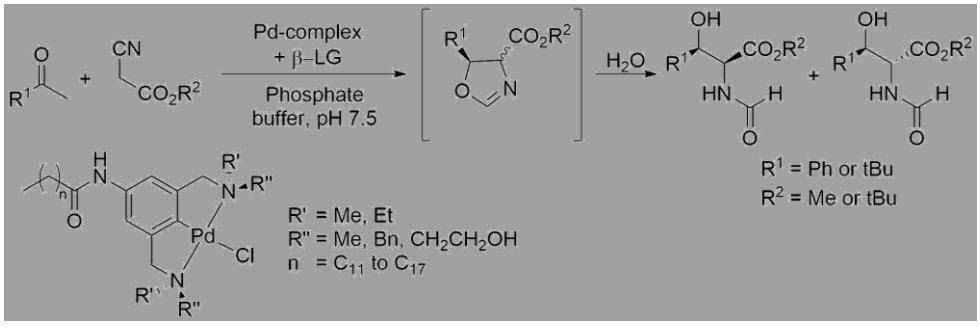

Figure 29: Aldol condensation catalysed by a Pd(II)-NCN pincer ligand gives *cis* selectivity compared to *trans* selectivity when catalysed by $Pd(OAc)_2$.

5.3. Hydrogenation

Hydrogenation is a reaction that is frequently applied in industry, and thus of interest to the biocatalytic community. Despite the efforts to eliminate transition metals for the catalysis of

hydrogenation reactions, they are still leading in the catalysis of hydrogenation [81]. Using artificial metalloenzymes containing Ru or Ir a large number of catalysts for transfer hydrogenation have been described, in comparison with fewer examples using gaseous hydrogen. Interestingly, the first described protein-transition metal hybrid was Pd based, it was built by reduction of $PdCl_2$ onto silk fibroin fibers and gave enantiomeric excess in the asymmetric hydrogenation of dehydroamino acid derivatives [90].

Chemically, heterogeneous catalysts are the Pd catalysts of choice for chemists for hydrogenation and the focus of biohybrid catalysts has been on providing an asymmetric environment for heterogeneous Pd catalysts. Ueno *et al* built a Pd nanocluster in ferritin protein cage to perform size selective olefin hydrogenation [91]. Ferritin is a protein complex comprised of 24 subunits with a cavity of 8 nm in diameter. Apo-ferritin was treated with K_2PdCl_4 and Pd(II) ions were reduced using $NaBH_4$ to give a Pd(0)-cluster inside the ferritin cavity. Each Pd-apo-ferritin contained a cluster of approximately 2 nm, with around 460 Pd atoms. Catalytic activity of Pd-apo-ferritin was determined by reduction of the alkene in acrylamide under a H_2 atmosphere in water at 7°C, 1 h and giving TOF of 72 mol h^{-1} for one Pd atom (thus TOF of 33 000 per Pd-apo-ferritin molecule) (Figure 30). The three-fold channels on the surface of ferritin gave some substrate size discrimination as when the size of reactants was increased, TOF decreased. Notably, apo-ferritin was determined to be in a native form by polyacrylamide gel electrophoresis all through the Pd nanocluster formation and reaction. Chiral reduction of racemic dehydroalanine derivative Ala-OMe was attempted but no enantioselectivity was observed.

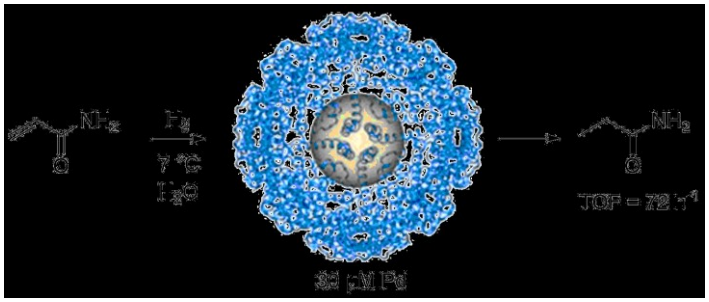

Figure 30: Size selective hydrogenation reaction using a Pd nanocluster encapsulated in a ferritin [91].

5.4. Oxidations

Oxidation reactions are an important part of organic chemistry and are frequently applied in industry. Alcohol oxidation is a fundamental reaction in organic chemistry on preparative and industrial scale. Common challenges include controlling site-, chemo- and enantioselectivity [92].

The quest for more sustainable and selective oxidation systems in the context of biological systems is ongoing. Hybrid catalysts for oxidations of alcohols were designed as seen previously with the ferritin nanoparticles [93]. Pd(II) was reduced with H_2 to Pd(0) nanoclusters (5 nm, 200 Pd atoms) inside the apo-ferritin cavity. Due to harsh conditions required for heterogeneous oxidations, a ferritin was used from the hyperthermophile *Pyrococcus furiosus* (*Pf*-ferritin) [93]. Oxidation of benzyl and vinyl alcohols to aldehydes proceeded selectively at 80 °C in water under 30 bar O_2/N_2 (8:92) with TONs up to 197 over 24 h, with no over-oxidation detected. The oxidation of non-activated and sterically demanding primary alcohols as well as secondary alcohols was much slower.

5.5. Reactions using kinetic resolution

The Bäckvall group designed a biohybrid catalyst built from Pd nanoparticles incorporated into cross-linked network of CalB by addition of $Pd(OAc)_2$ reduction with $Na(CN)BH_3$ (Figure 31) [94]. In this example the Pd catalyses the cycloisomerisation of 4-pentynoic acid and CalB provides an enantioselective transesterification (Figure 30). Pd(0)-CalB cross linked enzyme aggregate (CLEA)

catalyst contains 4.7 wt % palladium. The optimal conditions for the reaction were dry toluene at 60 °C with 1.0 eq of 4-pentynoic acid, 0.75 eq of Et_3N, and 2.5 mg of Pd(0)-CalB CLEA (1 µmol of Pd) for 3 h with the conversion of 50%, a theoretical maximum for kinetic resolution. The catalyst could be used in 6 repetitive cycles with the same isolated yields observed, while the measured concentration of Pd in the solvent was less than 1 ppm, suggesting that no Pd leaching is taking place in this example

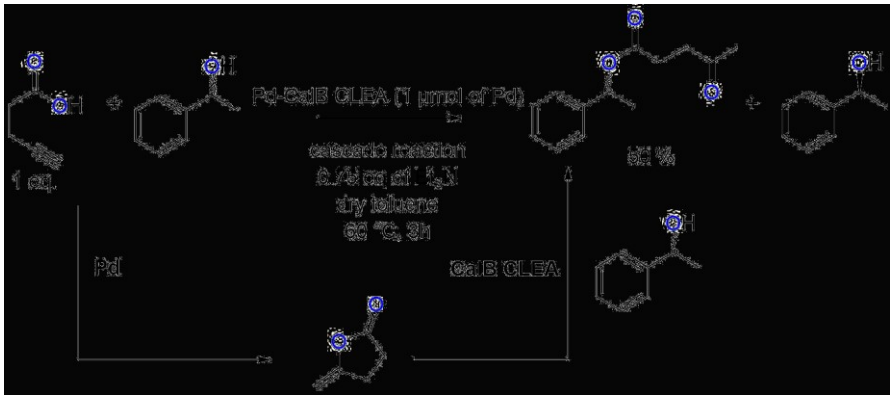

Figure 31: Cross-linked CalB with Pd(0) nanoparticles incorporated into the protein mesh was able to perform a Pd catalysed isomerisation, followed by an enantioselective transesterification [94].

The same catalyst was later used for dynamic kinetic resolution of primary benzylic amines (Figure 32) [95]. Pd(0)-CalB CLEA was further characterized and the effects of reducing agents on forming uniform Pd nanoparticles were investigated. $NaBH_4$ proved to be a better reducing agent that previously used $Na(CN)BH_3$ in [94], as the catalyst contained more reduced Pd. Analysis showed that the Pd(II) and Pd(0) species coexist in the catalyst [95]. Reuse of the catalyst resulted in drop of ee (when 1,4-dioxane was used as solvent, a drop from 95 to 86 % in the second recycling). The analysis showed that the leeching wasn't responsible for the reduced yield and drop in enantioselectivity. Instead the authors attributed to contamination with Cl ions, as formation of Pd-Cl or bonds similar to Pd-Cl was confirmed.

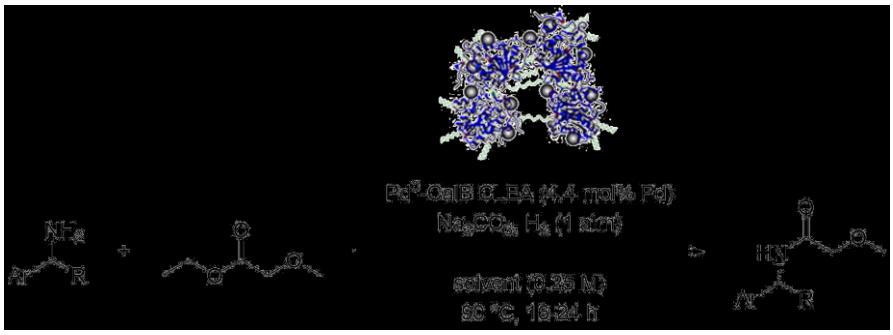

Figure 32: Dynamic kinetic resolution of an amide bond forming reaction, Pd(0) is used to racemise the secondary amine starting material, while CalB catalyses an enantioselective acylation. Adapted from Ref. [95].

6. Summary

Pd is an extremely important and versatile metal in the chemical sciences and yet while we have shown a broad range of applications so far in biological settings, we feel Pd has not reached its full

potential in this field. Throughout the review we have highlighted the issues that arise from using Pd in biological media and how researchers have minimised their impact on Pd catalysis. The main points are summarised below.

Toxicity – Heterogeneous vs homogeneous catalysis: It is clear that for *in vivo* chemistry, the use of heterogenous Pd(0) shows far less toxicity, better cellular uptake and better stability than homogenous Pd(0) complexes. By compartmentalising the Pd inside polymeric structures, catalyst poisoning is slowed and Pd toxicity is limited while still being catalytically active. However, the use of heterogeneous Pd in the preparation of biohybrid catalysis clearly limits the control the protein tertiary structure has in terms of enantioselectivity during the palladium catalysed step of a reaction. In comparison, the example from the Ward group shows the huge potential of a single Pd molecule 'active site' biohybrid catalyst (often refer to as artificial metalloenzymes).

Stopping non-specific metal protein interactions: Throughout many of the previous reports in the literature using homogeneous Pd, peptide bond hydrolysis through protein-Pd interactions [96] [14] is reported as a problem. However, this is not reported in any of the papers discussed in this review. A more prevalent problem is non-specific Pd-protein interactions which has been shown to cause protein aggregation, reduce ionisation in mass spectrometry or poison Pd catalysts. Aggregation is by no means reported in all papers but Kodama *et al.* found the addition of salts, especially $MgCl_2$, was key to prevent protein aggregation [31]. Filice *et al.* utilised protein aggregation to their advantage, creating protein-Pd aggregates, although it appears specific aromatic rich sequences were required for Pd binding [84]. As with organic phase homogeneous Pd-catalysis, ligands are also used to prevent aggregation, with water soluble ligands often providing better results due to increased solubility in the reaction media. Of particular note is the use of pre-formed Pd reagents for protein modification allowing these reactions to proceed with phenomenal rates and selectivity. Compared to the ubiquity of CuAAC methods, it is surprising this approach has not been extended to SM reactions and is not more widely used.

Which ligand? The use of strongly coordinating ligands such as electron poor phosphines or NHCs have shown activity both *in vitro* and *in vivo*, although *in vivo* reactions are still far more problematic due to the much greater complexity of the media. Indeed, it is difficult to compare the reactivity of complexes *in vitro* and *in vivo* as for the latter turn-on fluorescence reactions are often used to show reactivity, but due to the high sensitivity of fluorescence microscopy, activity could still be extremely low. For example, the Davis group found 300 µM of $Pd(ADHP)_2{\cdot}(OAc)_2$ **1**, was the lowest concentration that gave reactivity for a reaction on the cell surface, raising the question whether this was the point that non-specific interactions became saturated? Fortunately they found **1** was non-toxic up to 700 µM [23].

Which buffer? Interestingly, Tris, a multidentate primary amine buffer, is the buffer of choice for protein modifications when phosphine and NHC ligands are used. Whilst the binding of Tris to biological metals is well-described, binding to palladium is unclear, raising questions over the exact nature of the Pd catalyst, and if all unwanted Pd-amine interactions are prevented by the use of strongly coordinating ligands?

7. Conclusion

The use of palladium in biological environments has allowed for a range of orthogonal biocompatible chemistry to be performed for protein modification and intracellular chemistry. Combining Pd-catalysis and enzymatic catalysis in one-pot has allowed the development of synthetic methodology to quickly get to chiral building blocks. Palladium is one of the most valuable metals in synthetic

chemistry and yet to our knowledge is not used by Nature's remarkable enzymes. However, the well understood mechanisms of Pd-catalysis and its synthetic utility make Pd-biohybrid catalyst design an attractive goal. Initial results suggest that Pd-biohybrid catalysts has a future in the biocatalytic toolbox, especially if the issues highlighted in this review can be overcome by successful design. Work within the literature on other metals has shown this can be successful, for example glycosylated bovine serum albumin has been used to protect a Ru Grubb's catalyst from glutathione and other non-specific interactions [97]. Additionally new methods in protein engineering could design proteins that have minimal metal interactions [98]. Overall, we believe there is great potential for combining the Pd and proteins/enzymes to improve known reactions and possibly even discover new transformations.

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