Expert Opinion

Vitamin B_{12} in drug delivery: breaking through the barriers to a B_{12} bioconjugate pharmaceutical

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Importance of the field: Vitamin B_{12} (B_{12}) is a rare and vital micronutrient for which mammals have developed a complex and highly efficient dietary uptake system. This uptake pathway consists of a series of proteins and receptors, and has been utilized to deliver several bioactive and/or imaging molecules from ^{99m}Tc to insulin.
Areas covered in this review: The current field of B_{12}-based drug delivery is reviewed, including recent highlights surrounding the very pathway itself.
What the reader will gain: Despite over 30 years of work, no B_{12}-based drug delivery conjugate has reached the market-place, hampered by issues such as limited uptake capacity, gastrointestinal degradation of the conjugate or high background uptake by healthy tissues. Variability in dose response among individuals, especially across ageing populations and slow oral uptake (several hours), has also slowed development and interest.
Take home message: This review is intended to stress again the great potential, as yet not fully realized, for B_{12}-based therapeutics, tumor imaging and oral drug delivery. This review discusses recent reports that demonstrate that the issues noted above can be overcome and need not be seen as negating the great potential of B_{12} in the drug delivery field.

Keywords: B_{12}, cobalamin, haptocorrin, intrinsic factor, peptides, transcobalamin

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1. Introduction

The consumption of vitamin B_{12} (B_{12}; cobalamin) is essential for the survival of all living cells. B_{12} is produced naturally by bacteria and all other species must acquire the vitamin through their diet. There are two major biologically active forms of B_{12}: methylocobalamin and adenosylcobalamin. Methionine synthase uses methylocobalamin to produce the amino acid methionine from homocysteine, and methylmalonyl-CoA mutase uses adenosylcobalamin as a cofactor to produce succinyl CoA, an important molecule in the TCA cycle [1]. Mammals have developed a complex uptake pathway for B_{12} involving a series of transport proteins [1-3]. For the purposes of this review, the uptake pathway is discussed only superficially. For a more in-depth discussion of the transport proteins, the reader is referred to recent reviews by Banerjee *et al.* [1] and Randaccio *et al.* [4].

B_{12} is a water-soluble vitamin (molar mass 1355.38 g/mol as cyanocobalamin) with a highly complex structure, comprising a midplanar corrin ring composed of four pyrroline elements linked to a central cobalt(III) atom (see **Figure 2** later). The corrin ring is similar to the more commonly known porphyrin structure but with key differences. Corrin rings have a greater degree of saturation compared with porphyrins and the increased number of sp^3 carbons confers greater flexibility to the corrin. In addition, there is greater asymmetry to the corrin ring, the latter the result of a 15-carbon ring over the porphyrin 16-carbon structure [1].

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Article highlights.

- An overview is provided of B_{12} structure, chemistry, and uptake proteins and receptors.
- An overview is given of the use of the B_{12} dietary pathway in drug delivery.
- Methods and highlights in the use of B_{12} conjugates to utilize/target specific B_{12} proteins and associated receptors.
- In the section on general uses of B_{12} in drug delivery, oral delivery is discussed, especially of proteins and peptides, and general small molecule delivery by oral and invasive means is discussed.

This box summarizes key points contained in the article.

Several functional groups are readily available for modification on B_{12}. Only a few modification sites, however, maintain the recognition needed to utilize the full B_{12} uptake pathway necessary for oral delivery (see **Figure 1**). Other modifications can be used to target specific proteins while reducing affinity for others, a fact recently exploited to target haptocorrin (HC)-positive tumors [5]. An in-depth discussion of modification sites can be found in Section 2.

B_{12} is initially released from food by the action of peptic enzymes and the acidic environment of the gastrointestinal system [6]. It is then bound and transported by two glycoproteins, HC and intrinsic factor (IF) [2]. Haptocorrin is secreted by salivary glands and released also by the gastric mucosa. Haptocorrin has a high affinity for B_{12} under acidic conditions (pH < 3) and so protects B_{12} from acid hydrolysis. The HC:B_{12} complex travels from the stomach to the duodenum, where the increased pH (> 5) decreases the affinity of HC for B_{12} [7]. Haptocorrin is also enzymatically digested here. On release from HC, B_{12} binds to the second of the two gastric transport glycoproteins, IF.

Intrinsic factor is a 43.4 kDa glycosylated protein that is secreted from the gastric mucosa and the pancreas [2]. The IF protein facilitates transport across the intestinal enterocyte, which occurs by receptor-mediated endocytosis at the apically expressed IF–B_{12} receptor (cubilin) [8]. Cubilin works to transport B_{12} in concert with an anchoring protein amnionless (Am) [6]. Following transcytosis, and between 2.5 and 4 h after initial ingestion, B_{12} appears in blood plasma bound to the third trafficking protein, transcobalamin II (TCII) [3]. The holo-TCII is cellularly internalized by the TCII receptor (TCII-R). B_{12} is released by the degradation of TCII by lysozyme. Another receptor, megalin (MG), can reabsorb filtered holo-TCII from primary urine. The TCII-R and megalin receptors then ensure widespread delivery and maximal use of B_{12} [1].

Knowledge of the binding between B_{12} and its various transport proteins is critical if the system is to be successfully translated from bench to bedside. In the last 5 years there has been an explosion of critical structural data related to the B_{12} uptake pathway, with the publication of the IF [9], TCII [10] and cubilin$_{(5–8)}$-IF-B_{12} [8] structures. One missing piece in the B_{12} puzzle is the crystal structure of HC. Indeed, a real understanding of the functions of HC remains elusive, with suggestions it may play a bacteriostatic role in the mouth and bloodstream [11]. What is true now is that researchers have a better understanding of how B_{12} interacts with its transport proteins, and how these transport proteins interact with their receptors. The implications this can have on drug delivery and sites of potential conjugation can then be better detailed, rationalized and hence optimized.

Along with the wealth of knowledge from the experimental field is the recent use of modern theoretical approaches to B_{12}-based drug design. The foundational work for B_{12} molecular modeling studies has all but been completed with the publication of several recent papers providing both force field parameters and charge density information at levels of theory comparable to, or exceeding, the quality of the force fields within which the parameters have been used [12-17]. With very little modification, a family of B_{12} structures can be turned into topologies for force field parameter assignments. This has opened the door to a wider range of B_{12} molecular modeling studies, including studies of the periplasmic binding protein BtuF [16] and the TonB-dependent transporter BtuB [17]. Computational approaches have shown themselves to be a powerful interpretive tool for explaining the various structures involved in B_{12} transport and providing a useful tool for rational drug design.

Based on the results of recent years, it is clear there is great hope for a B_{12}-based bioconjugate to reach the marketplace sooner rather than later. This review details and expands on the points made in this introduction and discusses the future of the field.

2. Review of the B_{12} dietary pathway in drug delivery

2.1 Modifiable points – solvent-accessible pocket

The solvent-accessible surface of B_{12} is critical when considering B_{12}-based bioconjugates. The key point to be made upfront is that there *is* solvent accessibility. For TCII this solvent exposure is ~ 6.5% (~ 80 Å^2). For IF this exposure is twice as high at ~ 13% (~ 163 Å^2), with HC the least accessible at 3.2% (~ 40 Å^2) [18]. This exposure allows for select sites to be utilized when designing a B_{12} conjugate, for general pathway acceptance or for selecting specific parts thereof.

B_{12} and the molecule of interest can be: i) coupled directly together; ii) held apart by 'spacer' units to produce distance between the B_{12} and drug; or iii) carriers can be conjugated to B_{12} with the desired drug contained, unconjugated, within this carrier. Several functional groups are readily available for modification on B_{12}, including propionamides, acetamides, hydroxyl groups, the cobalt(III) ion and the phosphate moiety. Only a few modifications are capable of maintaining the recognition of all three transport proteins needed to utilize

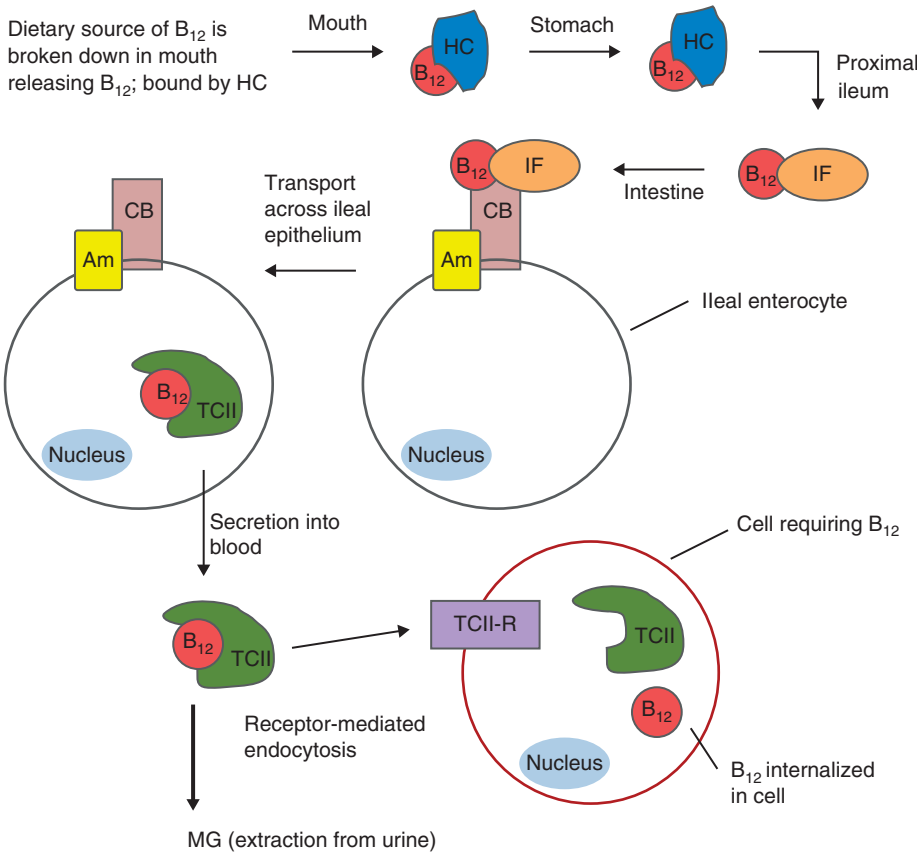

Figure 1. Uptake pathway for B_{12} from oral consumption to cellular entry. Although B_{12} has been cited as a potential carrier of drugs for several years, there are several limitations, including uptake capacity (~ 2.5 μg/day for an adult human [45]), that have limited its use in drug delivery so far.

Am: Amnionless; CB: Cubilin receptor; HC: Haptocorrin; IF: Intrinsic factor; MG: Megalin; TCII: Transcobalamin II; TCII-R: Transcobalamin II receptor.

the full B_{12} uptake pathway effectively in an oral manner. This limitation is a result of the manner by which the B_{12} is bound by the transport proteins and a strong indicator even in the absence of structural information on the similar solvent accessibility of B_{12} positions on binding in all cases. The size of the drug is important when considering the location for conjugation, as are the transport proteins being utilized, because the three transport proteins are not created equally. Conjugation of drugs resulting in the recognition of all three transport proteins has been successful with B_{12} at four major sites: i) to the peripheral corrin ring *e*-propionamide [19]; ii) through the 5′-hydroxy group of the ribose unit of the α-'tail' [20]; iii) through the 2′-hydroxy group of the ribose unit of the α-'tail' [21]; and iv) to the cobalt cation (see **Figure 2**) [22].

Examination of the recently published crystal structures of B_{12} bound with TCII [10] and IF [9] provides a better understanding of why such positions are available for conjugation without complete loss of recognition. TCII was the first transport protein crystallized [10]. The structure revealed several important details about points of modification on B_{12}, including the presence of hydrogen bond formation between various residues in TCII and B_{12}'s phosphate moiety and the side chains of the corrin ring. The further stability found to occur with the replacement of the Co's axial ligand with a histidine residue from TCII [10] was also rationalized. However, this latter substitution is not necessary for the binding of TCII and will not occur if a stable Co–C bond has been formed, such as the Co–CN in cyanocobalamin. The crystal structure [10] and molecular dynamics studies [14] have also shown that TCII does not completely encompass B_{12} on binding, and leaves an exposed section of the vitamin accessible to solvent. The 1.4 nm solvent-accessible pocket of B_{12} bound to TCII shows that the phosphate and ribose hydroxyl groups are left protruding into the solvent, but only the ribose 5′-hydroxyl tail is open to the environment enough such that it can easily accommodate the conjugation of large molecules (see **Figure 3**). Depending on the size of the conjugate, the three other sites have been shown to accommodate molecules without interference with TCII binding. It has been established that recognition and uptake of the vitamin still occur on modification of the β-axial position of the B_{12}'s cobalt, the α-axial ribose tail and several exterior carboxylic acid groups (produced by hydrolysis of side-chain propionamides) [10,19,22].

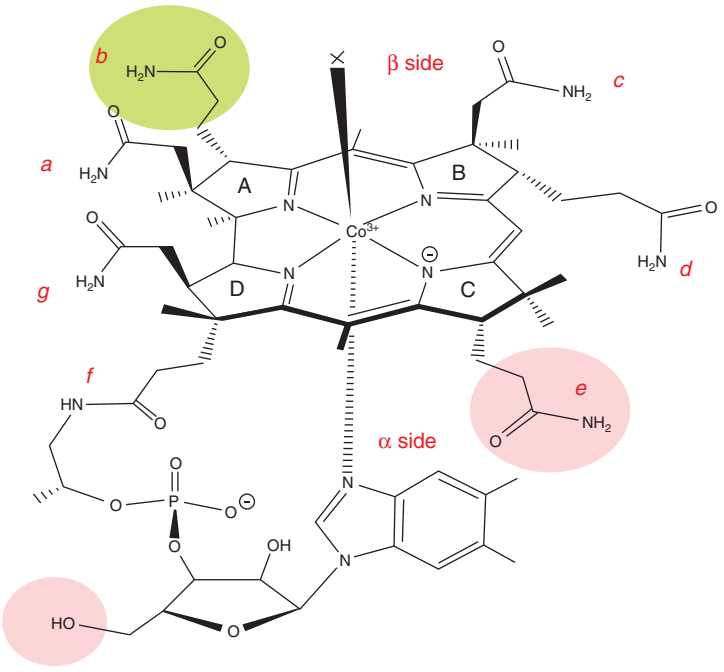

Figure 2. Structure of vitamin B_{12}. Molecular structure of XCbls: the central cobalt(III) atom is six-coordinated, with the equatorial positions filled by the nitrogen atoms of the corrin macrocycle. The (conventionally) 'lower', 'α'-axial site is occupied by an imidazole nitrogen atom from a 5′,6′-dimethylbenzimidazole base whereas the 'upper', 'β'-axial site can be occupied by various *X* groups (e.g., CN^-, CH_3^-, Ado^-, NO_2^-, SCN^-, $SeCN^-$, SO_3^- and thiourea). The corrin ring incorporates seven amide side chains, three acetamides (*a*, *c*, *g*) and four propionamides (*b*, *d*, *e*, *f*). The four pyrrole rings are usually indicated as A, B, C and D, as shown. Areas lightly shaded in pink have been commonly used for conjugates that maintain HC, IF and TCII binding. The area shaded in yellow, namely the *b*-propionamide, has been used, on conversion to the carboxylic acid, for HC-specific targeting. This HC-specific targeting is important where background TCII-R uptake prevents selectivity of cancerous tissue over healthy tissue.

Haptocorrin; IF: Intrinsic factor; TCII: Transcobalamin II; TCII-R: Transcobalamin II receptor.

The crystal structure of IF was published in 2007 [9]. This ~ 60 kDa protein is highly glycosylated and proved difficult to crystallize. The IF-B_{12} complex has several similarities to the previously discussed TCII-B_{12} complex, including several of the same hydrogen bond formations between the protein and the vitamin. The IF-B_{12} complex does not incorporate a histidine residue in the β-axial position, however, and ~ 13% of the B_{12} is solvent-accessible compared with only 6.5% of the B_{12} in the TCII-B_{12} complex [18]. The crystal structure revealed a possible mechanism, involving pH, for the transfer of B_{12} from IF to TCII, allowing researchers a better understanding of the last few steps of the B_{12} pathway [9].

While attention has concentrated on maintaining the binding between the transport proteins and B_{12} bioconjugates, little is known about what impact, if any, modifications to the B_{12}-transport protein complex might have on subsequent receptor binding. Until recently the structure of a holoprotein complex bound to a B_{12} receptor was unknown. In 2010, Andersen *et al.* published the structure of cubilin$_{(5–8)}$-IF-B_{12} (see **Figure 4**) [8]. This publication provided an outline of how the holo-complex interacts with the receptor, allowing researchers a new tool to determine the implications of conjugation on receptor binding.

2.2 Targeting specific transport proteins

On delivery to the bloodstream, a B_{12} conjugate typically will be acquired by the TCII protein. In cancer therapy/imaging, the hypothesis in B_{12}-based targeted delivery has historically been that increased TCII-R expression (as much as 3- to 26-fold in certain patients [6]) in a variety of cancers such as testis, breast, ovarian, thyroid, uterine, and brain cancer would provide sufficient selectivity over healthy tissue. This characteristic of B_{12} led to the production of both small molecule organic and inorganic B_{12} bioconjugates with various applications in medicinal chemistry. Although the bioconjugates are discussed in more detail in the next section, it should be noted here that the major problem associated with these bioconjugates has been background uptake by healthy tissue.

In an attempt to overcome background uptake, HC has been targeted more recently. Cytoplasmic or membrane-associated HC has been suggested to be *de novo* expressed [5] or overexpressed [23] in certain cancer lines. To target HC over TCII, Waibel *et al.* disrupted the interactions between

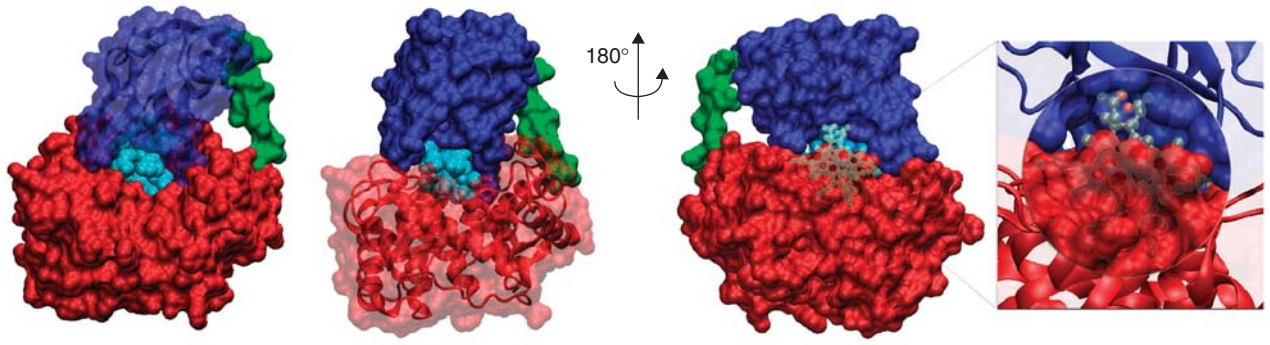

Figure 3. The solvent-accessible pocket of TCII bound to vitamin B_{12}. The left pair of images show the 5′ ribose and phosphate positions (cyan) accessible through a 1.4 nm gap in the solvent-accessible surface of TCII (red = α-domain, blue = β-domain, green = α/β linkage). The images on the right show the inaccessibility of the cobalt(III) coordination position in the crystal structure (a position at which functionalization has been shown to occur without a deleterious change to binding affinity in a few instances, indicating a TCII structural change at this position must occur to accommodate the steric bulk of a ligand. See text and [10]).

B_{12} and TCII by utilizing the *b*-propionamide site after modification to the *b*-monocarboxylic acid (the yellow shading seen in **Figure 2**) [5]. This method negates the possibility of oral uptake but demonstrated greater tumor targeting by reducing prevalent TCII-based uptake of the conjugates into healthy cells. This is a considerable breakthrough for B_{12}-based cancer therapeutics because it demonstrates that one of the oft-stated problems of using B_{12} systems, namely non-specific cell uptake, can be addressed, at least towards cancer cell lines with *de novo* HC expression. Images obtained with single-photon-emission computed tomography (SPECT) agents targeting HC and TCII are shown in **Figure 5**.

2.3 General uses of B_{12} in drug delivery

2.3.1 *Increased solubility*

The solubility behavior of a drug is one of the key physicochemical properties that can determine clinical efficacy and is often the cause of the failure of a drug to reach the market [24]. For orally administered drugs, the compound needs to be highly soluble or become immersed in the intestinal fluid to allow for absorption (assuming enterocyte passage is also feasible). For intravenously administered agents, sufficiently high solubility in the plasma is vital to diminish undesirable precipitation in the systematic circulation. In its native form, B_{12} is a highly soluble vitamin [10.2 mg/ml] that can be used to increase the solubility of compounds that would otherwise have little ability to be used as a drug.

2.3.2 *Oral delivery*

Few peptide/protein-based drugs have the ability to survive the gastrointestinal tract and/or cross the intestinal wall to make it to the bloodstream. Therefore, delivery of a pharmaceutically viable amount of peptide/protein is unachievable through simple oral dosing. At present, administration of these compounds is done through subcutaneous injections. This approach is time-consuming, painful, inconvenient, and can lead to allergic reactions near the sight of injection, as well as lower patient compliance. The improved ease of administration associated with the oral-enteric pathway provides an attractive means for the delivery of many pharmaceutical drugs because higher patient compliance is likely [25,26]. As a highly soluble, non-toxic vitamin with an extremely effective uptake pathway, B_{12} makes for an attractive vehicle for the oral delivery of drugs [26]. Although great strides have been taken in the field, the use of the uptake pathway is not without disadvantages, including a limited oral uptake capacity (1 – 2 μg of B_{12} per day [27]) and the inability to protect peptides/proteins from proteolytic degradation [28]. However, recent results, both experimental and theoretical, offer new strategies to overcome these issues, and these are discussed below.

2.3.3 *Delivery of small molecules*

For years, B_{12} has been utilized for the delivery of imaging agents and therapeutic drugs for the treatment and diagnosis of rapidly proliferating cells owing to the increased need for B_{12} in such cells (see **Table 1**). Early investigators demonstrated that cobalt radionuclides (^{57}Co, ^{58}Co, ^{60}Co) could be used to label B_{12} for imaging [29-31]. The half-life of the radionuclides, however, required the dosage for humans to be too small for successful external images and the radioactive compounds were shown to accumulate in the liver, pancreas and kidneys, leading to organ damage [32]. In an attempt to overcome these issues, some extra radionuclides, including ^{111}In (2.8 days; 0.245 MeV-γ) [32], ^{131}I (8 h; 364 keV-γ, 606 keV-β) [33] and ^{99m}Tc (6.02 h; 140 keV-γ) [34], have been conjugated to B_{12} for labeling purposes since these early days. Unfortunately, these experiments still resulted in

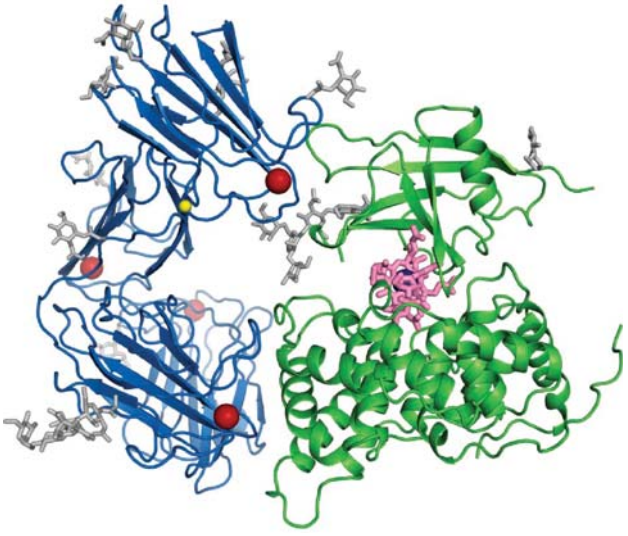

Figure 4. Cubilin$_{(5-8)}$-IF-B_{12} structure. This structure, published in 2010 (see Protein Data Bank accession code 3KQ4), shows for the first time the interaction between holo-IF (pink and green) and CUB_{5-8} domains (blue with calcium as red spheres). The structure provides a road map to study B_{12} conjugates bound to IF to explore the implications of B_{12} modification on IF binding and subsequent receptor interactions. The structure also offers up potential mutagenesis sites on IF for use of IF as a delivery agent itself. Neither of these two latter points has been studied extensively so far.
Image modified with permission from [8].
IF: Intrinsic factor.

undesired organ accumulation, limiting the amount of drug delivered to cancerous cells and demonstrating high background uptake in healthy cells [31-34].

In 2009, Siega *et al.* determined it was possible to deliver Gd^{3+} to cancer cells by conjugation to B_{12}. By using the metal chelating agent DTPA, a B_{12}–Gd^{3+} conjugate was constructed. The Gd^{3+} moiety conjugated to the 5′ ribose of the B_{12} did not affect the binding of the B_{12} transport proteins. Viability tests on human myelogenous leukemia K562 cells incubated with the conjugate showed a significant decrease in cell viability compared with those incubated with the B_{12} parent compound and/or the Gd^{3+} ion alone [35]. Given that this system utilized conjugation at the 5′-ribose position, allowing TCII recognition, it can be postulated that this system, if tested *in vivo*, will give poor tumor specificity.

Nitric oxide (NO) has been demonstrated as a potential candidate for antitumor therapy owing to its ability to cause both necrosis and apoptosis [36]. In 2002, Bauer *et al.* set out to demonstrate that conjugation of NO to B_{12} would result in a greater anticellular effect against malignant cells compared with normal cells. Nitrosylcobalamin (NO–B_{12}) is a B_{12} derivative with NO as the β-axial ligand. Once the conjugate was in the cell NO was detached from B_{12}, causing inhibition of cellular metabolism and directly damaging DNA, leading to apoptosis. The antiproliferative efficacy of the conjugate was determined in 22 human tumor cell lines and two non-cancer cell lines. It was determined that tumor cell lines were more sensitive to the conjugate than the normal cell lines. The conjugate was determined to inhibit tumor growth *in vitro* and *in vivo* by activation of the extrinsic apoptotic pathway [36].

Colchicine is a highly toxic compound that has been used as a therapeutic agent for a wide variety of diseases since the sixteenth century [37]. In recent years, it has been investigated as a potential anticancer drug. It has a similar mode of action as taxanes but is far more water-soluble, making it more accessible to biological applications. Unfortunately, colchicine does not have the ability to distinguish healthy from cancerous cells and colchicine chemotherapy results in overwhelming systematic toxicity [37]. In 2004, Grissom and co-workers used B_{12} to deliver colchicine to cancerous cells. To make the B_{12} conjugate, the colchicine analogue 2 (2-(4-acetylphenoxy)-*N*-acetophenocolchicine) was synthesized. To make the B_{12} conjugate, the colchicine analogue 2(2-(4-acetylphenoxy)-*N*-acetophenocolchicine) was synthesized. Compared with the unmodified colchicine, the analogue retained activity with only a minimal loss of toxicity. Using an acid labile hydrazone linker, the analogue was then attached to the β-axial ligand of B_{12}. The linker allowed for a pH-dependent release of colchicine in acidic conditions. Once taken into the cell by means of the B_{12} receptors, colchicine was released to interact with microtubules but showed a 10-fold decrease in toxicity [38]. As noted before, this system *in vivo* would again be predicted to suffer from TCII-based non-selective uptake/accumulation.

A progression of papers has recently appeared in the use of B_{12} to deliver Pt drugs, focusing on cisplatin and analogues thereof. In this instance it was shown that B_{12} can act as a ligand for cisplatin by the formation of a cyanide-bridged species between the β-axial ligand of B_{12} and Pt [39]. Alberto and co-workers showed that the B_{12}–cisplatin conjugate retains a labile chloride ligand that can be exchanged with ligands such as guanine, allowing the conjugate to behave in a similar way to cisplatin [40]. In 2008, Alberto and co-workers continued their initial work with the cisplatin derivatives and synthesized several prodrugs around the {B_{12}-CN-Pt-R} moiety (see **Figure 6**) [22].

Using an *in vitro* adenosylation assay from *Salmonella enteric*, the group was able to show that Co III was reduced to Co II and the Pt II complex was released [22]. As the Pt II complexes were shown to be released, B_{12}-mediated delivery of Pt complexes showed promise. In 2010, the *in vitro* cytotoxicity of the {B_{12}-CN-Pt-R} conjugates was published [41]. The preliminary results suggested a lower activity (IC_{50} between 8 and 88 μM) than for cisplatin alone. It is hypothesized that the limited B_{12} uptake capacity, discussed earlier, is most probably to blame. Studies on the effects of the uptake capacity on concentration are underway [41].

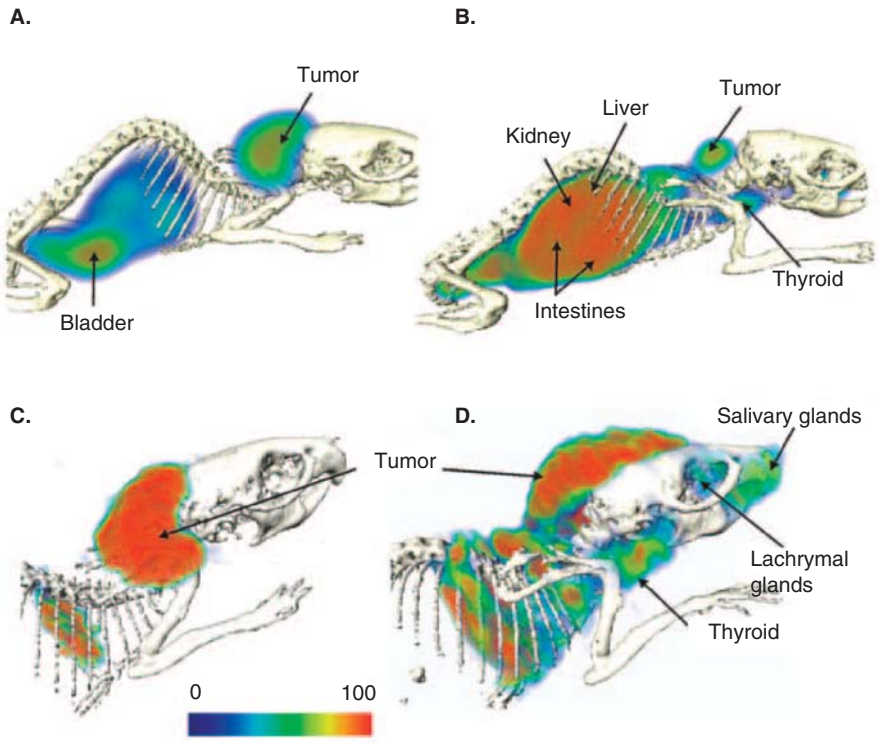

Figure 5. SPECT/CT scans of mice 24 h after intravenous injection of B_{12}-^{99m}Tc conjugates designed to be non-binders to TCII (HC binder) and binders to TCII. A, C. HC targeting compound. B, D. TCII binding compound. Note increased tumor specificity obtained with HC targeting. Color scale relates activity.

Reproduced with permission from [5].

HC: Haptocorrin; IF: TCII: Transcobalamin II.

2.3.4 *Delivery of peptides/proteins*

Table 2 lists the peptide/protein B_{12} conjugates reported so far. A comprehensive review of this area has been reported recently and the reader is referred there [42]. Instead, herein is discussed the use of molecular dynamics in the development and understanding of B_{12}-based drug delivery systems using a B_{12}-insulin conjugate system (see **Table 2**) as an example of the power of this approach.

3. Molecular modeling considerations

Molecular modeling serves invaluably in both predictive and interpretive capacities in modern drug design. The field is limited in application only by either the availability of proper theoretical descriptions of biologically relevant molecules (largely in the form of molecular force field parameters and topologies, limitations addressable by parameter generation and validation) or the absence of experimental data on which to base proper analyses. For molecular dynamics studies, the limitation is largely in the form of absent crystallographic or solution-phase structural information against which to identify structural candidates and test new designs. As applied to the study of bioconjugates, the atom-level description of molecular phenomena afforded synthetic chemists by even modest computational efforts reveals a great deal about the binding environment of a molecule. The potential difficulties of designs based on the placements of steric bulk (that prohibit binding of the native molecule) or physical inaccessibility of targeted functionalization (that changes the binding of the native molecule) can be visualized. Insights into possible molecular designs not readily obvious from either crystallography or macroscale sample characterization without synthetic efforts that survey possible candidate positions can also be highlighted.

As a complement to many of the experimental studies of functionalization positions [43,44], molecular modeling studies reveal the extent to which the modification potential of the B_{12} framework itself is greatly limited for new bioconjugate designs by the nature of its binding within its transport proteins. Using the binding of B_{12} to TCII (the complex for which the best structural information is available [1,3,10]) as a basis for all subsequent bioconjugate design work (as a disruption at any step in the transport pathway for B_{12} is probably deleterious for any bioconjugate design), several design considerations were revealed. These molecular modeling studies provide both a reinforcement of previous experimental work and insights into approaches for potential new B_{12}-based delivery designs based on functionalization positions deemed accessible, themselves based on the geometry of the B_{12}–TCII binding interaction.

Table 1. B_{12}-small molecule conjugates.

Molecule	Size	Conjugation site	Linker (coupling agent)	Use	Year	Ref.
Small molecules						
^{99m}Tc-DTPA	452.0	*b*-acid	1,4-diaminobutane (EDAC)	Imaging of TCII receptors	1997	[31]
^{111}In-DTPA	467.7	*b*-acid	1,4-diaminobutane (EDAC)	Imaging of TCII receptors	1997	[31]
b-nido-carborane	144.2	*b*-acid	Diaminobutane (HOBt/ EDAC)	Antitumor therapy	2000	[46]
d-nida-carborane	144.2	*d*-acid	Diaminobutane (HOBtf EDAC)	Antitumor therapy		
bis-nido-carbarane	288.5	*b*- and *d*-acid	Diaminobutane (HOBt/ EDAC)	Antitumor therapy		
NO	30.0	Cobalt atom	Direct	Antitumor therapy	2002	[36]
Cy5 dye	792.0	Ribose-5′-OH	1,6-diaminohexane (CDI)	Imaging	2003	[47]
Colchicine	399.4	Cobalt atom	4-chlorobutyricacid chloride (EDAC/NH5)	Delivery	2004	[38]
$[^{99m}Tco_4]^-$	162.0	Cyano ligand	Imidazolecarboxylic acid (NA)	Radiodiagnosis	2004	[48]
			picolinic acid (NA)	Radiodiagnosis		
			2,4-dipicolinic acid (NA)	Radiodiagnosis		
			serine (NA)	Radiodiagnosis		
			N,*N*-dimethylglycine (NA)	Radiodiagnosis		
$[Re(OH_2)(CO)_3]$	288	Cyano ligand	Imidazolecarboxylic acid (NA)	Linker for biomolecules	2004	[48]
			2,4-dipicolinic acid (NA)	Linker for biomolecules		
			serine (NA)	Linker for biomolecules		
			N,*N*-dimethylglycine (NA)	Linker for biomolecules		
Cisplatin	300.0	Cobalt atom	Cyano ligand (NA)	Oral anticancer	2005	[39]
Cisplatin-2′deoxyguanosine	585.3	Cobalt atom	Cyano ligand (NA)	Proof of principle		
Cisplatin-methylguanosine	598.2	Cobalt atom	Cyano ligand (NA)	Proof of principle		
^{131}I	126.9	Pt(ll) center	Cisplatin (NA)	Imaging	2007	[33]
trans-$PtCl(NH_3)_2$	263.7	Cobalt atom	Cyano ligand (NA)	Delivery	2008	[22]
trans-$PtCl_2(NH_3)$	281.8	Cobalt atom	Cyano ligand (NA)	Delivery		
Cis-$Ptcl_2(NH_3)$	281.8	Cobalt atom	Cyano ligand (NA)	Delivery		
$PtCl_3$	300.4	Cobalt atom	Cyano ligand (NA)	Delivery		
$[^{99m}Tc(CO)_3(OH_2)_3]$*	236.0	*b*-acid	Propyl-PAMA-OEt (EDACorTBTU)	Imaging	2008	[5]
			Ethyl-PAMA-OEt (EDAC or TBTU)	Imaging		
			Butyl-PAMA-OEt (EDAC or TBTU)	Imaging		
			Pentyl-PAMA-OEt (EDAC or TBTU)	Imaging		
			Hexyl-PAMA-OEt (EDAC or TBTU)	Imaging		
			3-hydroxy-2-methyl-l-propyl-lH-pyridin-4-one(NA)			
$V0_2$(OH/H)	118.0	Cobalt atom	3-hydroxy-2-methyl-l-propyl-lH-pyridin-4-one(NA)	Diabetes treatment	2008	[49]
VO_2	82.0	Cobalt atom		Diabetes treatment		
Fluorescence	475.2	Ribose-5′-OH	*trans*-1,4-diaminocyclohexane (CDT)	Imaging	2009	[50]
Khodamine 6G	479.0	RibosE-5′OH	*trans*-1,4-diaminocyclohexane (CDT)	Imaging		
Gd^{3+}	157.3	Ribose-5′-OH	Anbydrideof DTPA(NA)	Imaging	2009	[35]
			AnbydrideofTTHA(NA)	Imaging		
Rhodamine isothiocyanate	536.08	NA	HPMA(NHS/T5TU)	Imaging	2010	[51]

NHS: N-hydroxysuccinimide; EDAC: l-etbyl-3-(3-dimethylaminopropyl)carbodiimide; CDI: l,l′-carbonyldiimidazole; NA: Not available; CDT: l,l′-carbonyl-di-(l,2,4-triazole); DTPA: diethylenetriamine-*N*,*N*;*N*′,*N*′′, *N*′′′-pentaacetic acid; DCC: *N*,*N*′-dicyclohexylcarbodiimide; TTHA: Triethylenetetramine-*N*,*N*,*N*′,*N*′′,*N*′′′,*N*′′′′-hexacetic acid; TBTU: Q-(Benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TSTU: (*N*,*N*,*N*′,*N*′-Tetramethyl-0-(*N*-succinimidyl))uronium tetrafluoroborate; HPMA: Lysine-modified-hydroxypropyl-methacrylamide.

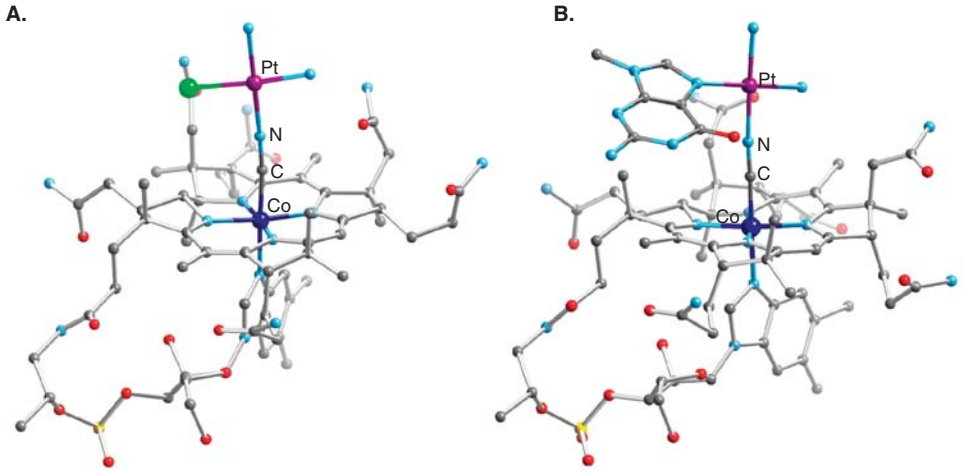

Figure 6. X-ray crystal structures of Pt conjugates of B_{12}. Images were generated from deposited CIF files CCDC-261001 and CCDC-261002. Both structures display the Co-C-N-Pt 'core'. Both compounds display micromolar activity but both were less effective than cisplatin directly, the result, it is hypothesized, of limited B_{12} uptake [41].

Any design of B_{12} bioconjugates must ultimately consider all of the steps in the B_{12} transport pathway. In these considerations, it is known that B_{12} binding to its transport proteins (HC, IF and TCII) must be conserved. The availability of partial or complete molecular geometries for TCII [10] and IF [9] reveals that these two proteins are largely similar in geometry and binding mode, and indicates that HC is likely to bind B_{12} in a similar manner. As the initial computational study of B_{12}-insulin [44] bioconjugate began with preliminary experimental results that indicated successful transport (as determined by cellular glucose uptake), the molecular modeling study focused on TCII. As part of any subsequent design based on B_{12} delivery, the characterization of the binding mode of B_{12} and the flexibility of the α/β domain 'clamshell' mechanism of binding by TCII (see **Figure 7**) produce an elegant picture of one set of readily accessible 'best-practice' approaches for bioconjugate design. This is a very straightforward model of how adhering to this set of approaches is likely to produce viable candidates for a wide variety of delivery targets.

In the previously reported molecular dynamics studies of TCII with B_{12} [15], B_{12}-insulin [44] and a B_{12} bioconjugate modified with a short peptide tether [14], the simulations revealed the extent to which the B_{12} is itself the hydrogen-bonding glue that binds the TCII into the encapsulation structure observed in diffraction studies (**Figure 7**). This work indicated that the interaction between side chains at the surface–surface interface between the α- and β-domains is not a major contributor to the stability of the complex. It also indicated that TCII in the absence of B_{12} probably consists of two, poorly interacting fragments linked by a short tether (obviously the design best suited to binding and protecting a large biomolecule that cannot easily migrate into the binding pocket of a less flexible or more inherently structured protein). As a tool for directing design, the result of the molecular dynamics simulation showed that modifications to the B_{12} are best performed for the generation of new bioconjugates at positions on the B_{12} molecule at the α/β domain interface. Appropriate modifications at this interfacial region are not expected to interfere with the individual B_{12}-α domain and B_{12}-β domain interactions that produce the encapsulated complex and, further, are made viable because the 'clamshell' design of this complex results in a α/β breathing motion in the MD simulations that makes this interfacial region accessible under ambient conditions and amenable to accommodating small tethering fragments to couple B_{12} to some arbitrary molecular beyond the solvent-accessible surface of TCII (see **Figure 8**).

The MD simulations provided atom-level explanations for all of the bioconjugate design work on B_{12} performed so far. This study specifically shows why certain chemical modifications produce viable candidates and some modifications produce B_{12} bioconjugates that, by their disruption of the B_{12}-transport protein binding interaction, do not. Modification at the cobalt (Co) is shown to be a reasonable approach for bioconjugate design because the Co coordination site lies at the α/β interface and small changes to side chains on the α-domain reduce steric congestion for coordinated ligands. This is obvious for small molecules, given the known binding of B_{12}-CN and B_{12}-CH_3. Whereas the cobalt is more embedded than the ribose 5′-hydroxyl position used as the point of conjugation in the B_{12}-insulin bioconjugate, the use of a small, flexible tether that is linked to one molecule and is Co-coordinated should serve as a reasonable mechanism for new Co-tether designs, a result supported by previous experimental work [44].

The MD simulation work also predicts that, owing to the mechanism of encapsulation and the solvent accessibility of

Table 2. B_{12}-protein/peptide bioconjugates.

Molecule	Size	Conjugation site	Linker (coupling agent)[§]	Use	Year	Ref.
Directly conjugated and encapsulate insulin delivery						
BS Albumin	66.0	Phosphate	Phosphaye-amine (EDAC)	NA	1971	[52]
YG-globulin	150.0	Phosphate	Phosphaye-amine (EDAC)	NA	1971	[52]
HS albumin	66.0	*e*-acid	GABA (EDAC)	antibody response	1979	[53]
IFN-con	22.0	ribose-5′-OH	glutaroyl (CDI)	24 – 28% activity*	1994	[54]
G-CSF	19.6	*e*-acid	disulfide (SPDP)	61 – 66% activity[‡]	1995	[55]
			Amide (EDAC)	29 – 85% activity[‡]		
			Hydrazide (EDAC)	ND-100% activity[‡]		
EPO	34.0	*e*-acid	Amide (EDAC) hydrazide (EDAC)	ND-34% activity[‡]	1995	[55]
				17 – 22% activity[‡]		
ANTIDE-1	1.6	*e*-acid	EGS (EDAC)	ND	1995	[56]
			Amide (EDAC)	30% IF recognition		
			Disulfide (SPDP)	65% IF recognition		
			Hindered thiol (SMPT)	54% IF recognition		
			Thioester (NHS ester of iodoacetic acid)	81% IF recognition		
			Transglutamase cleavable tetrapeptide (EDAC)	60% IF recognition		
ANTIDE-3	1.6	*e*-acid	EGS (EDAC)	ND	1995	[56]
			Amide (EDAC)	ND		
			Disulfide (2-iminothiolane)	ND		
			Hindered thiol (SMPT)	37% IF recognition		
			Thioester (NHS ester of iodoacetic acid)	65% IF recognition		
			Transglutamase cleavable tetrapeptide (EDAC)	48% IF recognition		
LHRH	1.2	*e*-acid	Amide (DCC/NHS)	45% absorbed	2000	[57]
DP3	0.9	*e*-acid	Amide (EDAC) hexyl (EDAC)	23% absorbed 42% absorbed	2000	[57]
Insulin	5.7	ribose-5′-OH	amide (CDI, CDT)	26% drop in glucose	2007	[20]
Encapsulated insulin delivery						
B_{12} coated-dextran nanoparticles	5.7	ribose-5′-OH	amide (CDI)	70 – 75% drop in plasma glucose	2007	[58]

*compared with native IFN-con.

[‡]compared with unconjugated G-CSF and EPO.

[§]linker chosen on basis of greatest yield and/or activity. ND = not determined.

BS, HS: Bovine and human serum; IFN-con: Consensus interferon; G-CSF: Granulocyte colony stimulating factor; EPO: Erythropoietin; ANTIDE: N-Ac-D-Nal(2)D, D-Phe (pCl), D-Pal(3),ser, Lys (Nic), D-Lys(Nic), Leu, Lys(IPr), Pro, D-Ala-NH2; LHRH: Luteinizing hormone-releasing hormone; DP3: Octapeptide (Glu-Ala-Ser-Ala-Ser-Tyr-Ser-Ala); GABA: *y* ami no butyric acid; EDAC: l-ethyl-3-(3-dimethylaminopropyl)carbodiimide; CDI: 1,1′carbonyldiimidazole; SPDP: *N*-succmimidyl 3-(2-pyridyldithiio)-propionate$_J$; SMPT: 4-[(Succinimidyloxy)-carbonyl]-α-methyl-α-(2-pyridyldithio)toluene; NHS: *N*-hydroxysuccinimide; DCC: *N*,*N*′-dicyclohexylcarbodiimide, CDT: l,l′-carbonyl-di-(l,2,4-triazole)$_T$; EGS: Ethylene glycol bis(succmimidylsuccinate; EDAC: l-ethyl-3-(3-dimethylaminopropyl)carbodiirnide.

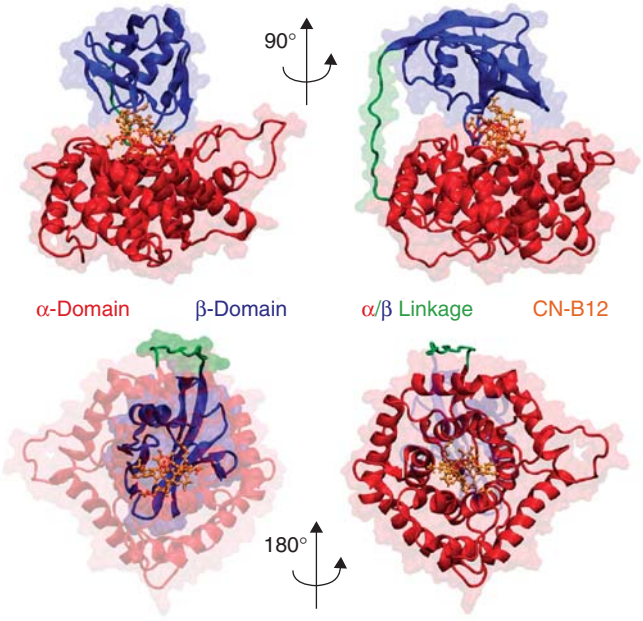

Figure 7. The binding of B_{12} within TCII. Substructures (domains) are differentiated by color. The α- and β-domains at the B_{12} binding position interact only through hydrogen bonding (and by way of a 10-residue covalent linkage), while numerous interactions between both domains and the B_{12} have been characterized experimentally and through previous molecular dynamics simulations. TCII structure based on [10].
TCII: Transcobalamin II.

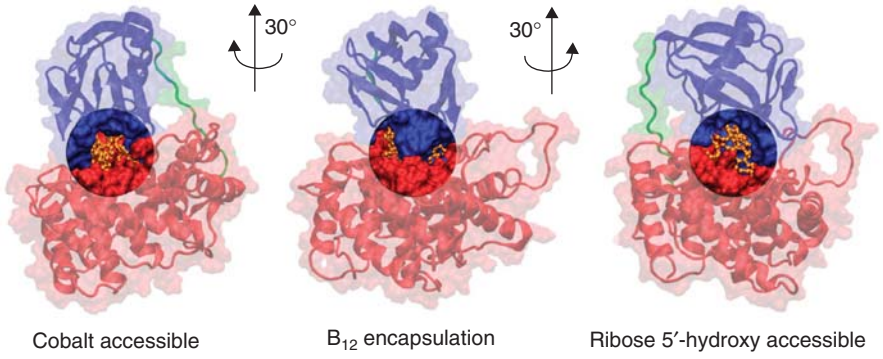

Figure 8. The time-averaged structure of B_{12}-TCII from a 50 ns MD simulation. The accessibility of the B_{12} for chemical functionalization but potentially limited B_{12} binding inhibition is shown by way of solvent-accessible surface representations at both the cobalt (left) and ribose (right) positions.

several regions of B_{12}, the cobalt and ribose 5′-hydroxyl positions need not be mutually exclusive as targets for bioconjugate design. Instead, with the accessibility of both positions attributed to the breathing motion of the α/β groups constrained largely by their strong binding to the B_{12} molecule, the possibility exists for three-component bioconjugates. This result was hinted at, but was not obvious from, the static model of the B_{12}–TCII binding interaction in the crystallographic study. The success of such designs is dependent on the modes of delivery and the differences in where and when the degradation of the combined product occurs. This route does offer a promising path for new work in B_{12} delivery approaches that are strongly supported from the synergy of experimental precedent and supportive computational work.

With the availability of force field parameters and the reported success of B_{12} MD simulations in several instances, one of the few issues in B_{12} bioconjugate design hampering continued and more thorough progress is the lack of structures for proteins within the B_{12} transport pathway. Although the success of the B_{12}-insulin bioconjugate indicates that, even in the presence of a large tethered molecule, the solvent-accessible binding positions on the

B_{12} are conserved in the members of the transport protein family, greater subtlety in the modes of chemical modification and targeted B_{12} design will come most easily through MD simulations that follow a B_{12} bioconjugate design through each step in the pathway, for which a modeling protocol is only as complete as the experimental characterization of all components. Within even the narrow selection of B_{12} modification positions hinted at by the solvent-accessible crystal structure maps and MD simulations of the native TCII-B_{12} complex, a wealth of bioconjugate design can be envisioned. Completeness in all-computational approaches to B_{12} bioconjugate design simply awaits adequate models of HC and IF, the members of the transport pathway either partially characterized (IF) or still unavailable (HC).

4. Expert opinion

B_{12} and the B_{12} uptake pathway play a fundamental role in biology and have been explored extensively from a structural and mechanistic viewpoint. The unique structure of B_{12} and the exquisite relationship with its binding proteins have driven researchers for several decades and continue to this day to offer up exciting new questions and new avenues of research. One of these avenues is the use of B_{12} in drug delivery, from small molecule *in vivo* imaging agents to the oral delivery of large proteins. Inherent restrictions of the B_{12} pathway, namely dose limitations and the demand across cells, healthy or otherwise, for the vitamin, have so far prevented the realization of a B_{12} bioconjugate pharmaceutical reaching the market. Despite this, several groups are working to address the challenges of the field, with recent successes including HC-specific targeting overcoming TCII background and the use of polymeric carriers attached to B_{12} to increase drug payload.

The future of the field then lies in expanding on these successes. The authors envisage building B_{12} bioconjugates with multiple payloads, using multiple sites on the B_{12} molecule simultaneously. The use of B_{12} to deliver protein vaccine components also has great potential. By targeting the immune response, potentially low doses need be delivered that require concentrations within the boundaries of the B_{12} uptake pathway capacity. The use of the B_{12} uptake proteins themselves has also not been explored. Coating a viral particle that requires an enteric IgA response with HC, for example, may offer gastrointestinal protection and ultimately provide for an oral vaccine for such a virus. The very investigation of the role of HC in blood serum and its structure will also be a highlight of the coming years in the B_{12} field. The take home message of this review is that this is an exciting time for B_{12}-based drug development, with many of the barriers thought to be road blocks to successful clinical trials being overcome.

Declaration of interest

The authors declare no conflict of interest and have received no payment in preparation of this manuscript.

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