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Ubiquitous metal, Difficult to track: Towards an understanding of the regulation of Titanium(IV) in humans

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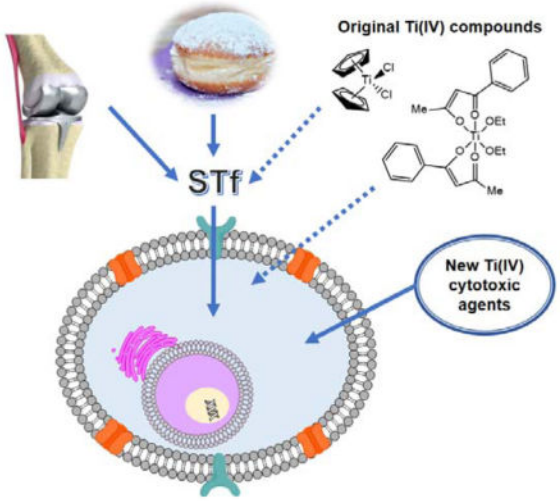
Abstract

Despite the ubiquitous nature of titanium(IV) and several examples of its beneficial behavior in different organisms, the metal remains underappreciated in biology. There is little understanding of how the metal might play an important function in the human body. Nonetheless, new insight is obtained regarding the molecular mechanisms that regulate the blood speciation of the metal to maintain it in nontoxic and potentially bioavailable form for use in the body. This review surveys the literature on Ti(IV) application in prosthetics and in the development of anticancer therapeutics to gain insight into soluble Ti(IV) influx in the body and its long-term impact. The limitation in analytical tools makes it difficult to depict the full picture of how Ti(IV) is transported and distributed throughout the body. Improved understanding of Ti function and its interaction with biomolecules will be helpful in developing future technologies for its imaging in the body.

Graphical Abstract



Molecular mechanisms in the body regulate the cytotoxic properties of titanium(IV) such that Ti(IV) could be bioavailable for cellular function.

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1. Introduction

Titanium is one of the most underappreciated metals in biology even though it is ubiquitous being the ninth most abundant element in the crust (4400 ppm)[1] and widely used industrially for human use in cosmetics, sunscreens, food, and prostheses. A common misconception is that the metal exists dominantly as inert and insoluble titanium dioxide (TiO_2) and thus serves no biological function. Much of this misconception is due to the limited research into the biochemistry of Ti, even in its prevalent TiO_2 form. Lack of appropriate tools to probe the metal further impedes our progress in understanding its biological function.

In our oxidizing atmosphere, the metal exists as Ti(IV), a d^0, diamagnetic species that makes it virtually blind to the standard spectroscopic and magnetic techniques traditionally used to study metal ions and their ligand coordination environment, namely Ultraviolet-visible (UV-vis) spectrophotometry and electron paramagnetic resonance (EPR). However, one could with adequate deconvolution software take advantage of ligand to metal charge transfer UV-Vis absorbances to study the physiologically relevant aqueous speciation[2] of potentially useful Ti(IV) compounds.[3] While there are nuclear magnetic resonance (NMR) active isotopes, ^{47}Ti and ^{49}Ti, their application is limited to small Ti(IV) compounds because of very low sensitivity and resolution.[4, 5] That said, a perusal of the literature reveals the application of state-of-the-art instrumentation to study Ti in living systems. These studies demonstrate clear examples of the metal's bioactivity although admittedly little comprehension of the mechanisms. Where identification of potential biological function has proven elusive and also little explored has been within humans. A survey of ~50 years research on the development of Ti(IV) compounds as anticancer therapeutics and on ~70 years application of Ti in the development of prosthetics has unlocked insight not only into the medical potential of Ti but more fundamentally, into the transport of the metal in the body and the regulation of its cytotoxic property. This review will focus specifically on the key highlights of this research that provide a revised understanding of the bioavailability of Ti in the human body and its activity. We will emphasize how analytical tools, especially in the imaging field, contribute to the changing depiction of Ti(IV) biorelevance but also highlight the significant limitations in what we can detect.

2. Understanding Ti(IV) bioavailability

For any element, in order to be bioavailable for consumption by organisms, it is important that it exists in a form that is easily accessible, is not toxic, and is either readily soluble or can be metabolized into a soluble form such as iron filings used to enrich cereals.[6] Ti(IV) is extremely prone to hydrolysis and in the absence of suitable ligands can irreversibly form insoluble oxide species especially under physiological conditions. Ti(IV) has extremely low solubility at pH 7.4 (0.2 fM using the proposed soluble species $TiO(OH)_2$ with $K_{sp} = 1 \times 10^{-29}$).[7, 8] Knauss et al. has recently reported much higher values for the solubility of rutile (TiO_2) over a broad pH ranges from pH 1 to 13 at different temperatures of 100 to 300 °C.[9] They measured the solubility by ICP-MS of about $10^{-7.7}$ mol/kg at 100 °C. Most recently, Schmidt et al. has determined the solubility of industrially produced anatase (TiO_2). The titania shows solubilities as low as about 1 nmol/L at 25 °C in the pH range 3 to 11.[10] They

have reported that no significant difference in the aqueous solubility of anatase and rutile were observed. Considering the very limited solubility of Ti(IV), it is surprising how abundant the metal is in humans. The human body contains approximately 700 mg of Ti, much more than cobalt and selenium, which are known to be essential metals.[1] In the blood (pH 7.4), Ti(IV) is present at low nM concentrations,[11] which is over a million times higher than solubility would predict. It is also found in other organisms at several orders of magnitude higher than their local environments, such as in the marine organisms the brown algae *F. spiralis* (308 ppm)[12] and the ascidian *E. ritteri* (1512 ppm),[13] and the terrestrial plants like horsetail, and nettle (up to 80 ppm).[1] The elevated concentrations of soluble Ti(IV) in these organisms is the consequence of chelation by small molecules and proteins but by no means suggests that an actual function exists for the metal. The profile for dissolved titanium in the open ocean is very indicative of a metal used biologically. At the surface, the metal is quite scarce, which implies consumption by organisms but becomes more abundant at greater depths where there are fewer organisms (Fig. 1).[14–17]

Interestingly, the requirement for solubility dictating Ti(IV) bioavailability is being challenged by recent findings on TiO_2. As an example, a study on TiO_2 nanoparticle uptake in cucumber plants (*C. sativus*) demonstrated transport of the nanoparticles from the roots to the leaves.[18] Micro X-ray fluorescence (micro-XRF) was used to track the movement of the Ti (Fig. 2).[18] Micro X-ray absorption near edge structure (micro-XANES) was applied to identify the Ti species in the different compartments of the plants. Micro-XANES showed that in all but one compartment the nanoparticles were present in the same ratio of anatase to rutile species as originally prepared. The plants experienced a dose-dependent elongation of the roots. Improved growth has been observed following TiO_2 uptake in other plants. In the *C. sativus* work, the root elongation was correlated with increased nitrogen levels and likely enhanced productivity of the nitrogen cycle.[18] The photocatalytic properties of TiO_2 bestow additional bioactivity such as an antibacterial effect.[19] These studies help to reconceptualise Ti inertness particularly when examined in a biological environment.

3. The biological functions of Ti(IV)

Valentine et al. recently wrote an excellent review postulating the potential biological functions that Ti(IV) might display given its chemical properties.[20] The main conclusions will not be repeated here other than to note what should be an indisputable characteristic of Ti(IV) and that is its structural function. The marine organisms diatoms accumulate Ti(IV) at very high levels. In some diatoms, part of this Ti(IV) pool becomes incorporated into their frustules as TiO_2.[21] Higher Ti(IV) content in the frustules examined in bioengineered experiments detrimentally impacts on the integrity of the structure provided by SiO_2 (Fig. 3).[22] In natural diatoms, the Ti(IV) in the frustules displays a bactericidal property that is photocatalytically activated,[23] which perhaps participates in a defence mechanism for the organism. The Ti(IV) content in the frustules is likely regulated to obtain an optimal balance between structural integrity and protective bactericidal activity.

In humans, Ti also exhibits a structural function. It features the property of osseointegration, a serendipitous discovery made by Dr. Per-Ingvar Brånemark in the 1950s.[24, 25] That is, the metal is able to integrate and be structurally accepted by bone without the requirement of

soft tissue connection. It aids in the healing and regrowth of some bone types. This finding led to the application of Ti in the development of prosthetics. While this property in itself is a clear example of titanium bioactivity, it is a solid state phenomenon that may have more to do with the surface of the metal serving as a biomineralization template. Nonetheless, it shows that the metal is biocompatible with the human body.

4. Ti(IV) in the body

4.1 Identifying the soluble Ti(IV) pool in blood

Although Ti(IV) in the blood is significantly higher than predicted solubility, there are no known natural roles that this metal plays in the body. This soluble Ti(IV) content probably comes from the daily dietary intake due to its general abundance and not from a specific Ti(IV) source. The metal is widely distributed in the body. In the kidney cortex and lungs, it is present at 1.3 μg/g.[26] It is most abundant in the lungs (3.7 μg/g) in the form of TiO_2 due to the white paint dust particles that we inhale.[26, 27]

In people with prosthetics developed with Ti typically in alloy form, Ti(IV) levels are even higher. Ti is one of the most utilized metals in prosthetics because of its numerous attractive features such as the aforementioned biocompatibility, corrosion resistance, low weight, and insignificant magnetism.[28] An additional attractive feature but one stemming from another misconception is the belief that the metal in its solid form is inert. Some of this thinking is due to the surface of Ti developing an oxide layer that serves as a passivating agent. As it turns out, the metal can escape from the implants as either insoluble TiO_2 due to grinding or implant failure, or as soluble Ti(IV) from corrosion or ligand chelation.[29–31] There are health concerns related to the release as TiO_2 especially if it is of nanoparticulate form[27, 32] because of the inherent risks of particles in the circulatory system and the material's ability to generate reactive oxygen species, however, this property has to be photo-induced. We will direct our attention to the release of soluble Ti(IV). The outstanding review by Golasik et al. reveals the alarming damage that soluble Ti(IV) could impose on the body such as lysing bone cells.[26] The studies presented in this review concerning the short-term effect of the elevated amounts of the ion in humans with implants, however, paint a more positive picture. Little of the toxicity that *in vitro* experiments suggest that soluble Ti(IV) is capable of is actually observed in people. Any harm reported appears to be more localized to the sites where implant failure occurs and not generally spread through the body. Ti(IV) reaches up to 0.25 μM in the blood of people with prosthetics, roughly as high as 50 times more than normal levels.[11] A cell study was performed in which cells were grown in the presence of a physiologically relevant source of Ti(IV), $[Ti(citrate)_3]^{8-}$, that was pre-treated with serum-containing culturing media.[32] This was proposed as a model for soluble Ti(IV) released in the body. It was observed that ~80 μM compound was required for any cytotoxicity to be observed. Viability studies performed by our own lab have shown that even at 100 μM, this form of Ti(IV) shows no cytotoxicity.[33] Using these concentrations as a benchmark above which soluble Ti(IV) in serum would exhibit cytotoxicity, the amount of Ti(IV) released into the body even by implant sources should not pose a cytotoxic threat.

It is quite interesting to consider what species in blood maintains Ti(IV) soluble and if for any particular reason. This information could give insight into the long-term effect of a

sustained influx of Ti(IV). A double-focusing inductively coupled plasma mass spectrometer (ICP-MS) was used to identify the soluble Ti(IV) speciation present in the serum of people exposed to implants and those who were not. The data reveals that in the people with implants the Ti(IV) was nearly 100% bound to the blood protein serum transferrin.[9] In those people lacking exposure, their Ti(IV) was also serum transferrin (sTf) bound but the low-level detection is at the limits of the technique so it is difficult to say if the protein is truly the only Ti(IV) binder in blood.[9]

The finding that Ti(IV) is endogenously bound to sTf is a significant finding because of the protein's metal ion transporting function. STf is a bilobal, 80 kDa glycoprotein responsible for transporting serum Fe(III) into cells via an endocytotic process and through this function maintaining the otherwise poorly soluble Fe(III) in a bioavailable form[34]. It is present at ~30 μM in blood[34]. All Fe that is ingested and metabolized is regulated by sTf immediately following the simultaneous transport of Fe across the enterocyte membrane and oxidation to Fe(III), and then release into the bloodstream. STf consists of N- and C-lobes that are divided into two subdomains (N1 and N2, and C1 and C2). There are two Fe(III) binding sites in each lobe consisting of an aspartic acid from the N1- or C1-subdomain, a tyrosine in the hinge near the N2- or C2-subdomain, another tyrosine in the N2- or C2-subdomain, and a histidine in the hinge near the N1- or C1-subdomain (Fig. 4)[35]. Carbonate serves as a bidentate synergistic anion to complete the Fe(III) coordination and aid the protein in stabilizing the metal ion.[36] Fe(III) binding results in the formation of a ligand to metal charge transfer absorbance at 465 nm (ε= 5,200 $M^{-1}cm^{-1}$ relative to protein concentration). By coordinating to residues that bring the two subdomains of each lobe close together, Fe(III) binding results in a major protein conformational change that converts the protein from an open to a closed form. Being only 30% Fe(III) saturated,[37] sTf also plays the important function of transporting non-iron metals. There is an evidence to demonstrate that it can transport, at least momentarily, chromium, bismuth, gallium, indium, ruthenium, vanadium, aluminium, lanthanides, actinides, and manganese.[38–40] STf binds metal ions with high Lewis acidic character.[41]

We decided to probe how sTf might bind Ti(IV). A few sources suggest that soluble Ti(IV) released from implants is chelated by citrate.[30, 31] Citrate is a bioactive anion that exists at 100 μM in the blood. An aqueous speciation study demonstrated that citrate has the capacity to form stable but labile Ti(IV) complexes that can exist at physiological pH,[42] which supports its implication in the speciation of soluble Ti(IV) released into the blood. A ^{45}Ti positron emission tomography (PET) imaging experiment showed that the metal ion from a ^{45}Ti-citrate complex injected into normal Sprague-Dawley rats and EMT-6 tumor-bearing BALB/c mice became exclusively bound to sTf.[43] Using a biomedical cyclotron, ^{45}Ti can be produced in excellent yields.[44] The decay properties of ^{45}Ti are well suited for small animal PET. A recent x-ray crystal structure we obtained of Ti(IV)-bound sTf, the first ever Ti(IV) protein structure, demonstrated sTf binding of Ti(IV) in a manner that is quite distinct from that of Fe(III).[33] The Ti(IV) is coordinated to only the two tyrosine residues in the metal binding site. The remainder of the coordination sites are fulfilled by carbonate and, in a surprise, citrate (Fig. 4). Ti(IV) binding results in the formation of a ligand to metal charge transfer absorbance at 321 nm (ε= 20,000 $M^{-1}cm^{-1}$ relative to protein concentration). In solution under physiologically relevant conditions, the Ti(IV) coordination remains identical

as revealed by a ^{13}C NMR experiment using isotopically labelled carbonate and citrate to show that they are coordinated to the Ti(IV) at the metal binding site.[33] The citrate coordination results in a metal-bound open conformation for the protein because in citrate blocking the histidine and aspartate from binding the metal, the subdomain containing these residues does not move. A metal uptake study was performed with A549 human lung cancer cells, which have an overexpression of the transferrin receptor, using ICP-optical emission spectroscopy (ICP-OES) to measure the levels of intracellular Ti(IV) following treatment with Ti_2-sTf. Ti(IV) is found to be transported into the cells via sTf. This is a significant finding because others have argued that a metal-bound closed protein conformation is a requirement for metal delivery into cells by sTf.[39] Also significant is the synergistic role that citrate potentially plays in sTf transport of Ti(IV) because while metal transport is a function that it exhibits in bacteria,[45, 46] it has been little explored in humans. Further insight into the contribution of citrate and sTf to the transport and potential function of Ti(IV) is obtained by examining the efforts to produce a Ti(IV)-based anticancer therapeutic.

4.2. Regulating Ti(IV) activity

The search for a Ti(IV) anticancer compound that can finally make the leap into the drug market has been a long endeavour. This mission has endured as long as it has because of the fascinating cytotoxic properties of Ti(IV) that make it more attractive than the platinum(II)-based therapeutics, which are one of the major anticancer drugs.[47–51] The Pt(II) drugs suffer from the lack of specificity, numerous side effects, a limited spectrum of activity, and cellular resistance during extended use. The original lead compounds budotitane and titanocene dichloride (Cp_2TiCl_2) (Fig. 5) displayed in cell studies a greater spectrum of activity. They were active against cancer cells not responsive to cisplatin, the only Pt(II) drug at the time, and not cross-resistant against cells that had grown resistant to the Pt(II) drug. Despite their *in vitro* promise, neither compound advanced far in clinical trials due to poor effectiveness.[51–55] One major problem related to their low activity *in vivo* is their poor stability in aqueous solution because of their high hydrolytic propensity, making the preparation of samples extremely challenging. The seminal work by Toney and Marks on the hydrolysis chemistry of Cp_2TiCl_2 showed that at physiological pH the compound immediately dissociated the Cp and chloride ligands, generating an insoluble precipitate.[56] Even though Cp_2TiCl_2 was not efficacious in vivo, it exhibited side effects after i.v. administration such as systemic moderate toxicity to some organs i.e., heart,[51] kidneys and liver.[53] These side effects were also observed for budotitane, which was also inactive in vivo. The maximum tolerated doses of Cp_2TiCl_2 was 240 mg/L every 3 weeks[52] and of budotitane was 230 mg/L twice a week.[51] This begs the question of how the lead Ti(IV) compounds could have demonstrated any activity at all.

Due to its greater promise in drug trials, the mechanism of Cp_2TiCl_2 has been more extensively characterized and provides a greater understanding of the fate of Ti(IV) in the body. The hydrolytic instability of the compound required that it be formulated during drug trials in such a way as to avoid metal precipitation. It has been argued that the formulation of the compound dramatically altered its solution speciation affecting its behavior during clinical trials.[57, 58] The reality is that regardless of the formulation used, the fate of the metal in blood was quite similar in that it became largely protein bound.[52] In other words, serum

proteins rescued the metal ion from precipitation. A popular belief was that sTf was the protein source that maintained Ti(IV) bound and directly contributed to its cytotoxic property. STf has been implicated in the therapeutic property of chromium, bismuth, gallium, indium, ruthenium, and vanadium ions, likely as a delivery agent.[38] *In vitro* studies demonstrated that sTf could specifically bind two Ti(IV) ions, even directly from Cp_2TiCl_2 with very high affinity (log K ~ 26 per site) demonstrated via a suite of techniques including UV-Vis spectroscopy, fluorescence, and calorimetrically.[59–63] STf could selectively deliver Ti(IV) to certain cancer cells because they overexpress the transferrin receptors, which facilitate the endocytotic entry of metal-bound sTf into cells.[64–66] Cancer cells have a higher requirement for Fe(III) and sTf is their main source of this metal ion. Sadler et al. showed that Ti_2Tf blocked uptake of Fe_2Tf into placental cells[61] suggesting that Ti(IV) has the capacity to interfere with cellular mechanisms that require Fe(III). However, numerous studies have shown that sTf may not be as important as thought in facilitating Ti(IV) cytotoxicity.[67–71] Even at 100 μM concentration Ti_2Tf does not exhibit any cytotoxic behavior against A549 and MRC (normal) human lung cell lines.[3, 33]

Serum albumin (SA), the most abundant blood protein at 600 μM, has also been implicated in the mechanism of action of Cp_2TiCl_2. A ^{45}Ti PET imaging study showed that an ascorbic acid complex of ^{45}Ti could exclusively bind to SA when it was delivered into rats.[72] Although human SA has four metal binding sites, none have the ability to specifically bind the Ti(IV) ion.[73] Depending on the nature of the ligand to which Ti(IV) is bound,[74] HSA is capable of binding Ti(IV) in a compound form at one of its numerous molecular binding sites.[75, 76] These sites have the impressive ability to bind a wide variety of hydrophobic and hydrophilic molecules. Using ^1H NMR and equilibrium dialysis, we were able to demonstrate that HSA can bind one equivalent of the titanocene moiety from Cp_2TiCl_2.[77] Nonetheless, the titanocene-bound HSA complex does not have not significant cytotoxicity. No cytotoxicity was observed when A549 cells were treated with the complex even at 100 μM concentration[3].

Several studies have speculated on the possible intracellular site of Ti(IV) attack. The common belief is that Ti(IV) attacks DNA by binding to the phosphate groups based on direct solution studies between Cp_2TiCl_2 and DNA.[78–80] Interaction of Ti(IV) with phosphate groups is believed to be important for its transport within cells. Sadler et al. proposed that ATP binds Ti(IV) from endocytotic sTf and moves it out of the endosome and into the cytoplasm.[61] ATP binding of Ti(IV) is quite stable and rapidly occurs at the acidic pH condition of the endosome.[61, 81] Cellular studies with Pt(II)-sensitive and resistant cells have confirmed DNA being the site of Ti(IV) attack from Cp_2TiCl_2.[82] It is proposed that DNA binding of Ti(IV) results in detrimental structural changes that lead to a block in the late S/early G2 phase of the cell cycle and also the induction of apoptosis at any phase of the cell cycle. An *in vivo* study gave further insight into Ti(IV) localization within cells and its site of activity. Mice xenografted with human tumors were treated with Cp_2TiCl_2. An electron-spectroscopic imaging (ESI) approach was taken to track Ti(IV) in the tumor (Fig. 6).[83] Within the first 12 hours, the Ti(IV) localized to the nucleus, specifically the nuclear chromatin and the amount increased up to 2 days. A continuing decrease was observed on the 3rd and 4th day. Parallel to this localization, Ti(IV) was found after 24 h to be associated with the cytoplasmic lysosomes particularly at sites rich in phosphorus. As the Ti(IV)

content decreased in the nucleus, it increased in the lysosomes. It is not certain what function the Ti(IV) might have in lysosomes but there are reports on its ability to inhibit proteases.[84]

It is highly likely that the promising activity observed for Cp_2TiCl_2 was due to the treatment of cells at very high concentrations, in large excess of 100 μM, and very high dosage of animals during animal model studies. At nearly 1 mM concentration even the generally non-cytotoxic titanium citrate can demonstrate a cytotoxic behaviour. This was observed for human erythrocytes.[85] To an extent, the case of Cp_2TiCl_2 may be one of overhype especially when considering how both SA and sTf can have an impact on regulating its cytotoxic behaviour. Tacke and Tshuva et al. performed a collaborative study that demonstrated that the high albumin binding of titanocenes (likely in a combination of Ti(IV) ion and compound form) can in fact lower the metal uptake into cells, which might lower its cytotoxic potency.[86] Recently we demonstrated that citrate and HsTf can work in synergism to regulate the blood speciation of Ti(IV) to maintain it in a nontoxic form. This suggests that the human body has an innate molecular mechanism for regulating the activity of Ti(IV). As such any influx of soluble Ti(IV) in a labile form can be quickly ligand exchanged by citrate and delivered to sTf to control its transport into cells (Fig. 7). This molecular mechanism to regulate Ti(IV) would also apply to the extremely labile Cp_2TiCl_2. The clinical failure of Cp_2TiCl_2 is probably due not just to its hydrolytic instability: Ti(IV) uptake by HSA and HsTf (in synergism with citrate) likely contribute to the attenuation of its cytotoxic property by converting the metal ion into nontoxic species. Clearly, these proteins might be involved in the cytotoxicity of the compound at very high concentrations just by virtue of delivering super elevated levels of the metal into cells.

Several new families of Ti(IV) compounds are being developed for anticancer applications (Fig. 5). Second generation titanocene complexes are being developed by several researchers such as the research groups of Tacke, Meléndez, McGowan, Gomez-Ruiz, Kaluderovic and Baird. These newer complexes aim to overcome the poor water solubility of the parent compound and its hydrolytic instability. Modifications made include substituting the chloro ligands with less labile and more soluble ligands,[87, 88] derivatizing the cyclopentadienyl rings[89–93], or both[94–97]. In some compounds, a bioactive moiety is incorporated to improve biocompatibility and selectivity. One of the most promising second generation titanocenes is titanocene Y, which demonstrates *in vivo* activity against different cancers (carcinoma, prostate, breast, and renal).[98, 99] Tshuva and Huhn have introduced an entirely different Ti(IV) family that contain the tetradentate diamino bis(phenolato) (salan) and N,N′-ethylenebis(salicylimine) (salen) ligands. Ti(IV) complexes of these ligands are far more soluble, stable, and cytotoxic than the original lead Ti(IV) compounds. The Ti(IV) salan and salen complexes are generally six-coordinate with alkoxide ligands occupying the remainder of the coordination sites. They demonstrate high cytotoxicity against colon and ovarian cancer cells.[100, 101] Huhn has prepared heptacoordinate versions of these compounds with the tridentate 2,6-pyridinedicarboxylate ligand [Ti(salan)(dipic)], which demonstrate superior stability and potent cytotoxicity in cells.[102] Tshuva has also synthesized a hexadentate versions of the salan ligands which include two additional phenolate moieties and coordinate Ti(IV) with high stability.[103] These complexes are also highly potent. Our laboratory has introduced another family of Ti(IV) compounds that incorporate the

heterogeneous ligands termed chemical transferrin mimetics (cTfm).[104, 105] These ligands mimic STf binding of Fe(III) in order to favor Fe(III) binding relative to Ti(IV).[33] The Ti(IV) cTfm complexes are expected to work by the two-prong method of intracellularly binding Fe(III) and simultaneously releasing Ti(IV) resulting in depletion of bioavailable Fe(III) and Ti(IV) delivery to its sites of attack. The FDA approved iron chelator deferasirox serves as an excellent cTfm ligand. It forms the most stable Ti(IV) complex in solution under physiological conditions (to date), is activated intracellularly in part by its Fe(III) chelation ability, and is highly potent.[105]

The new generation of Ti(IV) compounds outside of the titanocenes appear to work independent of serum proteins and are largely dependent on the nature of the ligands. The ligands assist in maintaining the Ti(IV) stable and soluble and help to deliver the metal to the cells. The cTfm ligands serve an active role in the mechanism of action of the Ti(IV) compounds they form by enabling the compounds to potentially inhibit molecular processes, such as Fe(III) dependence, that keep the cancer cells alive. Despite the significant advances made in the design of newer Ti(IV) anticancer complexes, there are still limitations such as low *in vivo* efficacy. A ^{45}Ti PET imaging study of isotopically labeled[Ti(salan)(dipic)] revealed that the half-life of the compound in rats was short-lived and resulted in very low tumor accumulation to the point where it would not make a viable drug (Fig. 8).[106] High dosages of the compound were needed in a separate study in order to reduce tumor size.[107] This study suggests that increased stability of Ti(IV) compounds may result in compounds too stable to exhibit any valuable cytotoxicity *in vivo*. Incorporating biomolecular moieties onto the ligands that would direct the compounds specifically to cancer cells may prevent this issue.

5. Conclusion

The current understanding of soluble Ti(IV) in the human body stands in stark contrast to the general consensus that it is not important. There are molecular mechanisms that can maintain it in a highly stable and nontoxic form, in particular, the synergistic regulation facilitated by sTf and citrate. A recent review indicates that Ti(IV) distribution in the body does not alter the levels of essential metals in different tissues further supporting a nontoxic behaviour for Ti(IV) in the body.[108] This knowledge illuminates on how Ti(IV) anticancer compounds can be designed to bypass these molecular mechanisms (Fig. 9). The question that persists is whether the nontoxic protein-regulated Ti(IV) speciation provides a bioavailable form of the metal ion that the body may find use for. Studies have shown that Ti(IV) can be biomineralized by ferritin but no work to date has actually demonstrated the ability of cells to store the metal ion.[109, 110] Storage of the metal ion in this manner would imply that it is being separated for a particular function. There is a tremendous need for more sensitive tools and improved workflows to specifically study the long-term impact of soluble Ti(IV) in the body and to unveil a potential function.

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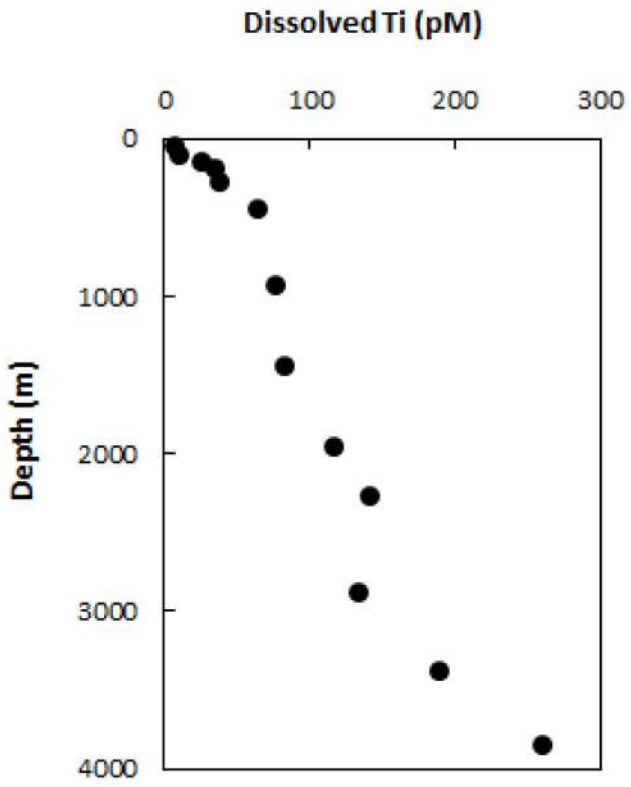

Fig. 1.

Concentration of dissolved titanium varies as a function of the depth in North Pacific Ocean (at 50° N,145°W. VERTEXVII, Station 7, July 1987). Adapted by permission from Macmillan Publishers Ltd: *Nature* **348**, 322–325, copyright 1990 (Ref. 14).

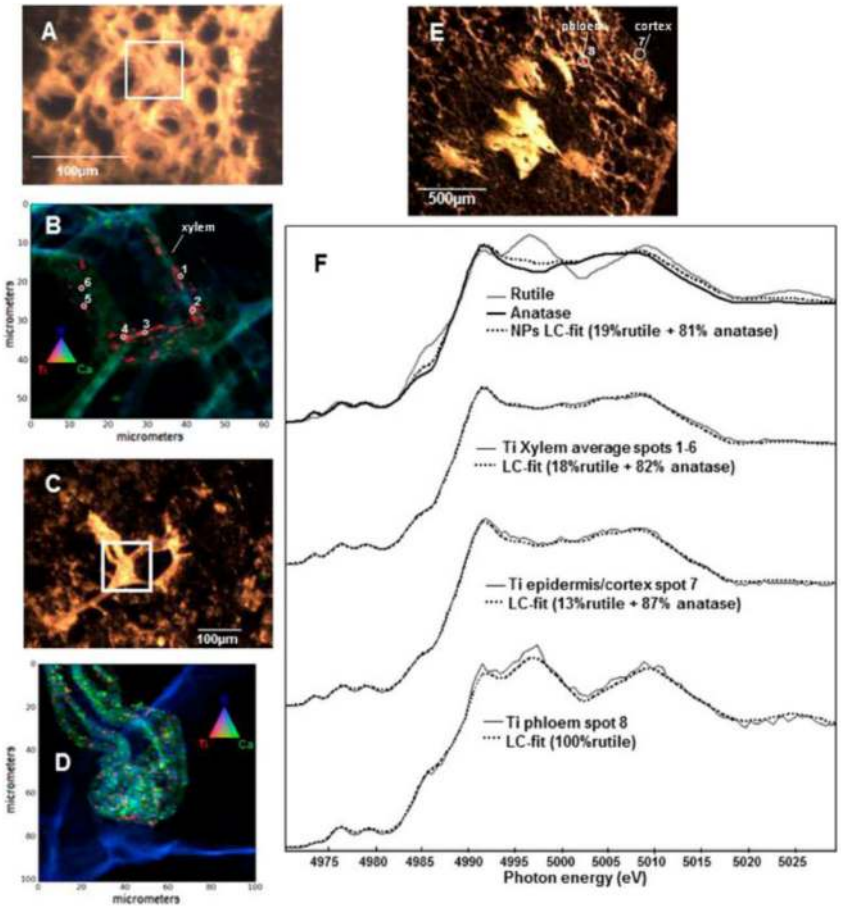

Fig. 2.

Images from Cucumber plant root cross section after treatment for 15 days with 500 mg L^{-1} TiO_2 NPs. Image of cucumber root vascular cylinder (A) (Video microscope) and a phloem cylinder (C). Tricolor micro-XRF images of cucumber root xylem (indicated in A) (B) and phloem cylinder (indicated in C) (D). Color code indicates titanium (red), calcium (green) and potassium (blue). (E) Cucumber root cross-section image (Video microscope) shows white circles (7 and 8) indicating the areas where micro-XANES was acquired. Map acquired at 5.1 KeV, 200 ms dwell time and 0.3 μm^2 pixel. (F) Micro-XANES spectra of marked spots in (B), (E) and reference materials (TiO2 NPs anatase and rutile). Spots 1–6 were acquired in focused mode (beam size 0.33 × 0.65 μm^2) and spots 7 and 8 with the use of a 50 µm pinhole. Reprinted with permission from Environ*mental Science &* Technol*ogy*, **46** (14), 7637–7643. Copyright 2012 American Chemical Society (Ref. 18).

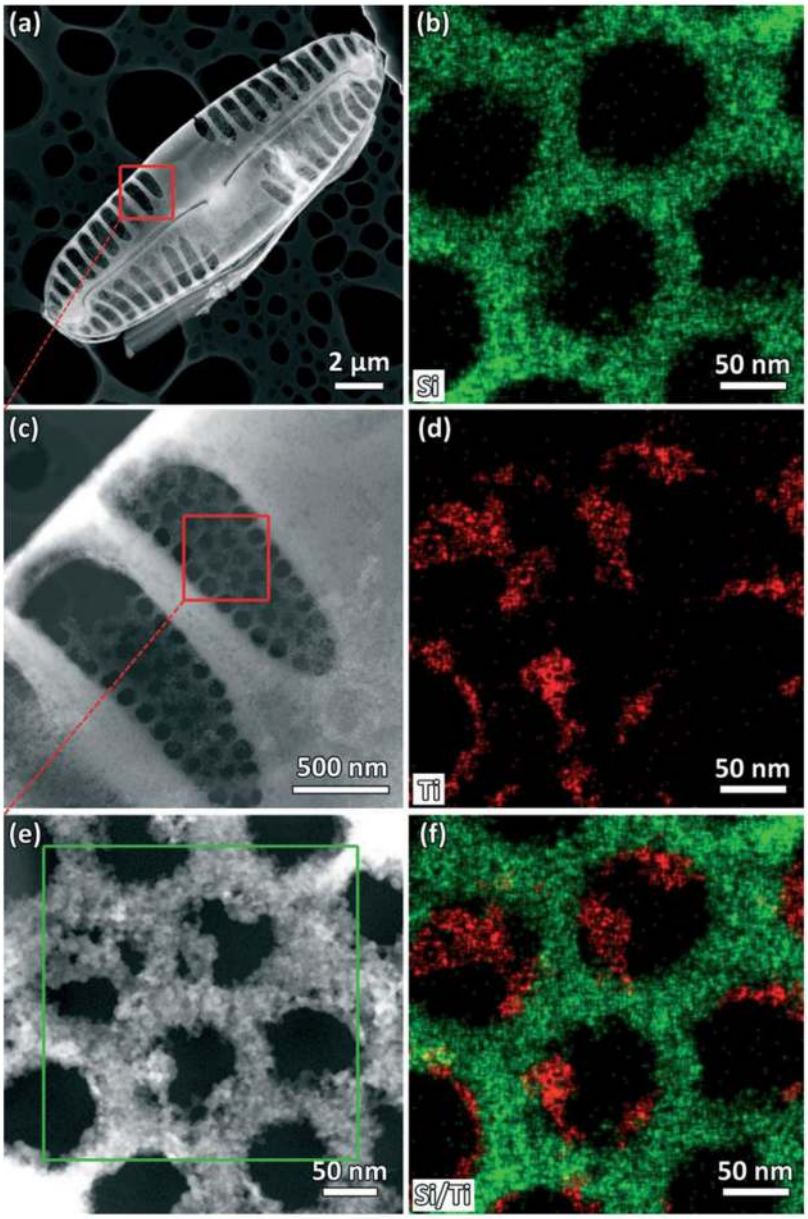

Fig. 3.

Titanium imaging in diatoms cultivated in the presence of a titanium source. (a, c and e) HAADF-STEM images, at different magnification of silica-titania frustules cultivated with 9 mg Ti-TiBaldH L-1, pre-treated with HNO_3 and calcined at 550° C; (b, d and f) EDX elemental maps of the area outlined in (e). Color code indicates silicia (green) and titanium (red). Reprinted from Ref. 22 with permission from the Royal Society of Chemistry.

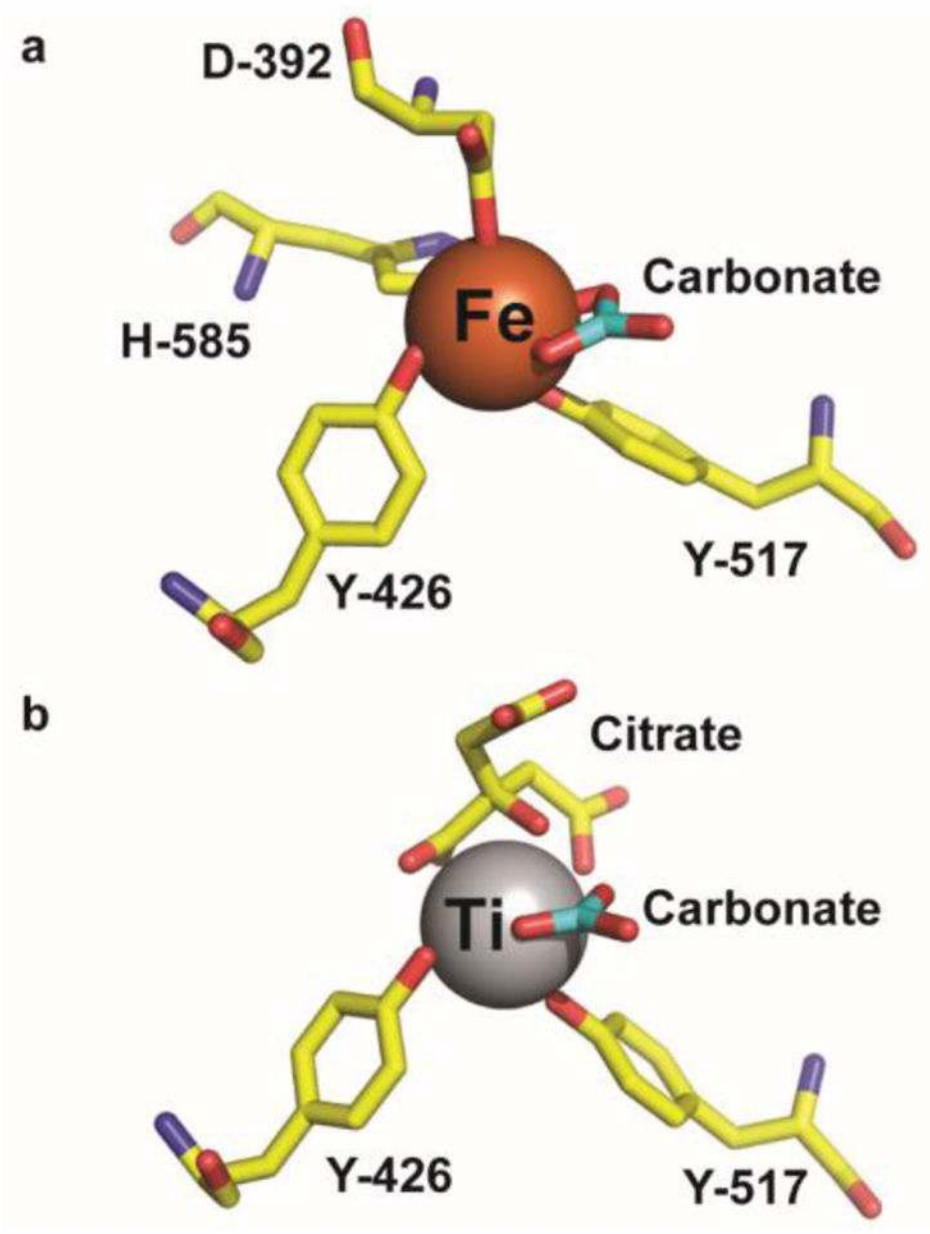

Fig. 4.

X-ray characterization of the coordination of Fe(III) and Ti(IV) by serum transferrin. (a) The C-lobe metal binding site of serum transferrin (3QYT) shows Fe(III) bound to two tyrosine, one histidine, and one aspartate residues and to the synergistic anion carbonate. Fe(III) is bound in a closed protein conformation. (b) Ti(IV), however, is bound by citrate in place of the histidine and aspartate residues resulting in an open conformation (5DYH). Adapted with permission from *Journal of the American Chemical Society*, **138** (17), 5659–5665. Copyright 2016 American Chemical Society (Ref. 33).

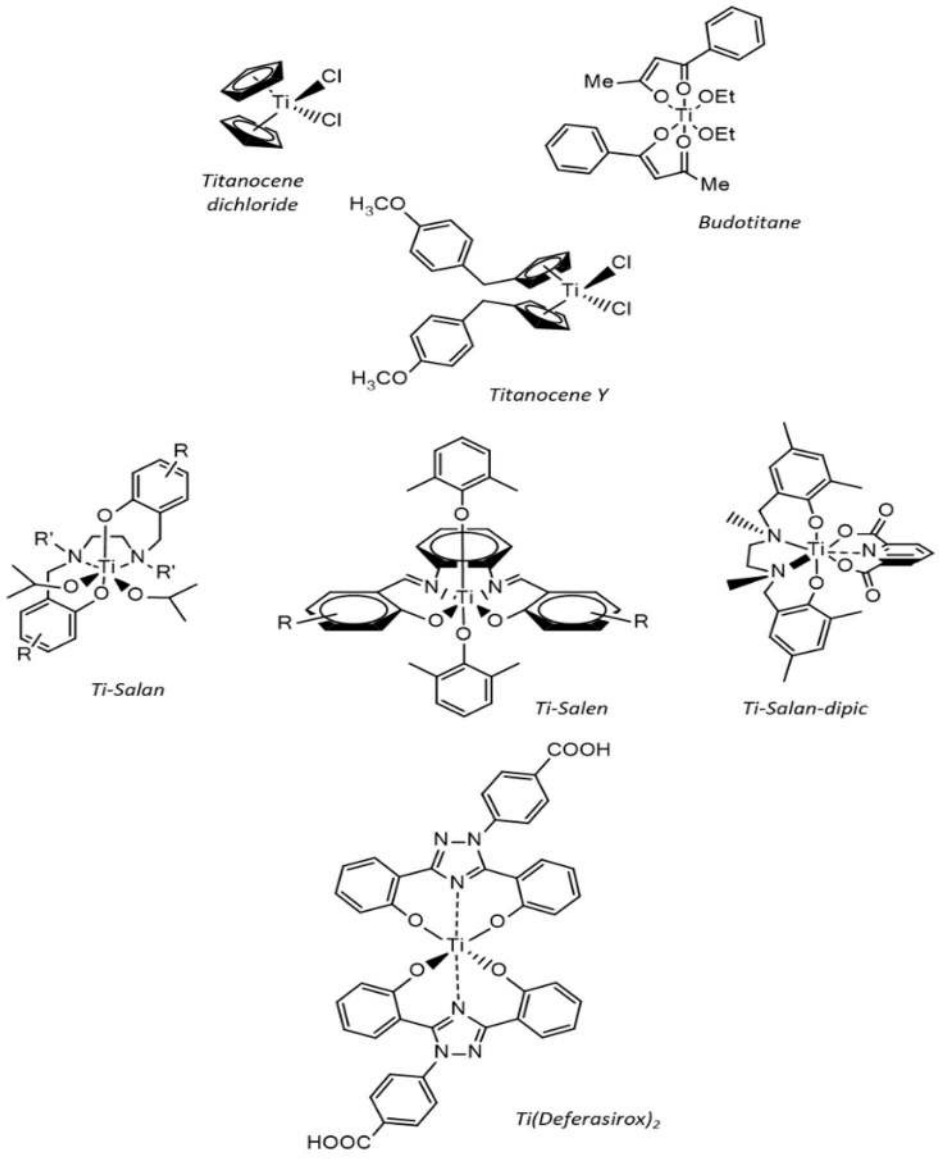

Fig. 5.

The original lead Ti(IV) compounds, titanocene dichloride and budotitane, for anticancer application and the new generation of Ti(IV) compounds.

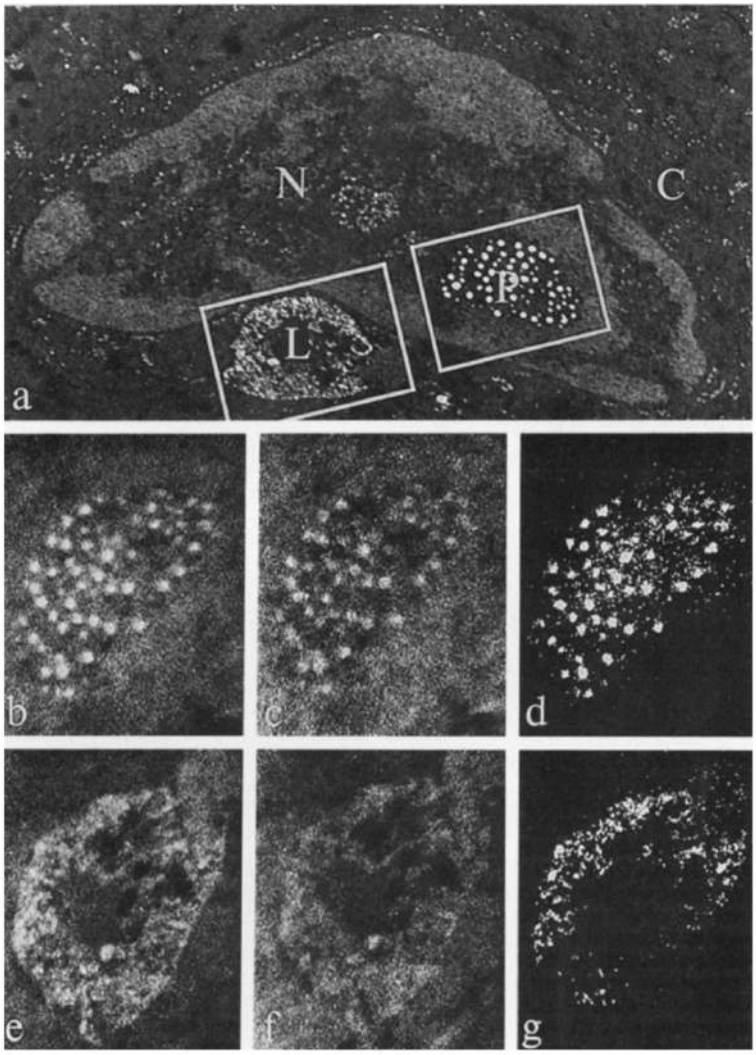

Fig. 6.

Electron-spectroscopic images showing intracellular localization of Ti and phosphorus in a hepatocyte 48 h following application of titanocene dichloride (80 mg/kg b.w.). Image at 150 eV (*a*) confirms phosphorus enrichment in nuclear particles (*P*) and in cytoplasmic inclusion body (*L*). Images at 465 eV (*b, e*), 410 eV (*c, f*), and the net distributions of Ti (*d, g*) in *P* (*b–d*) and *L* (*e–g*) show high concentration of Ti within both structures. Irregular deposition of Ti is noticeable in the cytoplasmic inclusion body, X 38,000 (*a*); X 58,000 (*b–g*). Reprinted from *Acta Histochemica*, **91**(1), Petra Kopf-Maier, Electron-spectroscopic imaging – a method for analysing the distribution of light elements in mammalian cells and tissues, 25–37, Copyright 1991, with permission from Elsevier (Ref. 83).

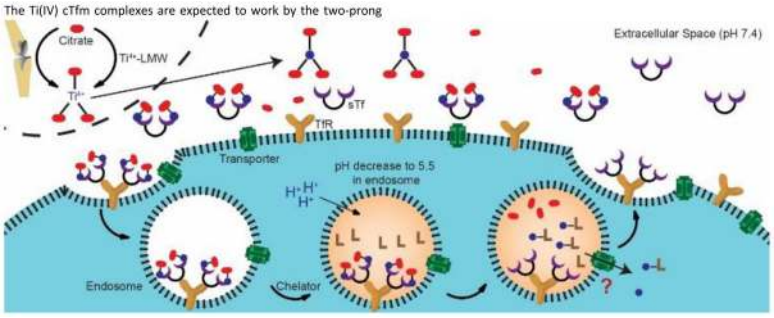

Fig. 7.

Tinoco et al. proposed mechanism of sTf mediated cellular delivery of Ti from Ti-implants and its low molecular weight (LMW) compounds as regulated by citrate. Citrate binds Ti(IV) released from implants or from LMW compounds that enter the bloodstream and then delivers the Ti(IV) to sTf. Ti(IV) bound sTf is recognized by the transferrin receptor (TfR), which transports the metal into the cell via endocytosis. Ti(IV) saturation of sTf may not be a requirement. A chelator (L, unknown) then removes Ti(IV) from the protein complex and the metal is released into the cytoplasm by a transporter (unknown). Reprinted with permission from *Journal of the American Chemical Society*, **138** (17), 5659–5665. Copyright 2016 American Chemical Society (Ref. 33).

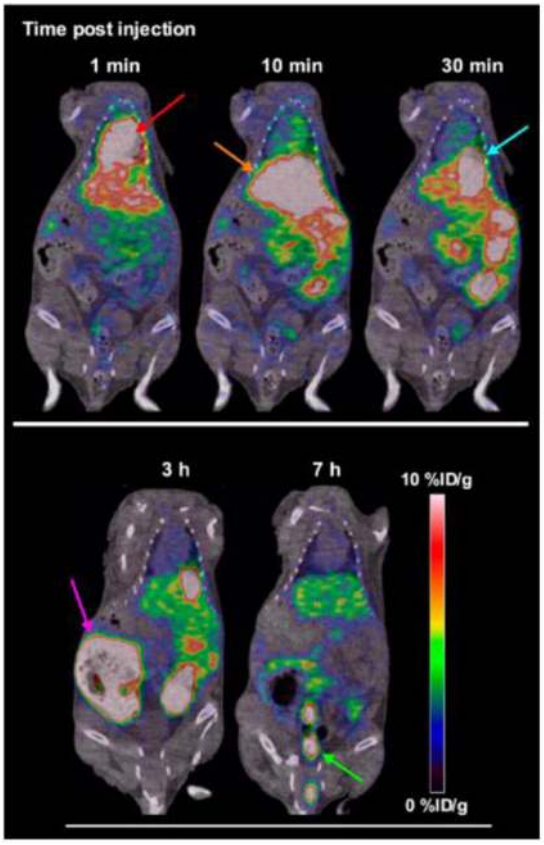

Fig. 8.

PET/CT images taken from female NMRI nude mice bearing dual-flank HT-29 xenograft tumors, following *iv* injection of 1.6 MBq [^{45}Ti(salan)(dipic)], *ncaiv*, at various time points after injection. The arrows point at the regions of interest corresponding to heart/blood (red), liver (orange), gall bladder (blue), cecum (pink) and colon/faces (green). Reprinted with permission from *Journal of Medicinal Chemistry*, **58** (18), 7591–7595. Copyright 2015 American Chemical Society (Ref. 106).

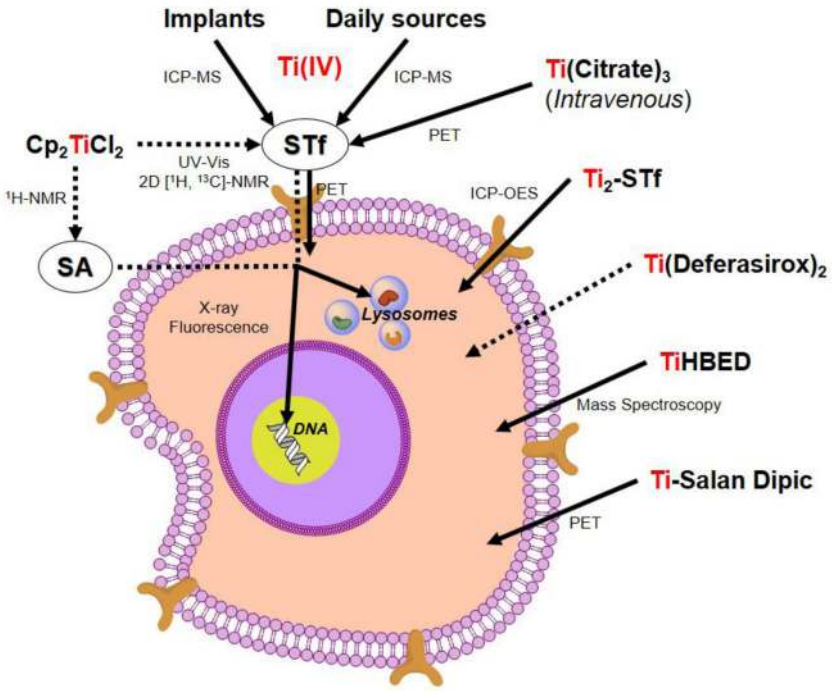

Fig. 9.

This figure highlights the analytical tools that have been applied to study Ti(IV) in the human body (or in appropriate animal models). These tools aid in our current understanding of Ti(IV) transport into cells as facilitated by biomolecular vehicles and small molecule ligands. Nonetheless, more sensitive tools are needed to gain further insight as to the factors that influence Ti(IV) cytotoxicity and potential function and storage in cells.