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# Protein from Tunicates as Bioingredient: Structure Identification, Application and Regulation

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## Abstract

Protein–cellulose moieties, also referred to as protein–cellulose complexes (PC-tun complexes), have been investigated in numerous studies; however, their precise bio-arrangement within tunicate cells remains unclear. Chemically, the principal constituents of the tunics are proteins with amino groups and tryptophan, scleroproteins, collagen and elastic fibers as well as high quantities of acid mucopolysaccharides and neutral polysaccharides. PC-tun complexes are also immerged in a matrix that contain secondary metabolites as fatty acids. To identify, measure, and characterize proteins, various techniques from protein chemistry have been adapted for cytological applications. A range of methods exists for protein separation and identification, each with its own specific protocol.

Traditional qualitative methods of identification well knowing as staining, will be explored in this review based on the colorimetric and fluorescence properties. Four (4) colorimetric principles and their derivations as: (1) Coomassie Brilliant Blue (CBB R-250 and CBB G-250); (2) Silver staining; (3) Zn2+ reverse staining; (4) Biuret method and their derivations that include Cu-protein staining as Lowry method and BCA assay (Bicinchoninic acid - BCA); and fluorescent stains fall into two general categories: (a) fluorogenic stains and (b) intrinsically fluorescent stains will be reviewed as strategy to identify the proteins considering their aspects for application in health and care, food and materials and their regulation accordingly FDA and EU was discussed in this literature background review Enhance safe design and application became the most important requisite for a material like T-complex since well-established and characterized accordingly EU and FDA regulations agencies. This both agencies were the most informative and clear about new materials, their definition and application concerns were discussed in this review study.

**Keywords:** Protein; Cellulose; Staining; Regulations; Moietes

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## Introduction

Understanding the biocomposition and bioarrangement of chemical components in the raw material can provide unique properties to final products that incorporate these bio-based ingredients. In this context, the T-complex emerges as a novel bioingredient that has been physically, chemically, and structurally characterized in this study. T-complex is made from fibrils extracted from tunicates from Styela plicata. Tunicates are a class of marine invertebrates present throughout the globe and have been used to obtained many different states of cellulose like materials for bioinspired application [1]. They grow in coastal communities, and they present themselves as residues and invaders in the marine ecosystem. These organisms contain cellulose and protein in their structural framework and chemical composition, forming a novel chemical structure known as protein–cellulose complexes (PC-tun complexes).

The main challenge for producing nanocellulose from tunicates have been the lack of raw material available for large-scale harvesting and the limited amount of literature available on their isolation and their characterization T-complex extraction can be done using production routes that involve a combination chemical, aseptic, enzymatical and mechanical methods. Wood has been modified *via* various top-down approaches to take advantage of these cellulose nanofibres [2]. The big part of

 

this processes involve homogenization, refining, mill grinding and their combination with chemical or enzymatic reactions [3]. In this study, the primary areas of impact for applying this new bio-solution include materials, cosmetics, and biomedical applications. As a novel bioingredient, several safety and regulatory aspects still need to be clarified. However, the unique potential of biocompatibility linked to the renewability character will contribute for the existent technical requirements of defined application products and will bring new functionalities.

## Cellulose-protein moieties (PC-tun complexes)

Protein-cellulose moieties or also called protein-cellulose complexes (PC-tun complex) have been investigated in many studies without clear elucidation of their bio-arrangement in the tunicate cell. The ultra and suprastructure of cellulose is well known for wood-derivated materials, as well cellulose-lignin complexes in the wood. In case of the tunicates the binding of protein in the cellulose fibrils is still unknown. The protein content in tunicates is secreted and form a complex, called gelatinous filter feeding house. The house comprises cellulose [4,5] and at least 30 proteins [4,6,7] and is secreted by an epithelium [4,8]. Recently studies [9] showed that protein content of the tunic in Styela plicata was 25.82%.

Chemically, the principal constituents of the tunics are proteins with amino groups and tryptophan, scleroproteins, collagen and elastic fibers as well as high quantities of acid mucopolysaccharides and neutral polysaccharides [9,10]. If it is assumed that the nitrogen content is originated from protein, 25.82–38.08% of the dry weights of SP (Styela plicata), CI (Ciona intestinalis) and HR (Halocynthia roretzi), while the lowest protein content (17.74%) can be found in AS (Ascidia sp) [9].

To enhance and hold the links involved in the PC-tun complex, some sugar residual was identified in different classes [11,12]. Three fractions of different molecular weights: one fraction has a molecular weight of 100,000 (C1: Class 1) or more and more two with approximately 20,000 (C2: Class 2) and 8,000 (C3: Class 3) Daltons. In each class, some residual sugar could be identified as: C1 (Mainly Galactose, Gal); C2 (Mainly galactose and glucose, Gal and Glu); C3 (Mainly hexoses and sulfated esters, Gal, Glu, Manose-Man and Xylose-Xly) [11,13]. These sugars are considered to be the link between cellulose and protein in the PC-tun complexes as shown in **Figure 1**. It is important to mention that the binding interface of protein and cellulose is governed by specific linkages and the most frequent are O-linked glycans [9]. For C1, hexosamines are classified as N-glycans components (GalNH and GlcNH) linked β-1→4 0-glycan [1,2] and possible OH terminals in the cellulose for this binding are in the carbons C2, C4 and C6. For classes C2 and C3, sulfated esters, sulphated glycans and N-terminal units are classified as R-$OSO_3^-$ linked at carbons C3 and C4 [12,13].

Sulfated glycans are mainly classified as α-L→galactopyranose [5]. Finally, N-end-units are mainly classified as Gal and Xly major monosaccharides at the reducing terminal of glycans [13]. A unique property of the PC-tun complex is its composition of three classes of sugars, where linkages at the C1 position can be considered neutrally charged, while substitutions at the C2 and C3 positions with R–$OSO_3^-$ groups confer anionic character [5]. PC-tun complexes are also immersed in a matrix that contain secondary metabolites. 0.28–4.25% of the tunic consists of fatty acids such as lipids [9]. This class of components in a study for Ciona intestinalis, Ascidia sp., Halocynthia roretzi and Styela plicata showed 0.48; 4.25; 0.28 and 0.53% respectively in the chemical composition [9] of the tunicates. Secondary metabolites are normally recognized also in other materials as wood as easily extracted. Pre-treatments using water or solvents at high temperatures could remove these components. A great example is the lipids content in tunicates. Prehydrolysis step removed all lipids and ash without largely reducing the protein content [9]. Future attention in these studies is essentially in these determinations once a few information was founded in the literature. Since 1940`s various techniques have been adapted from protein chemistry to the cytological demonstration of proteins (e.g., ninhydrin reaction [14] Millon reaction [15] and Sakaguchi reaction [16,17].

Electrophoresis is one of the separation techniques usual applied for the protein separation and measurement, following by the staining techniques. The solution to check purity issues can be tackled by running a denaturing Sodium Dodecyl Sulfate (SDS)-polyacrylamide gel. This electrophoresis process separates proteins from a mixture based on size [18]. Protein electrophoresis is used to separate proteins based on their size and charge. Electrophoresis uses an electric field to move charged molecules through a gel matrix. Proteins, being charged molecules, will migrate towards the electrode of opposite charge. The rate at which they migrate depends on their size, shape, and net charge. Exist two types of protein electrophoresis: (a) Native PAGE: Proteins are separated based on their native conformation and charge and (b) SDS-PAGE: Proteins are denatured and separated based on their molecular weight [19].

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a specific type of electrophoresis that denatures proteins and coats them with negative charge, such that they can be separated primarily by their molecular weight and not their shape and charge [19]. Considering protein as the second major component of protein-cellulose and their complexes linkages and organizations at cell level in tunicates, find feasible techniques to characterize this material is one of the challenges. However, a traditional qualitative method of identification well known as staining, could allow the fast identification based on their colorimetric and fluorescence properties.

## Colorimetric or chromogenic detection

The simplicity of detection by visual inspection and widespread familiarity among users make Coomassie Brilliant Blue (CBB) staining the most used method for total protein detection in gels [20]. **Table 1** presents traditional colorimetric methods of stain and their target compound in the sample, detection method and mechanism of interaction with the reactional surface. In this study the protein portion is the target compound considering all the possible samples although the visual detection method by optical microscope. This review considered four principles and their derivations as: (1) Coomassie Brilliant Blue (CBB R-250 and CBB G-250); (2) Silver staining; (3) $Zn^{2+}$ reverse staining; (4) Biuret

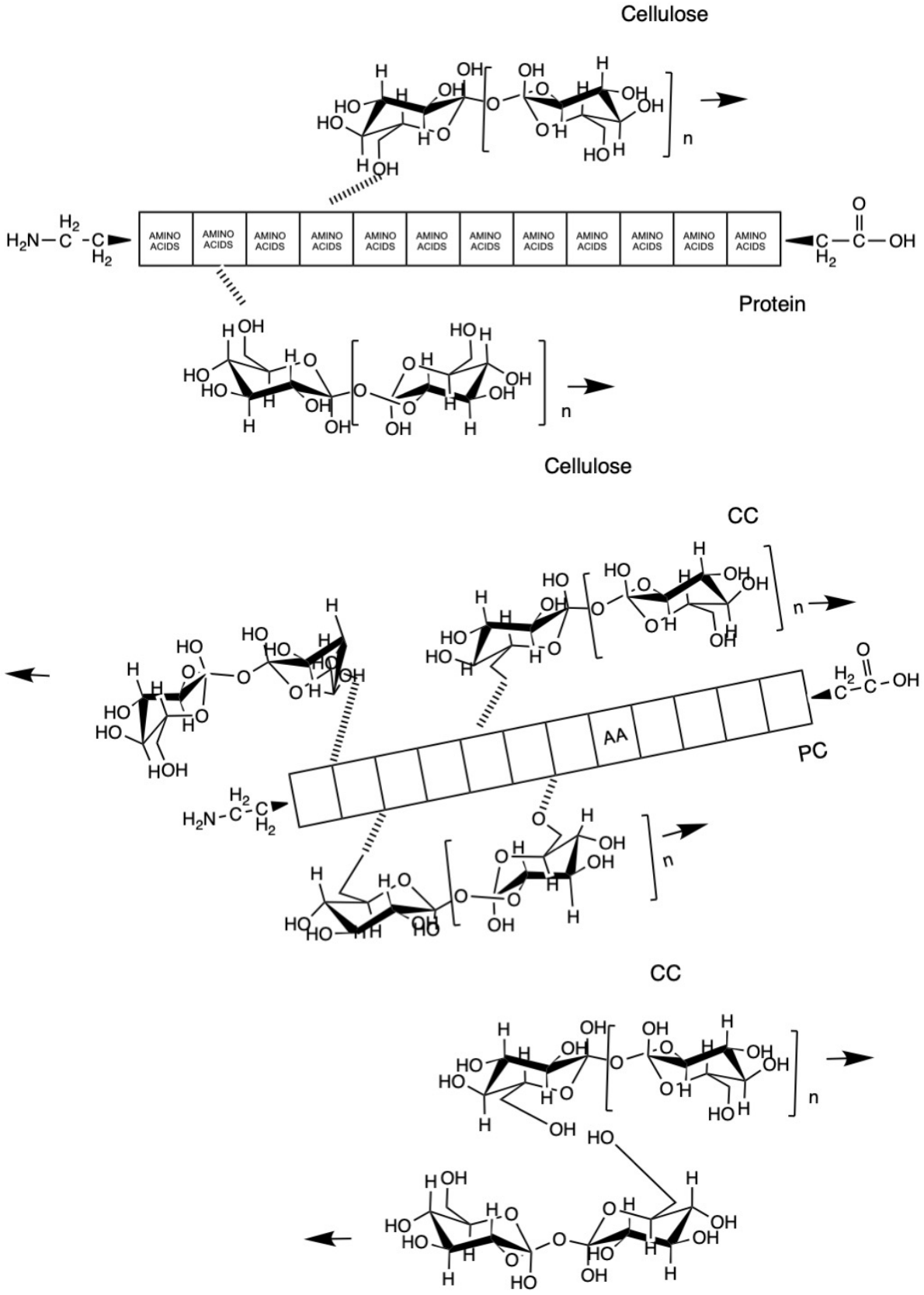


**Figure 1** Proposed model of PC-tun complexes, showing cellulose-protein possible interactions. A – Plan proposed model and B – Rotate proposed model; PC – protein chain composed by β-catenin protein primary general structure of central domain consisting of 12 MER repeats and amino-carboxylic acid end groups (R-N and R-COOH); AA – amonoacids repeating unit, each square represents an AA; CC -Cellulose chains starting their synthesis being grafting proteins and lipocompounds to form the filter feeding. The arrows representing the chain direction.

**Table 1.** Colorimetric total protein stains detection methods and their action via chemical interaction activated by bio-mechanism with the surface target compound.

| Colorimetric total protein stains | Target | Detection | Mechanism | Reference |
|---|---|---|---|---|
| CBB R-250 | Total protein | Visual inspection | Dyes bind via electrostatic interaction with protonated basic amino acids (lysine, arginine, and histidine) and by hydrophobic associations with aromatic residues | Steinberg TH [20] |
| CBB G-250 | Total protein | Visual inspection | Dyes bind via electrostatic interaction with protonated basic amino acids (lysine, arginine, and histidine) and by hydrophobic associations with aromatic residues. | Steinberg TH [20] |
| Silver staining | Total protein | Visual inspection | Reduction of protein-bound silver ions to metallic silver and, to a lesser extent, in some protocols, localized deposition of silver sulfide. | Steinberg TH [20] |
| Zn2+ reverse staining | Total protein | Visual inspection | Zinc ion ''reverse'' staining utilizes the ability of proteins and protein–SDS complexes to bind and sequester Zn2+ in a milieu where the precipitation reaction between imidazole and zinc ion produces zinc imidazolate (ZnIm2) to create an opaque background, contrasting with transparent protein–SDS–Zn2+ zones | Steinberg TH [20] |
| Biuret method | Total protein | Visual inspection | Copper sulphate is added to a protein solution in strong alkaline solution. A purplish-violet colour is produced, resulting from complex formation between the cupric ions and the peptide bond. | Sapan CV, et al. [21] |
| Lowry method | Total protein | Visual inspection | Based on the amplification of the biuret reaction by subsequent reaction with the Folin phenol reagent. | Sapan CV, et al. [21] |
| BCA assay (Bicinchoninic acid - BCA) | Total protein | Visual inspection | Uses BCA to detect the cuprous ions generated from cupric ions by reaction with protein under alkaline conditions. | Sapan CV, et al. [21] |

method and their derivations that include Cu-protein staining as Lowry method and BCA assay (Bicinchoninic acid - BCA).

## Fluorescence detection

For the purposes of discussion, excitation and emission light are commonly categorized into broad wavelength ranges: ultraviolet (UV), 250-400 nm; blue, 400–500 nm; green, 500–550 nm; yellow/orange, 550–580 nm; red, 580–650 nm; near infrared, 650–850 nm [20]. The underlying process of fluorescence involves the absorption of light energy (a photon) by an indicator followed by the emission of some of this light energy (as another photon) a few nanoseconds later. Because some energy is lost in this process, the emitted photon has less energy than the absorbed photon. Light with a short wavelength (toward the blue) has higher energy than light with a long wavelength (toward the red). Therefore, light emitted from an indicator usually has a longer wavelength than that of the absorbed (excitation) light. This change is called the Stokes shift. **Figure 2** shows the molecular transitions explaining these processes that can be depicted in terms of Jablonski energy diagrams.

Finally, fluorescent stains fall into two general categories: (a) fluorogenic stains that show significant fluorescent enhancement corresponding to localization with protein bands and (b) intrinsically fluorescent stains that bind selectively to protein bands and do not bind to the gel matrix present excitation peaks in different wavelengths as showed in **Table 2**. Detection is instrumentation dependent, requiring a monochromatic excitation light source, selective optical filtration to separate the longer wavelength emitted light from the shorter wavelength (and much brighter) excitation light, and a detection mode. For many fluorescent stains, detection can also be by visual inspection, but with reduced sensitivity in comparison to photographic or instrumentation method as microscope [20]. Table 2 shows fluorescent protein gel stains. Fluorescent protein gel stains discussed in the text are summarized regarding stain specificity, excitation, emission maxima and vendor supplier.

## Bioingredient application: Health and care, food and materials

T-complex is a new bioingredient that can potentially be safely used in many areas, such as cosmetics, special coatings and biomedicine. According to Cosmetics Regulation (EC) No 2009/1223 of the European Parliament and of the Council [27] since 2013, to regulate some chemical materials with different, classes and functions the following definitions shall apply: "(a) 'cosmetic product' means any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours"; "(b) 'substance' means a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition"; "(c) 'mixture' means a mixture or solution composed of two or more substances".

Considering the (EC) No 2009/1223 [27], it has been created an online database (CosIng) to search and find regulated ingredients. CosIng is an information-only database that provides

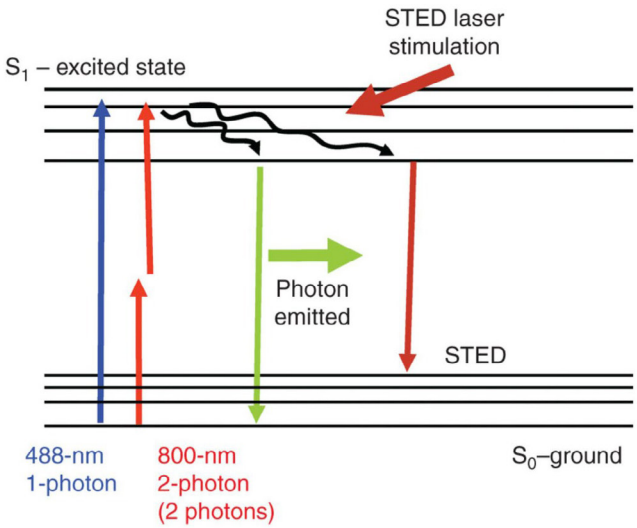


**Figure 2** Changes in electron state of fluorescent indicators during photon excitation and emission (Jablonski profiles). Excitation (from S0 to S1) induced by 488 nm laser light (blue) requires one photon or by two-photon 800 nm light (red) requires two photons. After relaxation to the lowest energy levels, the reverse transition (from S1 to S0) releases a photon of longer wavelength (green). The incidence of photons at 592 nm (STED wavelength) induces the transition from S1 to high levels of S0 and suppression of fluorescence [20].

**Table 2.** Fluorescent protein gel stains.

| Fluorescent protein gel stain | Target | Excitation peaks (nm) | Emission peaks (nm) | Vendor | References |
|---|---|---|---|---|---|
| Nile red | Total protein | 270,550 | 600 | Sigma-Aldrich | Steinberg TH [20,22] |
| SYPRO Orange | Total protein | 280,470 | 569 | Life technologies | Steinberg TH [20,24] |
| SYPRO Red | Total protein | 280,547 | 631 | Life technologies | Steinberg TH [20,24] |
| SYPRO Tangerine | Total protein | 280,490 | 640 | Life technologies | Steinberg TH [20,24] |
| SYPRO Ruby | Total protein | 280,450 | 610 | Life technologies | Steinberg TH [20,24] |
| Deep Purple | Total protein | 400,500 | 610 | GE Healthcare | Steinberg TH [20,24] |
| Krypton | Total protein | 520 | 580 | Thermo Fisher | Steinberg TH [20,25] |
| Krypton Infrared | Total protein | 690 | 718 | Thermo Fisher | Steinberg TH [20,25] |
| Flamingo | Total protein | 270,512 | 535 | Bio-Rad | Steinberg TH [20,26] |
| LUCY 506 | Total protein | 505 | 515 | Sigma-Aldrich | Steinberg TH [20,26] |
| LUCY 569 | Total protein | 569 | 580 | Sigma-Aldrich | Steinberg TH [20,23] |
| C16-FL | Total protein | 470 | 530 | Life technologies | Steinberg TH [20,24] |
| Pro-Q Diamond | Phosphoprotein | 555 | 580 | Life technologies | Steinberg TH [20,25] |
| Phos-tag 300/460 | Phosphoprotein | 300,460 | 630 | Perkin Elmer | Steinberg TH [20,25] |
| Phos-tag 540 | Phosphoprotein | 540 | 570 | Perkin Elmer | Steinberg TH [20,25] |
| Pro-Q Emerald 300 | Glycoprotein | 288 | 533 | Life technologies | Steinberg TH [20,25] |
| Pro-Q Emerald 488 | Glycoprotein | 512 | 525 | Life technologies | Steinberg TH [20,24] |
| Krypton glycoprotein | Glycoprotein | 654 | 673 | Thermo Fisher | Steinberg TH [20,24] |
| Glycoprofile III | Glycoprotein | 430 | 480 | Sigma-Aldrich | Steinberg TH [20,24] |
| TAMRA alkyne | O-GlcNAc | 545 | 580 | Life technologies | Steinberg TH [20,24] |
| Dapoxyl alkyne | O-GlcNAc | 370 | 580 | Life technologies | Steinberg TH [20,25] |

a distinction between ingredients and substances. By convention in CosIng [28]: ''Ingredients are considered all entries included in the CosIng inventory for labelling purposes and are displayed in capital letters (e.g. ETHANOL) and with the symbol "I".

The inclusion of an ingredient in the CosIng inventory is not an indication of authorization, only the Cosmetics Regulation (EC) No. 1223/2009 has legal value in this respect" and "Substances are considered only those chemical elements and compounds that are regulated by the Cosmetics Regulation (i.e. listed in the Annexes to this Regulation). They are included in the CosIng inventory for information purposes (only the Cosmetics Regulation has legal value) and displayed in small letters (e.g. Formaldehyde) and with the symbol "S"".

Thus, considering the definitions above, T-complex is a renewable bioingredient that have their functions listed in the 65 functions of cosmetic ingredients classified between 35 classes of products

as natural cellulosic material. In the CosIng inventory the ascidian tunic extract, descript as "ascidian tunic extract is the extract of the tunic, or outer skin, of ascidians (sea squirts)" was listed for safe cosmetic applications [29]. The T-complex still offers promising application opportunities, both as part of ingredient mixtures and as an active ingredient. When applied to material surfaces, it can form and stabilize edible films, which have the potential to replace non-renewable plastic and non-plastic films **(Figure 3)**.

According regulation [30] (EC) no 1935/2004 of the european parliament and of the council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC some definitions shall apply to the application of this new ingredients as: "(a)'active food contact materials and articles' (hereinafter referred to as active materials and articles) means materials and articles that are intended to extend the shelf-life or to maintain or improve the condition of packaged food. They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food"; "(b)'intelligent food contact materials and articles' (hereinafter referred to as intelligent materials and articles) means materials and articles which monitor the condition of packaged food or the environment surrounding the food".

The design of new eco-friendly coatings based on biodegradable polymers, such as the T-complex, not only reduces packaging demands but also promotes the conversion of food industry byproducts into value-added, film-forming components [31,32]. Diverse biological materials used in edible packaging formulations generally fall into the categories of polysaccharides, proteins, lipids and resins; combined materials can also be used [31].

Bioingredients can be obtained from marine and terrestrial sources. The importance of ingredients with bioactive substances has been well recognized in connection with health promotion, disease risk reduction and reduction in health care costs. Because of its unique functional properties, these compounds are widely used in food industries, pharmaceutical preparations, cosmetic [33] and material industries in the case of food contact and food additivation.

According to the [34] Chapter I —Food and Drug Administration, Department of Health and Human Services, Subchapter B —Food for Human Consumption, Part 170 —Food Additives, when comes to new bioingredients some definitions shall apply the discussion: "(m) Food includes human food, substances migrating to food from food-contact articles, pet food, and animal feed";"(1) Food additives include all substances not exempted by section 201(s) of the act, the intended use of which results or may reasonably be expected to result, directly or indirectly, either in their becoming a component of food or otherwise affecting the characteristics of food. A material used in the production of containers and packages is subject to the definition if it may reasonably be expected to become a component, or to affect the characteristics, directly or indirectly, of food packed in the container. "Affecting the characteristics of food" does not include such physical effects, as protecting contents of packages, preserving shape, and preventing moisture loss.

If there is no migration of a packaging component from the package to the food, it does not become a component of the food and thus is not a food additive. A substance that does not become a component of food, but that is used, for example, in preparing an ingredient of the food to give a different flavor, texture, or other characteristic in the food, may be a food additive";."(2) Uses of food additives not requiring a listing regulation. Use of a substance in a food contact article (e.g., food-packaging or food-processing equipment) whereby the substance migrates, or may reasonably be expected to migrate, into food at such levels that the use has been exempted from regulation as a food additive under § 170.39, and food contact substances used in accordance with a notification submitted under section 409(h) of the act that

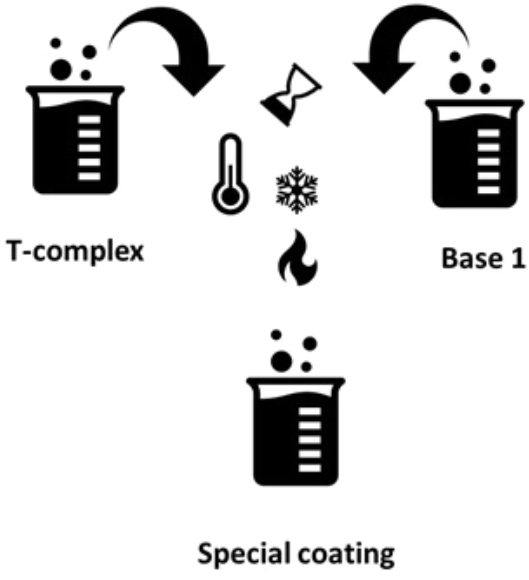


**Figure 3** Special coating preparation flowchart for application in materials.

is effective"; "(3)A food contact substance is any substance that is intended for use as a component of materials used in manufacturing, packing, packaging, transporting, or holding food if such use is not intended to have any technical effect in such food".

Thus, considering the definitions, T-complex is a renewable bioingredient that in the future could have applications as a nutraceutical ingredient and their functions are listed in the 32 functions of food additives list [34] classified between 43 classes of food products [34]. Nutraceuticals have been used not only for nutrition but also as a support therapy for the prevention and treatment of various diseases, such as to reduce side effects of cancer chemotherapy and radiotherapy [35]. Finally, one of the important function of nanoparticles as bioingredients, that have been studied, is the [36] Drug Delivery (DD) system enables the release of the active pharmaceutical ingredient to achieve a desired therapeutic response **(Figure 4)**. A drug (API) is a substance (recognized in official pharmacopoeia) intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease as per the FDA. Drug delivery is a technique of delivering medication to a patient in such a manner that specifically increases the drug concentration in some parts of the body as compared to others [34,35].

The Biopharmaceutics Classification System classifies drugs into four types based on their permeability and solubility [36-38].

(1) Class I drugs possess high permeability and high solubility, and are well absorbed; their absorption rate is greater than excretion (e.g., metoprolol, paracetamol, etc.)

(2) Class II drugs have high permeability, but low solubility and the bioavailability is restricted by their rate of solvation (e.g., glibenclamide, aceclofenac, etc.)

(3) Class III drugs possess low permeability but high solubility where the drug solvates quickly; nevertheless, absorption is limited by the rate of permeation. If the formulation does not change the permeability or gastro-intestinal duration time, then class I criteria can be applied (e.g., cimetidine)

(4) Class IV drugs have low permeability and low solubility and are poorly absorbed through the intestine; thus, they have poor bioavailability with high variability (e.g., Bifonazole) [36-38].

Many properties of the new bioingredient will be important considering drug-delivery systems, and on based on the pharmacokinetics, the movement of drugs into, through and out of the body—the time course of drug absorption, distribution, metabolism, and excretion. In simple terms, it is what the body does to a drug [31]. Considering this definition, T-complex is a type of nanocarriers in controlled of drug delivery of active substances in or out of their own composition. T-complex is a nanofiber, nanofibers are a nanocarriers in controlled of drug delivery. Nanofibers have a higher surface to volume ratio which is well suited for using them as a carrier for drug delivery. The properties of nanofibers, like diameter, morphology and porosity, can be modulated to obtain a wide range of drug release kinetics. High loading efficiency and spatial distribution of drugs can be achieved with nanofibrous delivery systems [39-41].

## Regulations and safe assembly

Nanomaterials needs to be regulated and classified due to the possible effects and interactions with the matrix applied, the human body and the environmental. Based on this discussion exist a number of definitions of "Nanomaterial", however some definitions are starting to be regulated for more than one agency, dependent on the country and type of material. The European Commission (EU) adopted on 18 October 2011 Commission Recommendation 2011/696/EU on the definition of Nanomaterial. This recommendation is based on the ISO term standard, a Reference Report of the European Commission's Joint Research Centre (JRC), an opinion of the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), on the 195 contributions received during public consultation 14 in October – November 2010, as well as on a number of other sources [42].

General definition of "Nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound

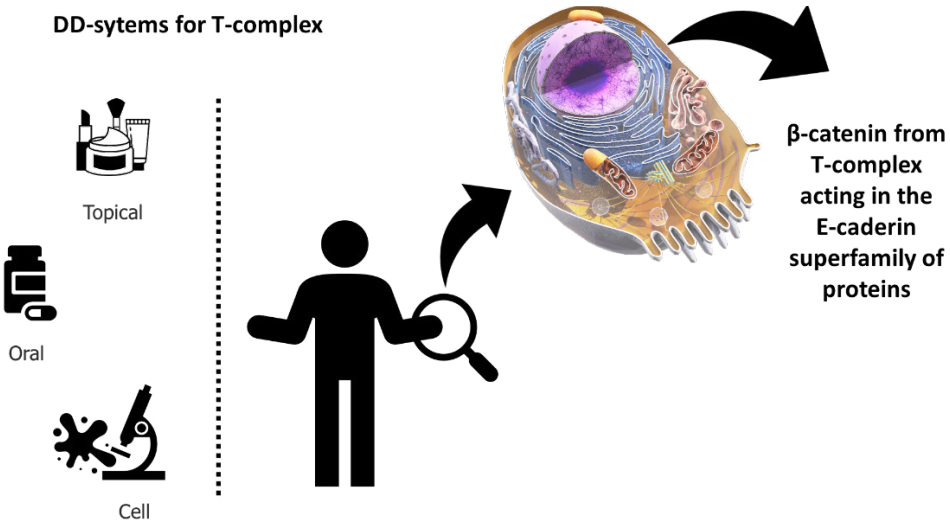


**Figure 4** Flowchart mechanism of DD-systems for β-catenin from T-complex.

state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and were warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50% [42].

The types of Nanocellulose are subdivided into 2 categories: cellulosic nanoobjects and cellulosic nanostructures [38]. The nanoobjects would be composed of discrete materials in the form of CNC crystals (nanocrystalline cellulose) or alternatively of CNF nanofibers (cellulose nanofiber); nanostructures comprising MFC (microfibrillated cellulose) and nanocomposites containing cellulose. T-complex is a CNF in this case classified between these two classes of nanometric structures, with at least one dimension expected to be closer to values below 100 nm, which falls into the category of nanoobjects. Another important aspect related to the new bioingredient T-complex is their chemical composition as already said, mainly protein and cellulose. The protein and cellulose are bonded in the fibril structures forming the complex called T-complex. It is relevant to mention that proteins and their derivates as aminoacids are regulated for a policy well established mainly in the food industry. These regulations are a strong argument that allow studies of this macro, oligo and molecules applied in other sectors as cosmetics and pharmaceutical.

An important role for proteins is their "GRAS" is an acronym for the phrase Generally Recognized As Safe. Under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act (the Act), any substance that is intentionally added to food is a food additive, that is subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excepted from the definition of a food additive [43].

Exist many substances could be checked using the GRAS database called SCOGS (Select Committee on GRAS Substances) [44]. The SCOGS database allows users to search for the SCOGS opinion and conclusion and includes the United States Code of Federal Regulations (21 CFR) citation for those GRAS food substances that have been codified in the CFR. For example, a search using the term “protein” in the SCOGS platform bring three main results as: acid hydrolyzed proteins, enzymatically hydrolyzed protein and soy protein isolate all classified as type 1 of SCOGS. This means that there here is no evidence in the available information on [substance] that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when they are used at levels that are now current or might reasonably be expected in the future [45]. Many of the SCOGS reports reviewed more than one GRAS substance and each substance was evaluated and assigned its own individual type of conclusion on safety. The SCOGS conclusions were made by scientific experts outside of FDA.

Identify the protein portion in the system is also important for some application mainly when are related to protein-delivery systems. Although much information is available on this topic, it is crucial to emphasize the protein interactions with other substances and their corona-effect needs to be carefully evaluated jointly other number of hazardous properties, when applicable. Once NPs (Nanoparticles) interact with biological fluids and come into contact with tissues, they are exposed to active biomolecules that form a ‘crown’ (corona in Latin) around them, thus transforming the bare NP into an NP that has a biological component: the so-called protein corona (PC) [46]. T-complex bioingredient naturally shows a protein enhancement in their structure and the listed applications in the cosmetic, coatings and pharmaceutical areas will take advantage of this structure. **Figure 5** shows the potential of T-complex *vs.* a regular CNF and open a large path of opportunities to introduce this new concept in the material sector [47,48].

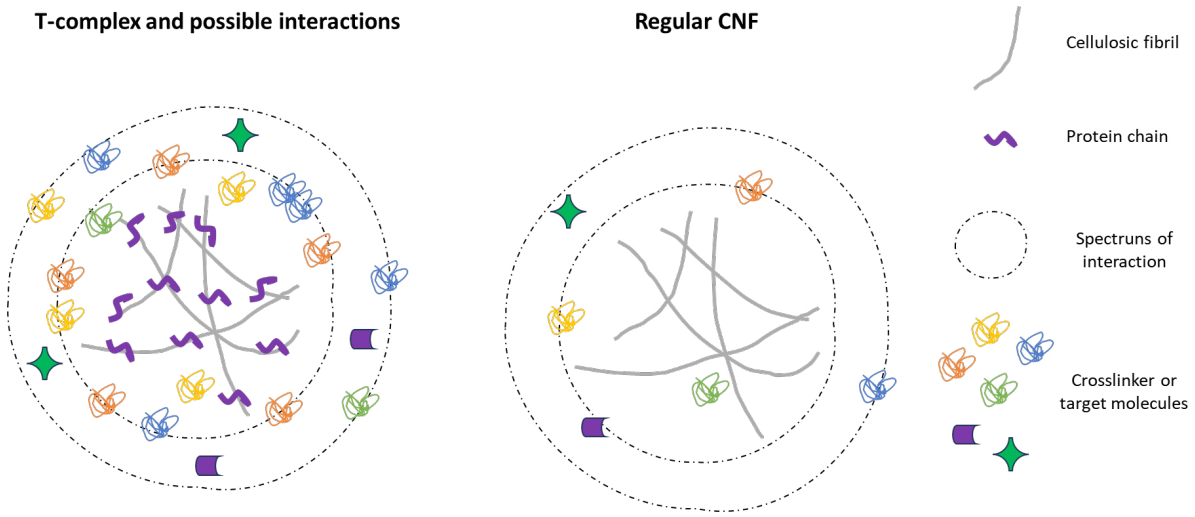


**Figure 5** Proposed spectrum of interactions between the T-complex nanocellulose and a regular CNF with different molecules available in the medium. The spectrum and interactions proposed for T-complex evidence that two levels of interaction could be leaded due to the protein content. This phenomenon is called protein-corona.

## Remarks and Conclusions

PC-tun complexes have a unique portion of protein and their naturally occur in marine tunicates. The extraction of this active compound has been studied without properly elucidation and application. PC-tun complexes are composed of a cellulosic and protein part. The cellulosic part forms the filter feeding tunic in the tunicates and the protein part also attached to the cellulose could have carcinogenic activity. Staining techniques as electrophoresis based on protein staining and fluorescence was shown as one of the key aspects in the development of materials for bioingredients. Enhance safe design and application became the most important requisite for a material like T-complex since well established and characterized accordingly EU and FDA regulations agencies. This both agencies was the most informative and clear about new materials, their definition and application concerns was discussed in this review study.

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