Graz University of Technology

Master's thesis

Enzymatic synthesis of phenolics functionalized chitosan microspheres for iron overload therapy

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Abstract

Excess “free” iron which occurs under certain physiological conditions participates in the formation of toxic reactive oxygen species via the Fenton chemistry. The reactive oxygen species oxidize biomolecules and have been implicated in many oxidative stresses related diseases. However, the ideal therapy for treating iron overload problems in humans has not yet been developed. In this study, the phenolic molecules catechol, caffeic acid and 2,5-dihydroxybenzoic acid were successfully coupled to glucosamine as model substrate for chitosan in a 1:1 ratio using laccase as confirmed by HPLC-MS and FTIR analysis. Furthermore, coupling of these molecules was demonstrated onto chitosans of different sizes, resulting in decrease in $-NH_2$ groups as quantified via derivatization. A concomitant increase in iron chelating capacity from below 3% to up to 70% upon phenolic functionalization was measured for the chitosans based on reduced ferrozine/Fe^{2+} complex formation. Interestingly these phenolic compounds also participated as cross-linkers producing characteristic microspheres. This work therefore opens-up new strategies, which can be explored, aimed at developing a new generation of iron chelating biomedical polymers.

Kurzfassung

Ein Übermaß von freiem Eisen, das sich unter bestimmten physiologischen Bedingungen entsteht, trägt zur Bildung von toxischen Sauerstoffradikalen über die Fenton-Chemie bei. Die reaktiven Sauerstoffspezies oxidieren Biomoleküle, erzeugen oxidativen Stress und sind somit für viele Krankheiten verantwortlich. Denoch gibt es keine optimale Therapie für die Behandlung von Eisenüberladung beim Menschen. In dieser Studie wurden die phenolischen Molekülen Catechin, Kaffeesäure und 2,5-dihydroxybenzoesäure erfolgreich an Glucosamin als Modellsubstrat für Chitosan in einem Verhältnis von 1:1 mit Laccase gekoppelt was durch HPLC-MS-und FTIR-Analyse bestätigt wurde. Weiterhin wurde die Kopplung dieser Moleküle auf Chitosane in verschiedenen Größen bewiesen, was zu einer Abnahme in NH_2-Gruppen führte und über Derivatisierung quantifiziert wurde. Interessanterweise wurde eine gleichzeitige Erhöhung der Eisen-Chelat-Kapazität von unter 3% auf bis zu 70% bei phenolischen Funktionalisierung für die Chitosane über Ferrozine/Fe^{2+} Komplexbildung gemessen. Weiters wurde durch Quervernetzung mit diesen phenolischen Verbindungen Mikrosphären erzeugt. Diese Arbeit eröffnet damit neuer Strategien basierend auf enzymatisch funktionalisierten Chitosanen zur Behandlung von Eisenüberladung beim Menschen.

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1. Literature review

1.1. Iron in human body - positive effects

1.1.1. Iron importance in human body

Iron in the body is located in the blood hemoglobin and muscle myoglobin. Iron is a part of hundreds of enzymes in each cell and it participates in many chemical reactions. There is 3 to 4 grams of iron in the body, from which the 2/3 are the hemoglobin in the red blood platelets with precursors in the bone marrow. Also the content of the muscle is important, liver and macrophage system (Fig. 1). Part of the iron is used for the blood, muscles, enzymes, and ¼ can be stored in reservoirs (liver and macrophages). Generally, men have more iron - 4 g of iron is bound to carriers in the blood, the amount is recycled 5 times per day (Andrews, 1999; Ponka, 1997).

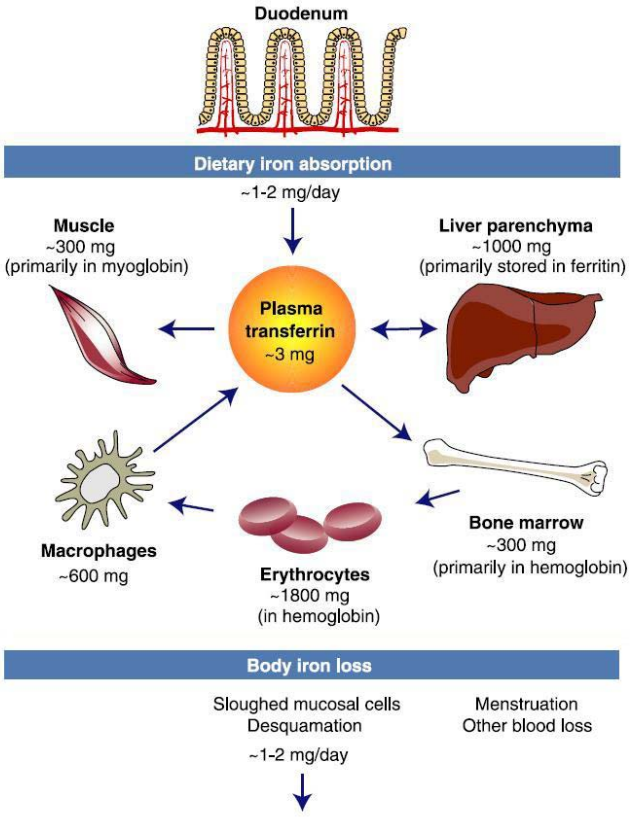


Fig. 1. *Iron distribution in the adult human body (G. Papanikolaou, K. Pantopoulos 2005)*

1.1.2. Iron absorption

The main source of iron is hem in the meat, cca. 2/3 of iron intake. Besides heme iron, there is as well non-heme iron in the food. It is primarily a trivalent iron (but mainly absorbed is the divalent iron). Iron absorption occurs mainly in the front segment of the small intestine - in duodenum. This process is regulated.

- Through resorption of iron controls the amount of iron in the body
- Conversely, elimination of iron is not regulated

Physiological loss of iron occurs through cells peeling, by women during the menstrual bleeding (birth, breastfeeding), etc. Every day 1-2 mg of iron is lost. The organism is not able to remove the excess iron physiologically. Under physiological conditions, adult individual absorbs 1/10 of iron intake from the food. This represents about 1-2 mg of iron which is to replace iron losses. When the need of the iron is increased, for example after previous bleeding, this ratio (1/10) can increase. On the contrary excess of iron and some conditions cause the amount of absorbed iron to decrease.

Absorption of the iron in the apical side of enterocyte is provided by the carriers. From all the different mechanisms of iron absorption in the intestine, the resorption of non-heme divalent iron is the most investigated. Trivalent iron, which is more common in food, is being reduced to divalent iron. The gastric acid and ascorbic acid (vitamin C) play important role in the reduction. The reduction is catalyzed by the enzyme in the enterocyte brush border. Divalent iron then enters into the cell trough DMT1 (divalent metal transporter). DMT1 is able to transport other divalent metals too. Absorption of the hem iron is less explored. To the present day the carrier is not exactly known. Iron is released from hem under the influence of the hemoxigenasy enzyme. Little is known about iron transport inside the intestinal cells.

Iron is stored in the enterocyte in ferritin. Iron leaves the cell on bazoteral membrane due to the cell exporter called feroportin. Iron enters into the blood where its specific carrier transferrin is present. Divalent iron is during the exiting oxidizing into the trivalent iron. This oxidation is done by the molecule cell wall in the enterocyte, called hefestin. In the blood, the trivalent iron is bound to transferrin. 1mol of transferrin can bind two molecules of the trivalent iron.

1.1.3. Recycling of iron

Because iron absorption is a complicated process and iron is potentially toxic, the organism handles it sparingly. The metabolism of iron is largely a closed cycle, where the iron used for the metabolism is already present in the organism. The basis of this cycle is the iron recycling from dissolved red blood cells. Daily, this represents around 20 mg, which is about ten times more than the iron absorbed. Central role in the iron recycling play macrophages which phagocytate the old erythrocytes. Erythocytes are after the phagocytosis dissolved in the macrophage cells and hemoglobin is divided into the globin and the hem, and from the heme iron is released. Iron leaves the macrophage through feroportin, i.e. the same molecule which provides its exit from the intestinal cells. Oxidation from divalent iron to the trivalent iron is done by the plasma protein - celuloplasmin. Celuloplasmin is similar to hefestin, membrane protein in the enterocyte. Both these proteins contain copper. Trivalent iron is binding again to its transporter, transferrin. Absorbed and recycled iron bound to the transferrin is then directed to the cells in the body which need it.

1.1.4. Overview of iron circulation

How does the circulation of iron in the body look like and which are the participants? Bone marrow produces red blood cells, which contain the largest part of iron in the body in the form of heme. The old erythrocyte is removed in the macrophage system. The macrophages release iron which binds to transferrin and transferrin again carries iron to mature erythroblasts in bone marrow. This is the part of closed iron metabolism which represents the largest part of its quantitative cycle, about 20 mg daily. This is several times the amount of the iron contained in the plasma. Also other cells in the body need iron and they are getting it from the transferrin. Moreover, the liver serves as an important reservoir of iron. If there is an iron deficiency in the body, it can be released from the liver reservoir. This cycle of iron is not completely closed. Daily loss of iron occurs due peeling of cells 1-2 mg per day. This amount or any other increased need of the iron is compensated by equivalent absorption of the iron in the duoden. The body is

capable to regulate the absorption process of the iron in the metabolism exactly in the duoden, because there is no regulation of its dispensation.

1.1.5. Entry of iron into cell

The cell which is in need of the iron has on its surface large amount of receptors for the transferrin. On the surface of the precursors of the red cells (which are the main consumer of the iron) several tens or hundreds thousands can be found. Transferrin carrying the iron is with high selectivity bonding onto the transferrin receptor. After it is bonded, it is transferred into the cell by the process known as mediated endocytosis. Receptor with bonded transferrin and iron is traveling from the surface of the cell to the endosome. There the iron will be released - trivalent iron is in endosome reduced to bivalent iron and leaves endosom into the cell through the channel DMT1, which is the same channel iron using to enter the transferrin. The transferrin receptor does not return back to the surface of the cell and releases the transferrin to the bloodstream. Iron can inside the cell, iron can enter the mitochondria where it is inserted by the enzyme ferochelate into the protoporfine. The synthesis of the hem for the hemoglobin or enzymes is thus finished.

iron can be as well reserve-stored. The reserve protein is ferritin and it can store over 4000 atoms of iron. Iron is stored in less reactive trivalent form. Ferritin its self is able to oxidize the iron and the cell can regulate the intake of iron according to its need.
transferrin receptors are used for the transfer of majority of the iron. These receptors bind the transferrin with bonded iron. A cell with the lack of the iron and in need of it - e.g. the precursor of the red cell (has on its surface large amount of transferrin receptors which bind the transferrin) through which iron is transferred into the cell. Surplus of iron is stored into the ferritin. During lack or surplus of iron on the other hand, the number of the transferrin receptors will decrease and the amount of stored ferritin will increase.

1.1.6. Regulation of iron metabolism in cell

Regulation of iron transport and its utilization in the cell is carried out on a post-transcriptional level. It is based on the stability of mRNA which afterwards affects subsequent translation and thus the synthesis of appropriate proteins. For this way regulated proteins mRNA has special hairpin structures at its end which are called iron responsive elements - IRE (Fig. 2). For proteins which acquire iron (for example for transferrin receptors) mRNA has the IRE at the 3'end. mRNA for the proteins connected to the iron usage has the IRE at the 5'end. Example of such proteins is ferritin. These structures can bond special proteins called iron responsive proteins - IRP. Amount of needed iron in the cell determines whether the proteins are active and whether they bond on IRE or not. Adequate amount of iron causes decay or deactivation IRP. mRNA for transferrin receptor is decaying without the bond on IRE. On the contrary mRNA for ferritin is stable, the translation is possible and new molecules of iron-storing ferritin develop. The situation is opposite with the lack of iron. IRP are stable and bond on the IRE on both types of mRNA. mRNA for ferritin is not able to translate and new molecules of ferritin do not develop. mRNA for the transferrin receptor on the contrary stabilizes and participates in the translation. Transferrin molecules are being developed and these then proceed into the cell membranes where they capture the transferrin and iron.

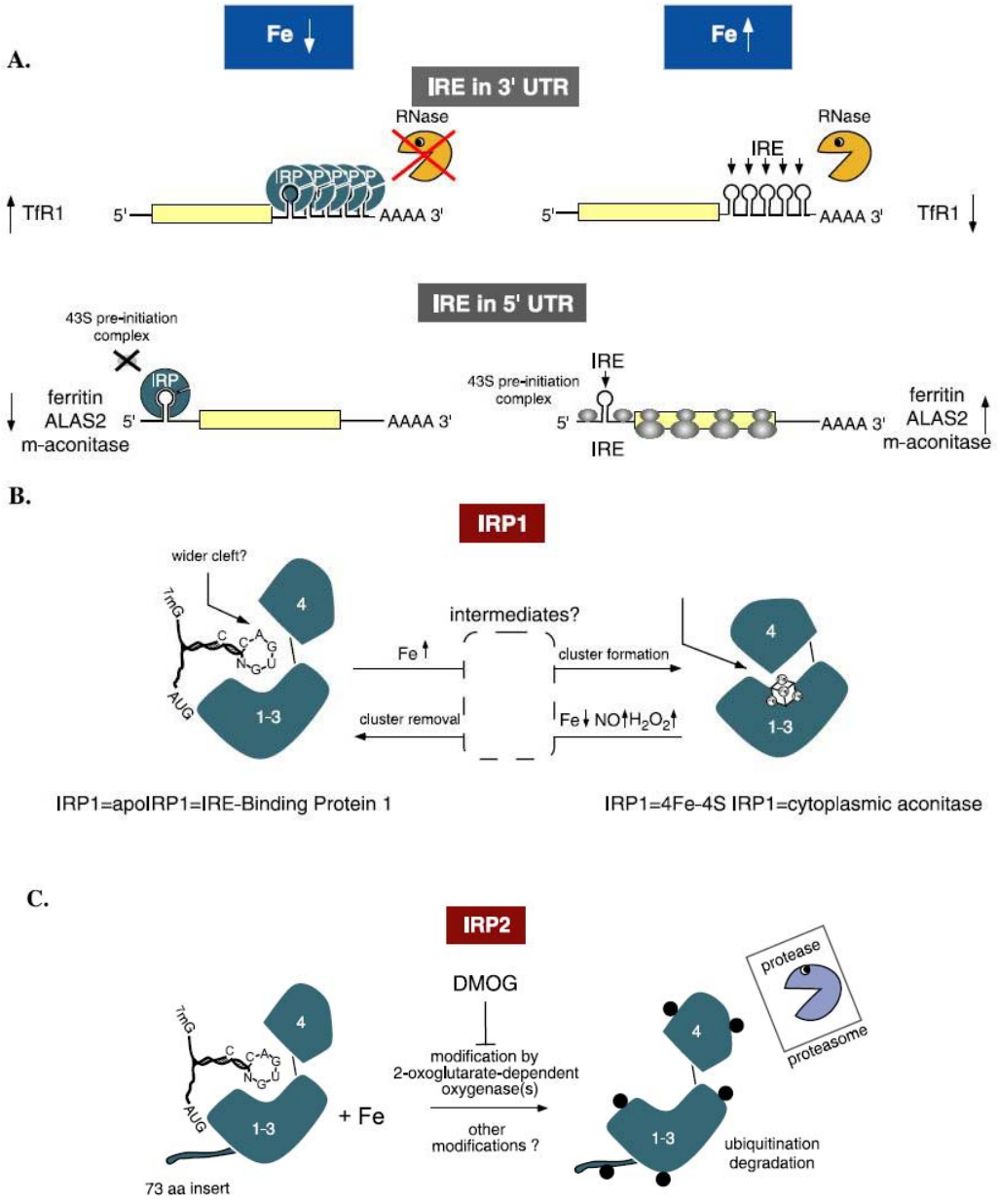


Fig. 2. *(A) Homeostatic responses to iron supply mediated by IRE-IRP interactions. Decreased iron supply activates binding of IRPs to the IREs, resulting in stabilization of TfR1 mRNA and translational inhibition of the mRNAs encoding ferritin. Conversely, IRPs do not bind to cognate IREs in iron-saturated cells, permitting degradation of TfR1 mRNA and translation of ferritin. (B) Posttranslational regulation of bifunctional IRP1 in response to iron, NO, and H2O2 via an iron-sulfur cluster switch. (C) Iron-dependent degradation of IRP2*
(G. Papanikolaou, K. Pantopoulos 2005)

1.1.7. Iron regulation with hepcidin

On the level of organism, the main iron metabolism regulator is hepcidin. Hepcidin is a protein composed of 25 amino-acids (Fig. 3A). It has a special hairpin structure, it contains 4 cystine bridges. The structure is similar to some anti-microbe peptides. Hepcidin is synthesized in the liver and operates in duodenum where it decreases the iron absorption. Therefore during increased iron concentration the production of hepcidin increases and absorption of iron decreases. Opposite situation occurs with the lack of iron. Except enterocyte hepcidin affects macrophages participating on recycling of iron from old erythrocytes. Increased production of hepcidin causes decrease of iron releasing from macrophages. Consequently iron accumulates in macrophages. This process can very quickly reduce the concentration of iron in the plasma, because the iron recycling is quantitatively significant.

What is the principle both of these regulations on the level of enterocytes and macrophages? Enterocytes and macrophages have the same molecule in their membrane, which provides the release of iron from cells. This molecule is iron exporter – feroportin. Feroportin is a final structure which affects hepcidin. Hepcidin bonds on feroportin and causes that the molecule of feroportin penetrates into the appropriate cell and is degraded there. If hepcidin production is high, cells are not able to export the iron into the blood due to the reduced amount of feroportin in the membrane. Iron accumulates in the enterocyte and macrophages. Enterocyte is desquamated after few days and absorption of iron in organism will not occur. On the contrary, the iron trapped in the macrophage stays in the organism but is not available for further use – iron sequestration in macrophages.

Hepcidin abundance has two effects. Firstly, rapid decrease of iron in serum due to its sequestration in macrophages and suppressed recycling. Secondly, long term absolute lack of iron in organism occurs due to its resorption in duodenum. Absence or lack of hepcidin results into the permanently increased iron resorption and subsequently its abundance and complications connected to that.

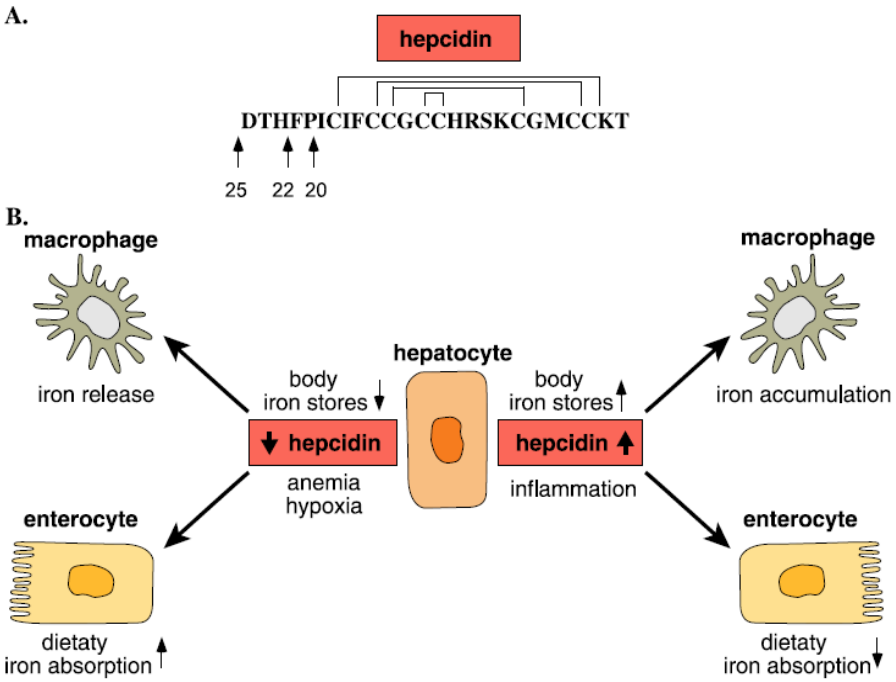


Fig. 3. *(A) The sequence of human hepcidin with cysteine disulfide bridges. Cleavage sites giving rise to three isoforms of 25, 22 or 20 amino acids, respectively, are indicated by arrows. (B) Model for regulatory functions of hepcidin. A decrease in plasma hepcidin levels, as a result of reduction in body iron stores, requirement for erythropoiesis or hypoxia, promotes dietary iron absorption and iron release from macrophages. An increase in plasma hepcidin levels in response to iron loading or inflammation inhibits dietary iron absorption and iron release from macrophages (G. Papanikolaou, K. Pantopoulos 2005)*

1.2. Negative effects of iron in human body

1.2.1. Iron and inflammation

Inflammatory states significantly interfere with iron metabolism. During the inflammation in the body, there is large amount of inflammatory cytokine and mediator which affect numerous processes. One of the most important anti-inflammatory cytokine is IL6.

IL6 stimulates the production of acute phase reactant in the liver. One of the peptides which is synthesized in the liver due to the IL6 with increasing tendency is hepcidin. Thus, during the inflammatory states, hepcidin production increases. Hepcidin consequently decreases iron resorption in the intestine, and especially decreases releasing of the iron from macrophages. This leads to a rapid decrease of iron level in the serum.

Drop of the iron concentration in the serum, transferrin concentration drop and increase of the concentration of ferritin in the serum are typical changes in the metabolism of iron during the inflammatory states. These changes occur quickly and are characteristic for anemia of chronic diseases and thus contribute to their pathogenesis. This anemia occurs during numerous inflammatory states, e.g. cancer. Among others, this state is characterized by the lack of iron available for erythropoiesis.

The meaning of rapid drop of iron in the serum and its sequestration in the macrophages is, that during the inflammation, iron is an important and necessary element for human body but also for bacteria. Bacteria need iron for their own reproduction and metabolism.

During an infection bacteria must get the iron from the host tissue and they have various mechanisms how to achieve that. Human organism is trying to prevent the bacteria from iron consumption and thereby reducing their reproduction. One of these mechanisms can be rapid sequestration of iron and drop of its serum concentration as a consequence of increased production of hepcidin. As a result, the bacteria will then reproduce with smaller rate and can be easily removed by the immune system.

1.2.2. Iron deficiency – cause

More than a billion of people in the world suffer from the iron deficiency. There are many causes to that, and they can be divided into the four main groups. The first of them is the lack of iron in the diet and is often associated with economic situation. second is bad absorption of iron, which may be a result of the diseases of digestion system which results in mal-absorption syndrome. third is increased consumption of iron which is not sufficiently saturated by intake or reserve. Greater need of iron prevails mainly with pregnant women. Connected with the pregnancy and child birth, 1 g of iron is needed. It is the iron to be delivered to the infant and further iron is necessary during the changes in the body of pregnant woman. The birth itself is also accompanied by the loss of blood. Frequent cause of iron deficiency is bleeding, additionally to the obvious bleeding it may be also a smaller but repeated loss of blood from e.g. the gastrointestinal tract, or a gynecological bleeding. Lack of iron can thus be a warning of a serious disease of these organs. Mild iron deficiency is very common in developed countries as well and is dominant with women.

1.2.3. Iron deficiency – effect

Deep deficiency of iron adversely affects the health and performance of an individual. The most significant implication is anemia. Fewer red blood cells are being created in the bone marrow due to the iron deficiency.

Blood cells which arise have smaller amount of hemoglobin, are smaller and less colorful. Sieropenic anemia is typically microcitar and hypochromic. Anemia its self can reduce the performance. Those affected are often tired, asthmatic and pale. Due to the lack of oxygen, the functioning of most of organs is impaired. Cardiac performance can be seriously impaired.

Changes can be apparent on the skin, mucous and nails. Exhausting the iron reserve anticipates the development of anemia. The amount of the serum ferritin in the blood is decreasing and the amount of transferring is increasing. Due to Reduced amount of iron in the plasma transferrin saturation declines. The number of the red cells consequently drops and they shrink in size,

together with the hemoglobin drop. During the iron treatment (in the case of its lack) is therefore necessary to deliver iron until reserve refill.

1.2.4. Toxicity of iron

Toxicity of iron is largely based on Fenton and Haber-Weiss chemistry (Fig. 4A), where catalytic amounts of iron are enough to yield hydroxyl radicals ($OH^{\cdot}$) from superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2), collectively known as reactive oxygen intermediates (ROIs) (Halliwell and Gutteridge, 1990).

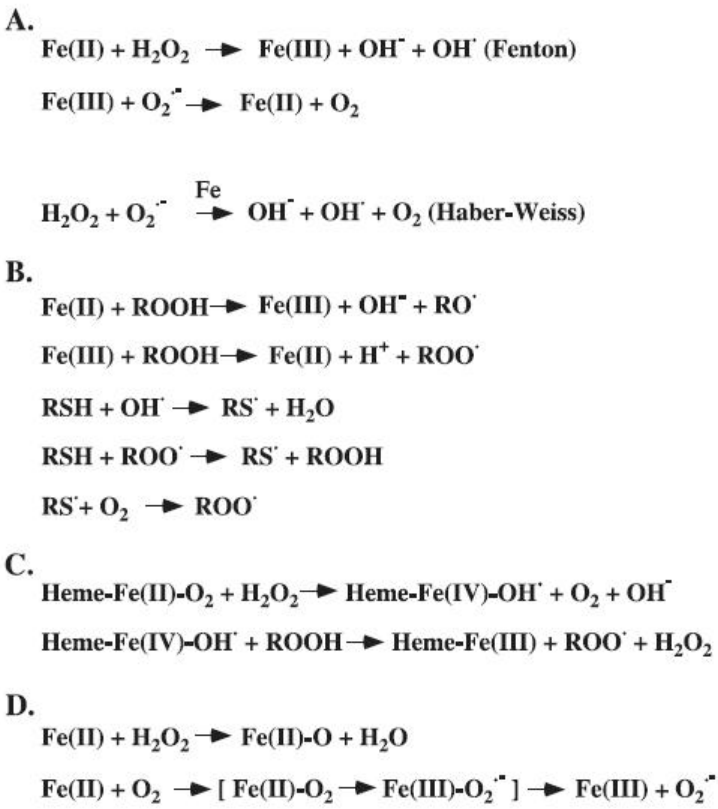


Fig. 4. *(A) Iron-catalyzed generation of hydroxyl radical via the Fenton reaction; the net Haber-Weiss reaction is also indicated. (B) Iron-catalyzed generation of organic radicals. (C) Heme-catalyzed generation of oxygen radicals via oxoferryl intermediates. (D) Direct interaction of iron with oxygen (Papanikolaou and Pantopoulos, 2005)*

ROIs are inevitable byproducts of aerobic respiration and emerge by incomplete reduction of dioxygen in mitochondria. ROIs can also be generated during enzymatic reactions in other subcellular compartments, such as in peroxisomes, endoplasmic reticulum, or the cytoplasm. ROIs are also produced by membrane-bound NADPH oxidase complex a multi-subunit enzyme primarily expressed in phagocytic neutrophils and macrophages, but also in other cell types. NADPH oxidase is an important tool for antimicrobial defense of an organism. The enzyme complex assembles upon infection and generates high levels of superoxide in a "respiratory burst", which is enzymatically and spontaneously dismutated into hydrogen peroxide. The reaction products give rise to more potent oxidants such as peroxynitride ($ONOO^-$) and hypochloride (OCl^-), which amplify the bactericidal and cytotoxic capacity of phagocytic cells and constitute major toxic species in vivo (Ischiropoulos and Beckman, 2003). The former is generated by the spontaneous reaction of superoxide with NO, while the latter is synthesized from hydrogen peroxide and chloride in a reaction catalyzed by myeloperoxidase. In this milieu, redox active iron catalyzes the generation of not only hydroxyl radicals, but also of organic reactive species, such as peroxyl ($ROO^\cdot$), alkoxyl ($RO^\cdot$), thiyl (RS), or thiyl-peroxyl ($RSOO^\cdot$) radicals (Fig. 4B).

Heme iron (either "free" or within hemoproteins) may also catalyze the formation of radicals, mainly via formation of oxoferryl intermediates (Ryter and Tyrrell, 2000) (Fig. 4C). Then the ferrous iron can also contribute as a reactant, either than as a catalyst, to free radical generation by a direct interaction with oxygen, via ferryl (Fe^{2+}-O) or perferryl (Fe^{2+}-O_2) iron intermediates (Fig. 4D).

It has been proposed, that when $[O_2]/[H_2O_2] > 100$, these reactions may represent an important source for free radical generation in vivo (Huang, 2003). Free radicals are highly reactive species and may promote oxidation of proteins, peroxidation of membrane lipids, and modification of nucleic acids. Likewise, reactive nitrogen species, such as peroxynitrite, may lead to protein damage via nitration. An increase in the steady state levels of reactive oxygen (and nitrogen) species beyond the antioxidant capacity of the organism called oxidative (and nitrosative) stress, is encountered in many pathological conditions, such as chronic inflammation, ischemia-reperfusion injury, or neurodegeneration (Ischiropoulos and Beckman, 2003). Excess of redox active iron aggravates oxidative (and nitrosative) stress and leads to accelerated tissue degeneration. This is evident in disorders of hereditary or secondary iron overload.

1.2.5. Iron overload – cause

Excess iron is accumulated in cells and organs and impairs whole organism (e.g. cells and tissues) due to the generation of oxygen radicals. skin may have apparently dark bronze color. Mainly impaired organs are those where the excess of iron is most pronounced, e.g. liver with the most difficult complication – cirrhosis and increased risk of hepatocellular carcinoma. Accumulation of the iron in pancreas leads to the extinction of Langerhans' islets and decreased insulin production, which are causes of the diabetes mellitus. Because the result of the iron accumulation is visible from skin darkening, it is often called bronze diabetes.
With serious cases of iron overload, myocardial is impaired. This can lead to cardiomyopathy with severe cardiac failures and arrhythmias. Accumulation of iron in the joints leads to pain and endocrine glands together with genital glands may also be affected.
Clinically, iron overload has many symptoms like fatigue, unspecific difficulties, joints pain, and numerous symptoms of organ impairment. The most typical are the symptoms of diabetes, liver cirrhosis and in severe cases of cardiac arrest. Iron overload can thus have lethal consequences and its early diagnosis and treatment can prevent the most serious complications.

1.2.6. Iron overload - effect

Iron overload can have several causes. It may be due to the excessive intake of iron through the oral route, overdose through parenteral route, or due to frequent transfusion in case of reduced haemopoiesis. The iron in transfused blood cannot be used for the formation of a new blood cells. Further reason can be excessive resorption of iron as a result of genetic disorders of its regulation. Such as disease is hereditary, i.e. genetic hemochromatosis. Here, the amount of iron absorbed in the duodenum is unusually high. The reason is insufficient regulation of this resorption. There is insufficient production of hepcidin in the liver. thus the resorption is not weakened, and daily the individual absorbs several times larger amount of iron. After several years or decades, this will result into obvious and often serious iron overload. Symptoms occur earlier and in more severe form, the more iron is absorbed.

. The reasons are the various disorders of hepcidin synthesis. The lower the synthesis is the more sever the disease is. Today, several molecules which participate in the regulation of hepcidin are known. Mutations of those lead to the hereditary hemochromatosis. The most common is a HFE molecule mutation that causes the first type hereditary hemochromatosis. The most serious is the hereditary hemochromatosis of second type called juvenile, where already in an early age severe symptoms are present. The hereditary hemochromatosis of the second type is caused by the mutation of hemojuvelin and very rarely directly by hepcidin mutation.

1.2.7. Treatment of iron disorders

Iron deficiency is treated by administration or adequate supply. Sometimes, simple improvement of the diet is enough (more meat, etc.). In case of increased deficiency, iron is usually prescribed in the form of tablets. Injection - intramuscular or intravenous (parenteral) administration is reserved rather for special cases.

Iron excess is treated in two ways.
By hemochromatosis, regular blood subscription. (venipuncture). The aim is to reduce the amount of hemoglobin and gradually also iron stores in the body that are used to create new blood cells.

If it is not possible to use (e.g. where the excess iron caused by the administration of transfusions, so it would be unreasonable to draw blood again), use special drugs, which take the iron in human body and it is then excretion (urine, bile and feces). These drugs are called iron chelator (siderophore). The use of these drugs is relatively difficult, have side effect and is uncomfortable for the patient. New drugs are still in developing (Crisponi and Remelli, 2008; Pradeep et al., 2007)

For official treatment are most commonly used: Exjade (with effect substance Deferasirox) which is submitted oral, and intravenously Ferriprox (with Deferiprone) and Desferal (with Deferoxamine) (European Medicines Agency).

2. The history and evolution of chelator therapy and their limitations

Due to using of poison gas in World War I were introduced chelating agents into medicine. Chelating agent, the organic dithiol compound dimercaprol (also called British anti-lewisite or BAL), was first more used as antidote for the poison gas based on arsenic (lewisite). The sulphur atoms in BAL's mercaptan groups have strong bond with arsenic atoms in lewisite, is forming a water-soluble compounds entering to the blood in the organism and allowing it to be removed from the organism by the kidneys and liver. BAL had terrible side-effects.

After World War II, many navy personnel suffered from lead poisoning as a result of their jobs painting the ship's hulls. The using of ethylenediaminetetraacetic acid (EDTA) as a lead chelating agent was introduced. Unlike BAL, it's a synthetic amino acid which doesn't contain mercaptans. The side effects of EDTA weren't considered as harmful as BAL.

BAL was modified (in the 1960's) into DMSA (Dimercaptosuccinic acid), a related dithiol with much fewer side effects. DMSA quickly substitute BAL and also EDTA and became US standard of care for the treatment of lead, arsenic, and mercury poisoning. More recently, the esters of DMSA were developed and they are reputedly more effective. For example, the monoisoamyl ester is reputedly more effective than DMSA in cleaning mercury and cadmium.

The research in former Soviet Union led to the introduction of DMPS (2,3-dimercapto 1 propanesulfonic acid), another dithiol, as a mercury-chelating agent. They also introduced ALA (Alpha Lipoic Acid), which is transformed by the organism into the dithiol dihydrolipoic acid, a mercury- and arsenic-chelating agent. DMPS has experimental status in the US FDA, whereas ALA is a current nutritional supplement.

Since the 1970's, iron chelation therapy is using to substitution of regular phlebotomy to the treatment of excess iron stores in people with hemochromatosis.

All the other chelating agents which were discovered, function through making a number of chemical bonds with metal ions, thus rendering them much less chemically reactive. The final complex is water-soluble, it can enter to the blood in the organism and to be excreted harmlessly. Calcium-disodium EDTA chelation was approved by U.S. Food and Drug Administration (FDA) to the treatment lead poisoning and heavy metal toxicity. The U.S. Federal Trade Commission decided that there were not enough of scientific studies to support these claims and that the ACAM's statements were false. In 1999, ACAM agreed with the stopping of false chelation therapy as effective in the treatment of heart diseases and to avoidance of legal proceeding (Kalia 2005).

There must be three requests in the development of effective and non-toxic iron chelators which are - the route of administration, the iron chelation efficiency and the toxicity profile of ligand. In ideal case a drug should be orally applicable, highly specific and while action in organism without adverse side effects. Although many chelators designed to orally application were created, next research aim to increase the efficiency and decrease the toxicity of these drugs. The molecular activity should be the starting point for developing a new generation of strong iron chelator (Kalinowski and Richardson, 2005).

2.1. Iron chelators

To treat or protect patients from the consequences of iron toxicity, iron chelating drugs have been introduced in clinical practice. The first generations of drugs were based on siderophores, iron-chelating molecules produced by nearly all microorganisms (Renshaw et al., 2002). Of the 500 characterized siderophores (Drechsel and Winkelmann 1997), only desferrioxamine introduced in 1962 and produced by *Streptomyces pilosus* is the current drug of choice (Aouad et al., 2002). Nevertheless, desferrioxamine is associated with several drawbacks including narrow therapeutic window and lacks bioavailability orally (Pradeep et al., 2007). In addition, the vast array of chelators that have been artificially designed and synthesized (Liu et al., 2002, Ding et al., 2008) have been reported to be clinically ineffective (Kalinowski and Richardson, 2005).

2. The history and evolution of chelator therapy and their limitations

Iron overload can be defined as a pathological situation in which plasma iron concentration exceeds many times the total iron binding capacity of transferrin.

This has been observed in the following cases:

- an excessive dietary iron intake. An example is the severe Bantu siderosis, observed in the Bantu tribe of Africa who drink acidic beer out of iron pots
- inherited diseases: idiopathic hemochromatosis in which gut absorption of iron is abnormally high; congenital atransferrinemia, marked by a total absence of circulating transferrin
- the medical treatment of thalassemia. Thalassemias are genetic disorders in which the rate of synthesis of one or more of the hemoglobin chains diminished

Untreated patients die of anemia in infancy but can be kept alive by regular blood transfusions. Since each unit of blood contains about 0,2 g of iron, the patients become overloaded with iron (some transfused patients may accumulate 50-70 g of iron over a 10-year period). Thalassemia is widely distributed throughout the Middle East, the Indian subcontinent and South East Asia. The frequency of these gene defects is staggering with an estimated 100 million carriers and close to 100 000 babies being born each year with serious forms of thalassemia. It has been shown that, under such conditions, the low-molecular mass iron pool was drastically increased.
This is consistent with the following observations concerning the iron-overloaded plasmas:

- free iron is detected by the bleomycin assay
- “OH” radical formation is promoted
- peroxidation of phospholipid liposomes is stimulated
- transferrin is completely saturated
- addition of apotransferrin is completely protective

All these effects certainly participate to tissue damage (liver, spleen, heart), cardiac abnormalities, skeletal and cranial defects, death. Treatment of iron overload has used, for the past 20 years, desferrioxamine, the only clinically useful drug available for this purpose.

However, there is now interest for new chelators.

These chelators have to be:

- selective for iron (and poor ligands for essential Zn, Ca, Cu, etc.)
- non-hydrolysable (hydroxamates are sensitive to acid)
- nonoxidizable (catechols are rapidly oxidized)
- water-soluble (by introducing sulphonate functions for example)
- and absorbable from the gastrointestinal tract (Kalinowski and Richardson, 2005)

2.1.1. Desferrioxamine

Desferrioxamine (DFO), is the active substance in drug DESFERAL. Last 40 years is used as a basis of therapy in patients with iron-overload, caused by frequent blood transfusions. Desferrioxamine is hexadentate siderophore, which is islolated from Streptomyces pilosus and is used by treatment of iron overload diseases, for example thalassemia. Desferrioxamine binds iron in the blood, decrease the concentration in blood and increases the concentration of iron in the urine output. This causes the reduction of excess iron in the body. This has already a positive effect in healing of nerve damage. And also can help by expression and release of inflammatory mediators by specific cell types (Choi, et al., 2004). DF binds with high affinity to Fe (III) that is shown in (Fig. 5). Desferrioxamine is able decrease oxidative stress in in Iron overloaded cells, and alleviate the symptoms associated with iron overload disease (Olivieri and Brittenham, 1998). DFO has a hydrophilic character, it limits the effectiveness of this ligand. This hydrophilic character inhibits the absorption of iron form the gastrointestinal tract (Aouad et al., 2002).

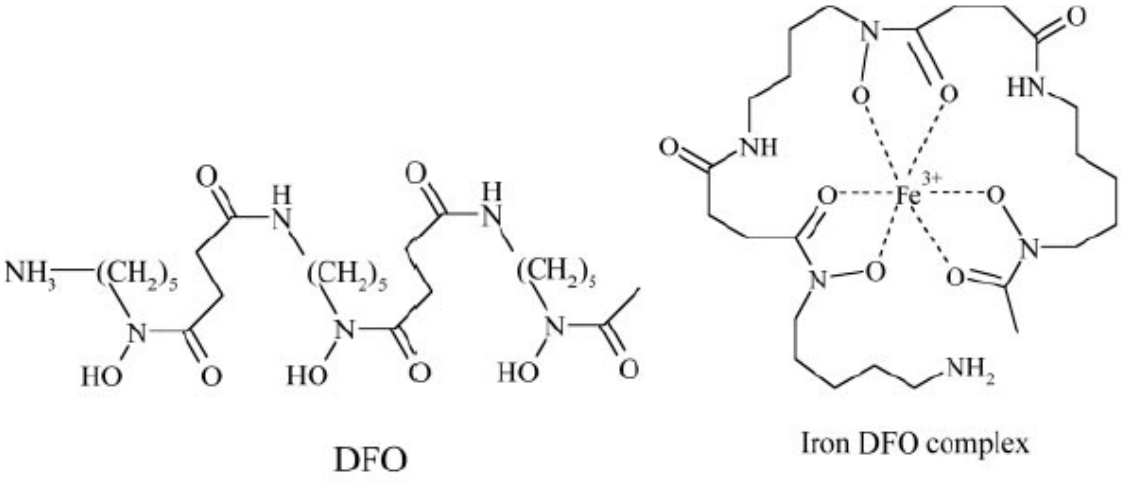


Fig. 5. *Chemical structures of siderophore ligands DFO and its iron complex*

2.1.2. Deferiprone

Deferiprone (L1, CP20), is the active substance in drug Ferriprox. Ferriprox is the first oral iron chelating drug (Fig. 6A) to be used in thalassemia and other iron-loaded patients (Kontoghiorghes 1987). Progress in its development has been very slow because it was mostly undertaken through research-orientated projects and supported by non-profit establishments.

It is very stable at room temperature and in solutions of physiological or acidic pH and forms red colour complexes with iron, similar to the red colour of the urine of patients treated with it. Its affinity for iron is higher than for Cu, Al, Zn and other metals (Sheppard 1993; Kontoghiorghes 1987). L1 is a neutral molecule forming a neutral complex with iron, whereas DF is positively charged and forms a positively charged complex with iron at physiological pH (Kontoghiorghes 1987). At low concentrations (10^{-6} M) the L1 iron complex is less stable than that of DF and the labile complexes of L1 with iron or copper may promote the formation of toxic oxygen-activated species (Motekaitis 1991). Both L1 and DF are hydrophilic and unlike lipophilic chelators they do not accumulate in lipids for example in cell membranes or in the brain. L1 is about 10 times more lipophilic than DF and may be easier to diffuse through cell

membranes than DF, unless other forms of membrane transport are involved (Kontoghiorghes 1987).

Both L1 and DF are unable to mobilize iron from hemoglobin and other heme containing proteins but DF can cause oxidation of hemoglobin. Ribonucleotide reductase which is an iron containing enzyme involved in DNA synthesis is also inhibited by both L1 and DF at high concentrations (mM) to about the same degree (Kontoghiorghes 1986).

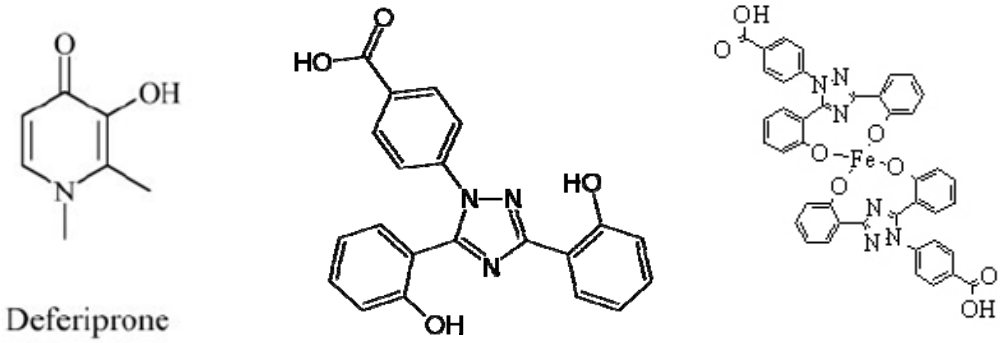


Fig. 6. *(A) Deferiprone (3-hydroxy-1,2-dimethylpyridin-4(1H)-one), (B) structure of deferasirox and (C) iron-desferiarox complex*

2.1.3. Deferasirox

Defarasirox is the active substance in drug Exjade. This is the first oral drug approved for this purpose. Is use to reduce chronic iron overload diseases in patients who are receiving long-term blood transfusions. associated with diseases such as beta-thalassemia and other chronic anemias. is also used when deferoxamine (another medicine used to treat iron overload) cannot be used or is inadequate, in patients who suffer from other types of anemia, in children aged from two to five years, and in patients with beta thalassemia major who receive less frequent transfusions. The structure and complex of Deferasirox with iron is in picture Fig. 6B,C (European Medicines Agency).

2.1.4. Tachpyridine

The structure of this chelator is shown in (Fig.7), Tachpyridine is a hexadentate chelator with triaminocyclohexane scaffold that used three secondary aminenitrogens and pyridyl nitrogens to bound iron (Buss et al., 2003c). It was found that under anaerobic conditions bind Fe (II) more efficiently than Fe (III) (Planalp et al., 2002) Was also found that the bound Fe (III) is subsequently reduces to the Fe (II). In this direction, is perhaps also a certain connection with the biological cytotoxicity of tachpyridine against cancer cells. Under aerobic conditions, iron-tachpyridine complexes are exposed to iron-mediated ligand oxidation, forming an inseparable mixture of mono- and diimino Fe (II) complexes (Park et al., 1998; Zhao et al., 2004). The disadvantage is that tachpiridine except Fe (II) and Fe (III) binds also Zn (II), Cd (II), Hg (II) (Park et al., 2000), Cu (II), Ga (III) and In (III) (Hilfiker et al., 1997). It can cause major health problems in the body, and causing cytotoxic effects. (Zhao et al., 2004).

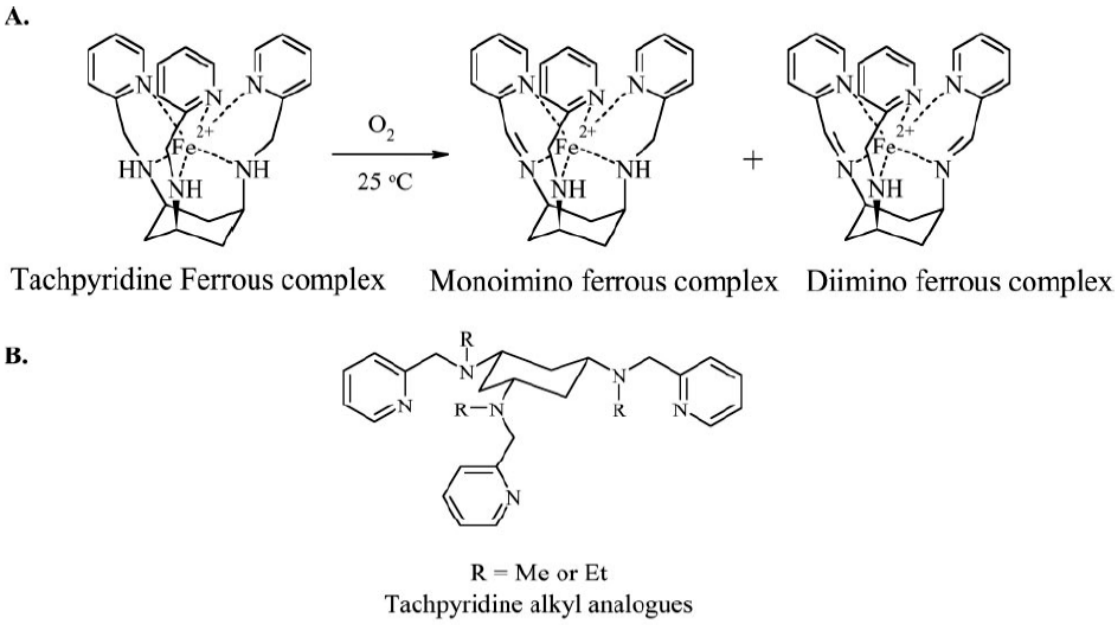


Fig. 7. *Structure of Tachpyridine (B) and iron-tachpyridine complex (A)*

2.1.5. Aroylhydrazones

Aroylhydrazones, shown in (Fig. 7) are an orally active, tridentate chelator, which binds iron. This connection is made by phenolic oxygen, imine nitrogen, and carbonyl oxygen (Buss et al., 2002b, Richardson and Ponka, 1998). The tridentate structure of the ligand was demonstrated through the synthesis of its iron complex, this can be seen in Fig. 7 (Ponka et al., 1979a). Aroylhydrazones have high affinity for Fe (III), and with lower affinity bind Fe (II). Forming ferric complexes is possible in the presence of oxygen (Vitolo et al., 1990). The ability to bind Fe (II) is necessary for plasmid pUC-18 DNA protecting against OH-mediated strand breaks by scavenging Fe (II) and already in increasing its auto oxidation rate. In Fenton reaction, limiting of Fe (II) levels label to catalyze Fenton generated ROS (Hermes-Lima et al., 1998). Aroylhydrazones also formed divalent metals Ca (II), Mg (II), Zn (II) and weakly Ca (II) and Mg (II) (Richardson et al., 1989).

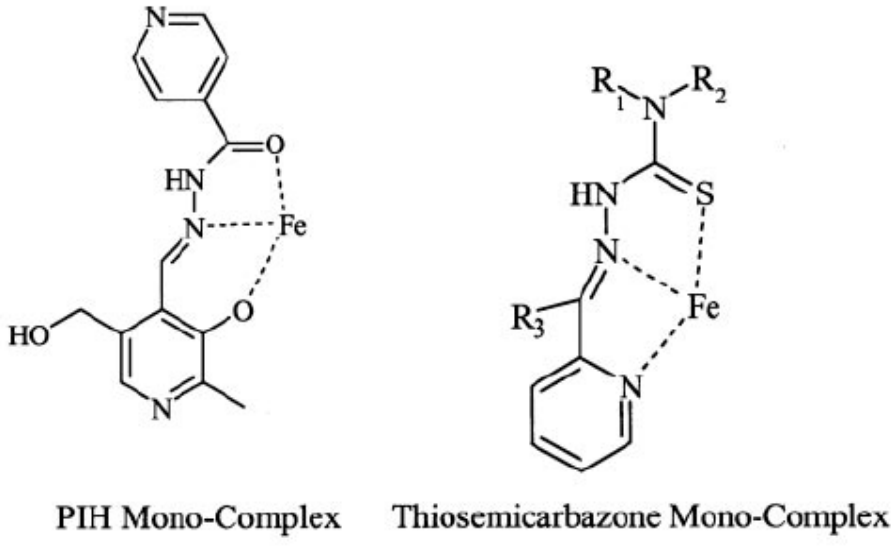
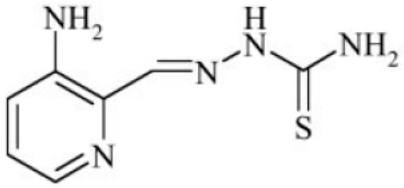


Fig. 8. *PIH (Pyridoxal isonicotinoyl hydrazone) complex with iron*

2.1.6. Thiosemicarbazones

Thiosemicarbazones and traipine, structure can be seen in the picture (Fig. 9) have the ability to bind Fe (II), Fe (III), Cu (II), Ga (III), Co (II) and Zn (II) were previously used to treatment of cancer (Finch et al., 2000). Thiosemicarbazones and traipine were the first chelators to be comprehensively assessed as anticancer agents (Finch et al., 2000).



Triapine

Fig. 9. *Structure of Triapine*

2.2. Toxicity of iron chelators

Most drugs have toxic side effects which may or may not be related to their pharmacological activity or toxicity findings in animals. A major aspect of toxicity in relation to iron chelation therapy is the removal or displacement of other essential metals. Two other iron chelating drugs namely DTPA and EDTA have been previously shown in iron-loaded patients to increase the excretion of Zn, Cu and Mn in addition to Fe resulting in toxic side effects. Increased excretion of Zn and Cu has also been observed in a few cases during treatment with DF and also with L1, especially increased Zn excretion in diabetic thalassemia patients (Kontoghiorghes, 1995). In addition to Zn deficiency the other major toxic side effects of L1, which have been identified in the last 10 years are neutropenia and agranulocytosis, joint/musculoskeletal pains and gastric intolerance.

Reports of significant increases in hepatic fibrosis or liver cirrhosis by a Toronto group have not been confirmed by any other group from any other country using L1 and by comparison to

patients taking DF (Tondury 1998, Kowdly 1998). The mechanisms involved in the toxic side effects reported by L1 are not yet known. European Medicines Agency mentions as a frequent side effects associated with chelate therapy (Exjade, Ferriprox, Desferal), side-effects such as: damage hearing, vision problems, dizziness, CNC disorders, aliphatic shock, headaches, seizures, loss of eyesight, asthma, nausea, urticaria, arthralgia, major kidney and liver damage, abnormal growth by children, pyrexia and others.

A chelator (Greek claw of a crab) is a naturally occurring or chemically designed molecule which has high specificity and affinity for a metal ion, forming a complex with it. An ideal chelator designed for the decorporation of a particular metal from the body should be able to bind, carry and remove the metal out of the body without causing any toxicity.
Iron chelating drugs could in principle reduce iron overload by causing a negative iron balance, which means an increase in iron excretion at levels higher than the amount of iron taken into the body from transfusions and iron absorption. The site of action of chelating drugs in vivo is not fully understood (Kontoghiorghes et al., 1987).

3. Proposed strategy

The ideal chelator for treating iron overload problems in humans has not yet been found (Crisponi and Remelli, 2008). The development of orally effective iron chelators is urgent and forms the basis of this work. In this study we aim at enzymatically incorporating catechol, caffeic acid and hydroxyl-carboxic acid moieties into chitosan to produce active iron chelating polymers. Hydroxycarboxylates and catechols are very attractive as compared with the other iron chelators which bind iron (II) because such ligands have affinity for other biologically important bivalent metals such as copper (II) and zinc (II) ions. An additional observed advantage of high-affinity iron (III) chelators is that, under aerobic conditions, they will chelate iron (II) cations and rapidly autoxidize it to the corresponding iron (III) species (Harris et al., 1973). Thus, high-affinity iron (III)-selective ligands bind both iron (III) and iron (II) under most physiological conditions. Therefore, novel chitosan-based microspheres (Fig. 12), functionalized with catechol or hydroxyl-carboxic acid could have high iron-chelating abilities while the formation of microspheres will provide the necessary stability for the transport through the changing gastrointestinal tract environment. Apart from enhancing the already inherent iron-chelating properties of chitosan (Burke et al., 2002), the presence of $-NH_2$ reactive groups on chitosan (Fig. 12) provides sites for enzymatic incorporation of catechol and hydroxyl-carboxic acid functional groups. In addition, chitosan has many remarkable properties among them, mucoadhesive properties, biocompatibility, non-toxic, antioxidant, antimicrobial, none immunogenicity (Prashanth and Tharanathan, 2007; Bhattarai et al., 2010; Agnihotri et al., 2004; Kumar et al., 2004; Vodna et al., 2007), and above all chitin (parent compound) is the second most abundant renewable polymer after cellulose (Rinaudo 2006). Further advantages of using chitosan is that it can be designed in many different forms (Faa and Crisponi, 1999) including highly functionalized microspheres (Fig. 12) as envisaged in this study. Microspheres will be made from the phenolic functionalized chitosans as illustrated in Fig. 12. These microspheres can potentially be used either prophylactically or therapeutically while the inherent mucoadhesive properties of chitosan will make it ideal for increasing the residence time of iron chelators.

For interest, just a few words about hydroxycarboxylates and catechols:

It was found that catechol, caffeic acid and hydroxyl-carboxic acid do not have just iron chelating activity. These molecules have also other properties, which are interesting in human healing.

Solutions of catechol react with iron (III) . Small amounts of catechol occur naturally in fruits and vegetables. Colorless catechol oxidizes to reddish-brown melanoid pigments, derivatives of benzoquinone. Benzoquinone is said to be antimicrobial, which slows the spoilage of wounded fruits and other plant parts (Yam et al., 2009).

Arthropod cuticle consists of chitin linked by a catechol moiety to protein. The cuticle may be strengthened by cross-linking, in particular, in insects, and of course by biomineralization (Briggs, 1999).

Caffeic acid have immunomodulatory and anti-inflammatory activity. Caffeic acid phenethyl ester (CAPE) is active component of propolis from honeybee hives (Demestre et al., 2008).

Another study also showed that CAPE have the possibility to suppresses acute immune and inflammatory responses and holds promise for therapeutic uses to reduce inflammation (Orban et al., 2000).

This anti-inflammatory and anti-cancer property has also been shown to protect skin cells when exposed to ultraviolet (UV) radiation, in particular UVC radiation (Neradil et al., 2003) and UVB radiation (Staniforth et al., 2006). Caffeic acid has been shown to be an inhibitor of the lipoxygenase enzyme that forms leukotrienes from arachidonic acid. This function has been useful in scientific experiments to elucidate the roles of the leukotrienes in various inflammatory responses (Mirzoeva and Calder, 1996).

2,5-dihydroxibenzoic acid is product of the metabolic break down of aspirin, excreted by the kidneys (Levy, 1972). As a hydroquinone, gentisic acid is readily oxidised and is used as an antioxidant excipient in some pharmaceutical preparations (Strupat et al., 1991).

2,3-dihydroxibenzoic acid strongly complex iron ions for absorption into bacteria. 2,3-dihydroxibnezoic acid have a catechol group, which upon deprotonation binds iron centers very strongly, and the carboxylic acid group by which the ring attaches to various scaffolds via amide linkages. Siderophore with high affinity is enterochelin, there contains three dihydroxybenzoyl substituents linked to the depsitripeptide of serine. It has s potential be useful iron-chelating drug (Young and Gibson, 1969, Gibson et al., 1970).

3,4-dihydroxibenzoic acid has mixed effects on normal and cancer cells in in vitro and in vivo studies. PCA could reduce or enhance tumor growth (Babich et al., 2002). Similarly, PCA was reported to increase proliferation and inhibit apoptosis of neural stem cells (Nakamura et al., 2000). In an in vitro model using HL-60 leukemia cells, protocatechuic acid showed an antigenotoxic effect and tumoricidal activity (Guan et al., 2009).

For us is now important, that studies on siderophores demonstrated that hydroxycarboxylates and catechols (d'Hardemare et al., 2006) are selective for tribasic metal cations including iron (III). Most of the other tribasic cations are not essential for living cells therefore their removal is of no consequence, which makes hydroxycarboxylates and catechols very attractive as compared to the other iron chelators which bind iron (II) because such ligands have affinity for other biologically important bivalent metals such as copper (II) and zinc (II) ions. An additional observed advantage of high-affinity iron (III) chelators is that, under aerobic conditions, they will chelate iron (II) cations and rapidly autoxidize it to the corresponding iron (III) species (Harris et al., 1973). Thus, high-affinity iron (III)-selective ligands bind both iron (III) and iron (II) under most physiological conditions. We believe taking advantage of the remarkable properties of these phenolic compounds and combining them with the excellent properties of chitosan (renewable polymer that can be molded into any shape) to synthesize chitosan phenolic functionalized microspheres will help treat iron related diseases.

3.1. Chitosan

3.1.1. Why chitosan?

Chitosan has many remarkable properties like, mucoadhesive properties, biocompatilibity, non-toxic, antioxidant, antimicrobial, none immunogenicity (Prashanth and Tharanathan 2007, Bhattarai et al., 2010; Agnihotri et al., 2004; Vodna et al., 2007; Ravi Kumar et al., 2004) and above all it is the second most abundant renewable polymer after cellulose (Rinaudo,2006). Chitosan molecule has three reactive centers: primary amino group, primary and secondary hydroxy group. Amino group easy susceptible to quaternization, what can increase the solubility

of chitosan in water and can be create complexes with metal ions. The primary hydroxy group is mostly substituted by spacer. which it connects the with active component, drug or group which is responsible for targeting drugs or increase the solubility in water. The secondary hydroxy group is modified especially to increase solubility in water. Because of its exceptional properties, chitosan is very interesting material and the bioactive agent in pharmaceutical and biomedical industries (Vavrikova and Visnova, 2009).

3.1.2. Chitosan as a carrier

Chitin is obtained from shrimp shells, crab and fungal mycelia. Production of chitosan is associated with food industries such as shrimp canning. With alkali is removed the protein and deacetylates chitin. Also some soluble glycans are removed, depend on concentration of alkali (Madhavan, 1992). Chitin is deacetylated in sodium hydroxide (40%) for 1-3 hours at 120°C and is produces deacetylated chitosan (70%) (Fig. 10). Chitin, is soluble in dilute acids such as acetic acid or formic acid and others.

In N-methylmorpholine N-oxide have chitosan gel forming ability and its application is in controlled drug release (Dutta et al., 1997). The hydrolysis of chitin under drastic conditions and with concentrated acids produces relatively pure D glucosamine. The presence of the substituent weakens the hydrogen bonds of chitosan. N-alkyl chitosans grow in water in spite of the hydrophobicity of the alkyl chains, and maintain the chitosan film forming property (Kalinowski et al., 2005).

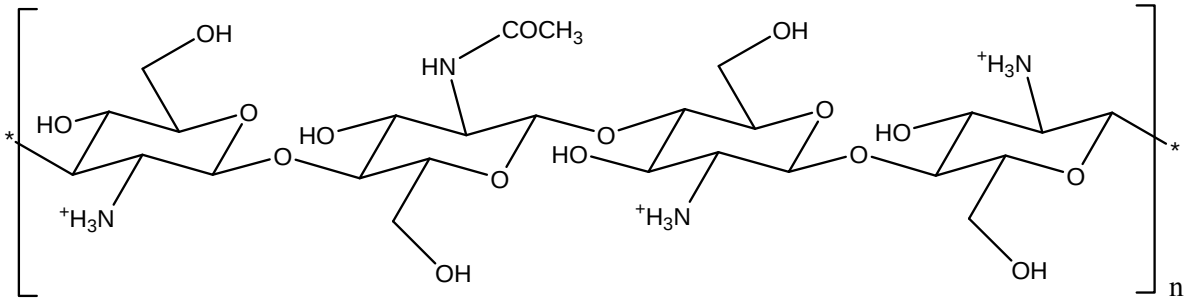


Fig. 10. *Molecular structure of chitosan*

3.1.3. Chitosan potential in various fields

Tab.1: Chitosan potential in various fields.

Engineering	Solid-state batteries (Arof et al., 1995), Paper finishing (Allan et al., 1972), Sorption of dyes (Kumar, 2000), Metal capture from wastewater Nair and Madhavan, (1984); Peniche Covas et al.,(1987); McKay et al., (1989), Water engineering (Kumar, 2000); Photography (Muzzarelli, 1997).
Chitosan as fat trapper	First chitosan takes fats in the stomach before they are digested this avoid absorption of fats in the digestive tract. The fat binds to chitosan fiber, this complex can be not absorb with the human body, and is removed through the digestive tract away. Chitosan's fibers have positive ionic charge, which gives him the ability to bind negative charged fat and bile acids. (Wadstein et al. 2000; Muzzarelli 1998, 1999).
Anti-throbogenic and haemostatic materials	Chitosan have antithrombogenic and antihaemostatic effect. Especially N-hexanoyl and N-octanoyl chitosan fibers are anti-thrombogenic. Chitosan Fibers can be used as a haemostatic material and as an antithrombogenic materials can be used N-octanoylchitosan and N-hexanoyl fibers (Hirano et al., 1996).
Antibacterial agents	By Gebelein et al., 1995 was inhibited growth of Escherichia coli in the presence of more than 0.025% chitosan. Helminthosporium, Fusarium and Alternaria growth is also inhibited by chitosan in presence. Mechanism is probably that the cationic amino groups of chitosan bind to anionic groups of these microorganisms, result is growth inhibition.

Cell-stimulating materials in plants and animals	Soya beans coated with a layer of depolymerized chitin like carboxymethyl chitin and hydroxyethyl chitin, this layer in teased the seed germination about 6%, the number was increased about 9%, plant dry weight increased about 8%, and yield was also increased about 10-12% (Hirano et al., 1996). Dressing with chitin increased chitinase activity in tree bark tissues around the wound for up to four times (Gebelein et al., 1995). In vitro cultures of several mammalian cells by treatment with chitin and its derivatives increased extracellular lysozyme activity. It was stimulated the connective tissue formation, and the self-defense function against microbial infection was enhanced at the cellular level (Kumar, 2000).
Preparation of biotechnological materials	Just as an example, the potential in new biotechnological material have chitin thanks to its structure. In chitin are two hydroxyl groups but chitosan has one amino group and in the repeating hexosamide residue has two hydroxyl groups. Modification of these groups gives rise to various fields of new biofunctional macromolecular products, which have the original molecular organization or new types of organization (Kumar, 2000). Potential for the playing with the structure of chitosan is enormous.
Food and nutrition	Acetylglucosamine moiety are even present in human milk support the growth of bifido bacteria, they block growth of other types of microorganism and generate the lactase which are necessary for digestion of milk lactose. Cow's milk has just a low content of the acetylglucosamine moiety, in humans cow's milk leads to indigestion (Knorr, 1991; Nicol, 1991).

Chitosan as an artificial skin	Skin treatment with chitosan is appropriate to Individuals who have extensive losses of skin, in fires, are ill and in danger of massive infection or to severe fluid loss (Malettas et al., 1986). It was proposed a design for artificial skin, for long-term chronic use, with focusing on a nonantigenic membrane, which performs as a biodegradable template for synthesis of neodermal tissue (Yannas et al., 1982). It seems that chitosan has the structural characteristics similar to glycosamino glycans could be regarded for development as a substrate for the skin replacement (Sandford and Stinnes, 1991; Le et al., 1996; Olsen et al., 1989).
Cosmetics	Chitosan can be used for cosmetic applications, chitosan have fungicidal, fungistatic and antimicrobial properties. Chitosan is "natural cationic gum" which becomes viscous when neutralized with acid. Chitosan is used in creams and permanent waving lotions and some derivatives can been used like nail lacquers (Mark et al., 1985).
Drug-delivery systems	Reacting chitosan with controlled amounts of multivalent anion results in crosslinking between chitosan molecules. The crosslinking may be achieved in acidic, neutral or basic environments depending on the method applied. This crosslinking has been extensively used for the preparation of chitosan microspheres. The particle size of chitosan microspheres can be modified approximately for the oral, nasal and parenteral delivery of drugs. Drugs are loaded by using the swelling properties of the microspheres in the drug solution. Release of drug from chitosan microspheres is dependent upon the molecular weight of chitosan, concentration of chitosan, drug content and density of crosslinking. Various therapeutic agents such as anticancer, anti-inflammatory, antibiotics, antithrombotic, steroids, proteins, amino acids, antidiabetic and diuretics have been incorporated in chitosan microspheres to achieve controlled release (Sinha et al., 2004).

3.1.4. Chitosan for biomedical usage

Chitosan have great potential for pharmaceutical applications due to its biocompatibility, high charge density, non-toxicity and mucoadhesion. Shown that it not only improves the dissolution of poorly soluble drugs but also exerts a significant effect on fat metabolism in the body. Gel formation can be obtained by interactions of chitosans with low molecular counterions such as polyphosphates, sulphates and crosslinking with glutaraldehyde. This gelling property of chitosan allows a wide range of applications such as coating of pharmaceuticals and food products, gel entrapment of biochemicals, plant embryo, whole cells, microorganism and algae (Sinha et al., 2004).

Chitosan properties such as biodegradability, low toxicity and good biocompatibility make it suitable for use in biomedical and pharmaceutical formulations (Chandy and Sharma, 1990), e.g. it is used for hypobilirubinaemic and hypocholesterolemic effects, antacid and antiulcer activities, wound and burn healing properties (Tachihara et al., 1997), immobilization of enzymes and living cell and in ophthalmology (Felt et al., 1999). Chitosan has a possibility to forming film, it has been suggested as a biopolymer of choice for the development of contact lens. Chitosan has been used as protective devices for acutely or chronically traumatized eyes (Markey et al., 1989). Chitosan membranes can be useful as "kidney membranes" because of their suitable permeability and high tensile strength (Amiji, 1995). Chitosan has been used as a vehicle for directly compressed tablets (Kristmundsdottir et al., 1995), as a binder, as a granulating agent, in ground mixtures, as a drug carrier as well as a co-grinding diluent for the enhancement of dissolution rate and bioavailability of water insoluble drugs (Sinha et al., 2004). Chitosan have mucoadhesive properties due to molecular attractive forces formed by electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces (Lehr et al., 1992).

These properties may be attributed to:

- strong hydrogen bonding groups like -OH, -COOH (Schipper et al., 1997)
- strong charges (Dodane et al., 1999)

- high molecular weight (Schipper et al., 1996)
- sufficient chain flexibility (He et al., 1998)
- surface energy properties favoring spreading into mucus (Lueβen et al., 1994)

Chitosan have no toxicity and can be applied also like nasal epithelium. Chitosan swells and forms a gel in aqueous environment (Felt et al., 1998). Positive charged chitosan polymer gives strong electrostatic interaction with mucus or negatively charged sialic acid residues on the mucosal surface. Chitosan shows bioadhesive characteristics and can reduce the rate of clearance of drug from the nasal cavity and increasing the bioavailability of drugs incorporated in it. Membranes prepared from chitosan have shown greater permeability for acidic drugs than basic drugs (Sawayanagi et al., 1982).

Chitosan possesses OH and NH_2 groups that can give rise to hydrogen bonding and the linear molecule expresses a sufficient chain flexibility, the conformation of which is highly dependent on ionic strength. These properties are considered essential for mucoadhesion (De and Robinson, 2003).

3.1.4.1. Chitosan membranes in wound dressing applications

Wound dressing is one of the most promising medical applications for chitin and chitosan. The adhesive nature of chitin and chitosan, together with their antifungal and bactericidal character, and their permeability to oxygen, is a very important property associated with the treatment of wounds and burns. Different derivatives of chitin and chitosan have been prepared for this purpose in the form of hydrogels, fibers, membranes, scaffolds and sponges (Jayakumar et al., 2010).

Wound healing is a complex process involving an integrated response by many different cell types and growth factors in order to achieve rapid restoration of skin architecture and function. From the various studies reported in literature, chitin and chitosan seem to be excellent dressing materials for wound healing. The fibrous materials based on chitin and its derivatives have the properties of high durability, good biocompatibility, low toxicity, liquid absorption, and

antibacterial activity. These properties lead to accelerate wound healing. Chitosan/collagen membrane could be used to hasten wound healing and induce cell migration and proliferation due to its better healing effect and antibacterial activity. Polypropylene-NIPAAm-collagen-chitosan membrane could be used as a temperature-sensitive material that would function as an automatic release of the dressing material once the wound is healed. Chitin and chitosan-based hydrogels may be considered as an occlusive dressing for wound management due to their ability to accelerate wound contraction and healing. Moreover, the moisture permeability of the hydrogels may prevent the accumulation of fluid in heavily exudating wounds.

To improve the wound healing properties, chitin and chitosan-based membranes can been developed with different types of polymers such as alginate, hyaluronic acid, polyethylene glycol diacrylate, poly (vinyl alcohol), γ-poly (glutamic acid) and 2-hydroxyethyl methacrylate. Due to their composite nature, these membranes were found to have the desired properties for wound healing applications. From the literature reported, it is confirmed that the Ag/ZnO-incorporated chitosan membranes are less cytotoxic than the traditionally used materials, and may be very potential wound dressings with antibacterial capability to prevent an injured skin from infections. Based on the improved antibacterial activity, cell attachment ability and oxygen permeability, it is concluded that chitin and chitosan scaffolds/sponges could be a promising candidate for wound dressing (Jayakumar et al., 2010).

3.1.4.2. Chitosan microspheres as a carrier for drugs

Sinha et al. (2004) write, the use of microsphere-based therapy allows drug release to be carefully tailored to the specific treatment site through the choice and formulation of various drug-polymer combinations. The total dose of medication and the kinetics of release are the variables, which can be manipulated to achieve the desired result. Using innovative microencapsulation technologies, and by varying the copolymer ratio, molecular weight of the polymer, etc., microspheres can be developed into an optimal drug delivery system which will provide the desired release profile. Microsphere based systems may increase the life span of active constituents and control the release of bioactive agents. Being small in size, microspheres have

large surface to volume ratios and can be used for controlled release of insoluble drugs. Chitosan microspheres are used to provide controlled release of many drugs and to improve the bioavailability of degradable substances such as protein or enhance the uptake of hydrophilic substances across the epithelial layers (Queen et al., 2000). Chitosan has also been used as a potential carrier for prolonged delivery of drugs, macromolecules and targeted drug delivery. Magnetic chitosan microspheres used in targeted drug delivery are expected to be retained at the target site capillaries under the influence of an external magnetic field. Also, strong interaction between cationic microspheres and anionic glycosaminoglycan receptors can retain the microspheres in the capillary region (Gallo and Hassan, 1988).

Some of method (Fig. 11) that can be used for preparation of chitosan microspheres for drugs delivery (Sinha et al., 2004).

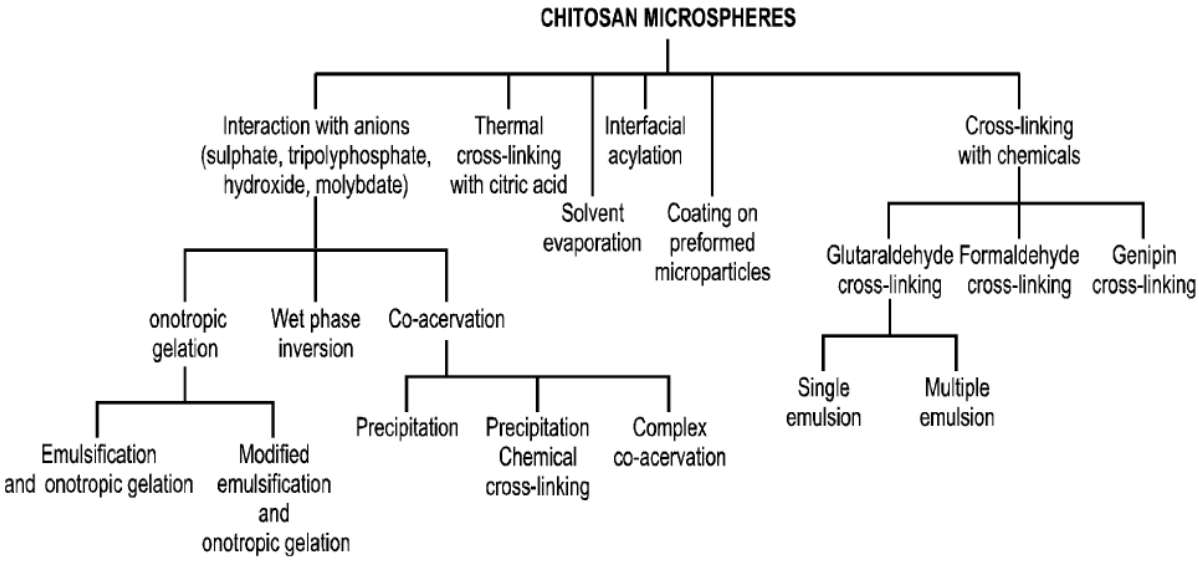


Fig. 11. *Method for preparation chitosan microspheres (Sinha et al., 2004)*

4. Our strategy

Therefore novel chitosan based microspheres (Fig. 12), functionalized with catechol or hydroxyl-carboxic acid could have high iron chelating abilities while the formation of microspheres will provide the necessary stability for the transport through the changing gastrointestinal tract environment. Apart from enhancing the already inherent iron chelating properties of chitosan (Burke et al.,2002), the presence of $-NH_2$ reactive groups on chitosan (Fig. 12) provides sites for enzymatic incorporation of catechol and hydroxyl-carboxic acid functional groups.

Further, the advantages of using chitosan is that it can be designed in many different forms including highly functionalized microspheres (Fig. 12) as envisaged in this study. Microspheres will be made from the phenolic functionalized chitosans as illustrated in Fig. 12.

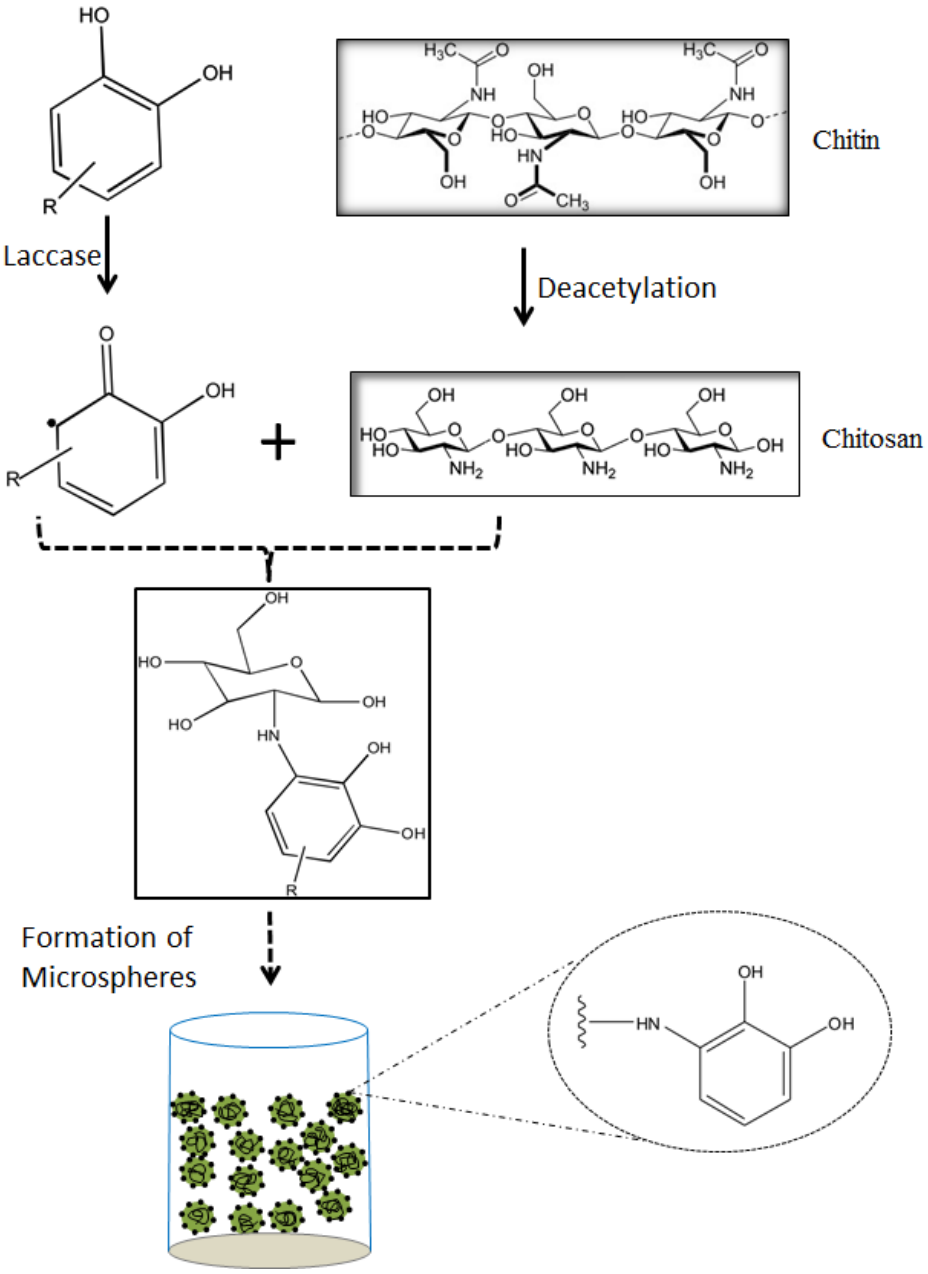


Fig. 12. *Envisaged strategy for the enzymatic synthesis of catechol functionalized chitosan microspheres*

We think that these microspheres can potentially be used either prophylactically or therapeutically while the inherent mucoadhesive properties of chitosan will make it ideal for increasing the residence time of iron chelators.

4.1. How to synthesize the phenolic functionalized microspheres?

4.2. Our tools: Laccase

Laccases are a group of oxidative enzymes whose exploitation as biocatalysts in organic synthesis has been neglected in the past, probably because they were not commercially available. The search for new, efficient and environmentally benign processes for the textile and pulp and paper industries has increased interest in these essentially 'green' catalysts, which work with air and produce water as the only by-product, making them more generally available to the scientific community. Consequently, a significant number of reports have been published in the past decade that have focused on the biochemical properties of these proteins and/or on their applications in technological and bioremediation processes in addition to their use in chemical reactions. Here, we provide a brief discussion of this interesting group of enzymes and their exploitation as biocatalysts, focusing particularly on their use in organic synthesis (Riva, 2006).

4.2.1. Laccases: What are they and how do they work?

Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) belong to the so-called blue-copper family of oxidases. They are glycoproteins, which are ubiquitous in nature - they have been reported in higher plants and virtually every fungus that has been examined for them (Thurston, 1994). An enzyme of this group was first described by Yoshida at the end of the 19th century as a component of the resin ducts of the lacquer tree *Rhus vernicifera* (Yoshida, 1883); more recently, proteins with features typical of laccases have been identified in insects (Kramer et al., 2001) and prokaryotes (Claus, 2003). The physiological function of these biocatalysts, which can be secreted or intracellular, is different in the various organisms but they all catalyze polymerization or depolymerization processes. It has been proposed that laccases are involved in cuticle sclerotization in insects and in the assembly of UV-resistant spores in Bacillus species. In plants they are involved in cell wall formation and, together with peroxidases, in lignification: there is no doubt that laccases are among the main enzymes involved in

delignification processes by white rot fungi (Mayer and Staples, 2002). Additionally, these enzymes can protect fungal pathogens from toxic phytoalexins and tannins, thus they are an important virulence factor in many fungal diseases (Mayer and Staples, 2002). The reactions catalyzed by laccases proceed by the monoelectronic oxidation of a suitable substrate molecule (phenols and aromatic or aliphatic amines) to the corresponding reactive radical. The redox process takes place with the assistance of a cluster of four copper atoms that form the catalytic core of the enzyme (Fig. 13a); they also confer the typical blue colour to these enzymes because of the intense electronic absorption of the Cu-Cu linkages (Piontek et al., 2002). The overall outcome of the catalytic cycle is the reduction of one molecule of oxygen to two molecules of water and the concomitant oxidation of four substrate molecules to produce four radicals (Fig. 13b) (Claus, 2004; Solomon et al., 1996). These reactive intermediates can then produce dimers, oligomers and polymers.

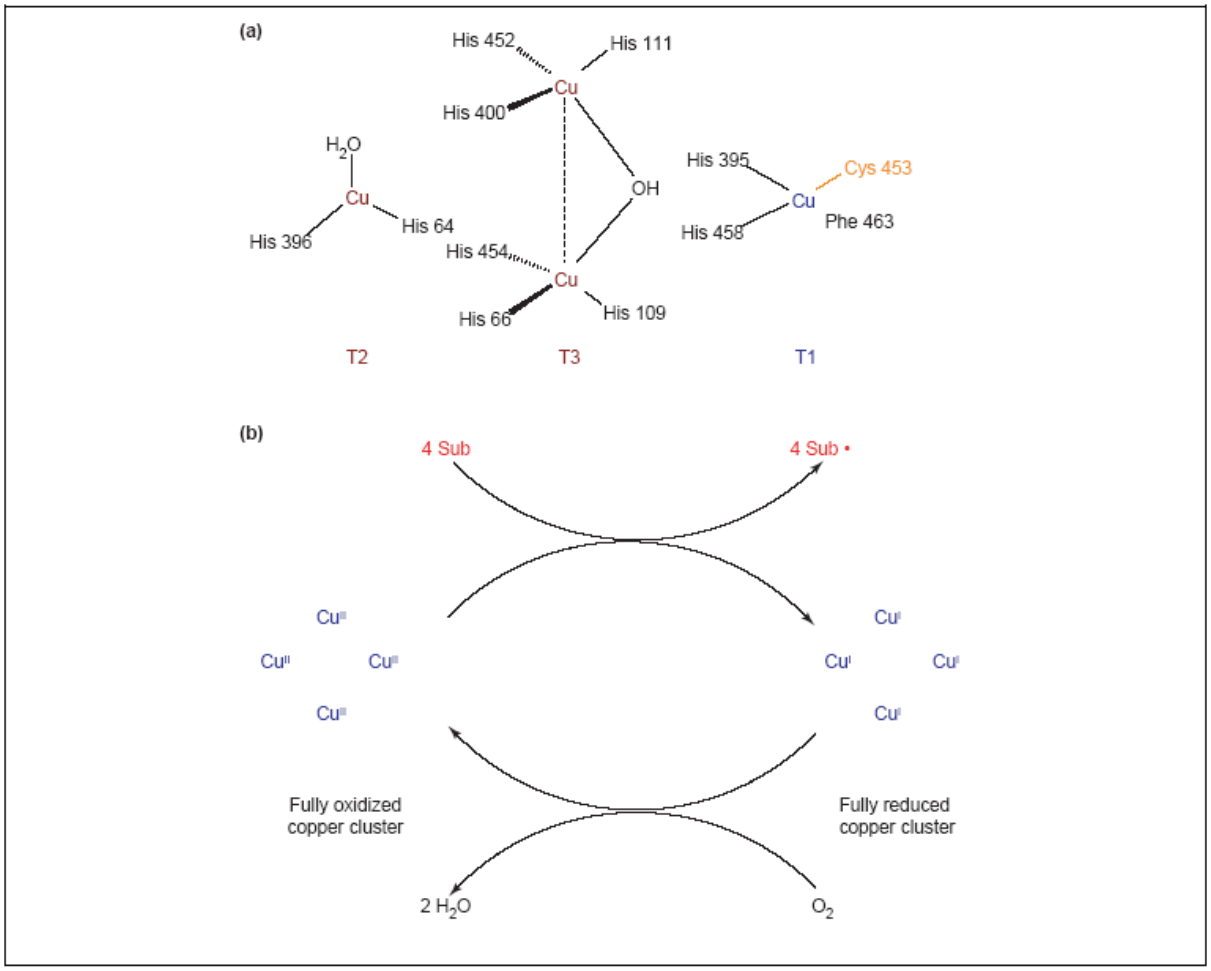


Fig. 13. *Laccases: active-site structure and catalytic cycle. (a) Model of the catalytic cluster of the laccase from Trametes versicolor made of four copper atoms. Type I (T1) copper confers the typical blue colour to the protein and is the site where substrate oxidation takes place. Type 2 (T2) and Type 3 (T3) copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place (Piontek et al., 2002). (b) Schematic representation of a laccase catalytic cycle producing two molecules of water from the reduction of one molecule of molecular oxygen and the concomitant oxidation (at the T1 copper site) of four substrate molecules to the corresponding radicals (Claus et al., 2004, Riva S., 2006)*

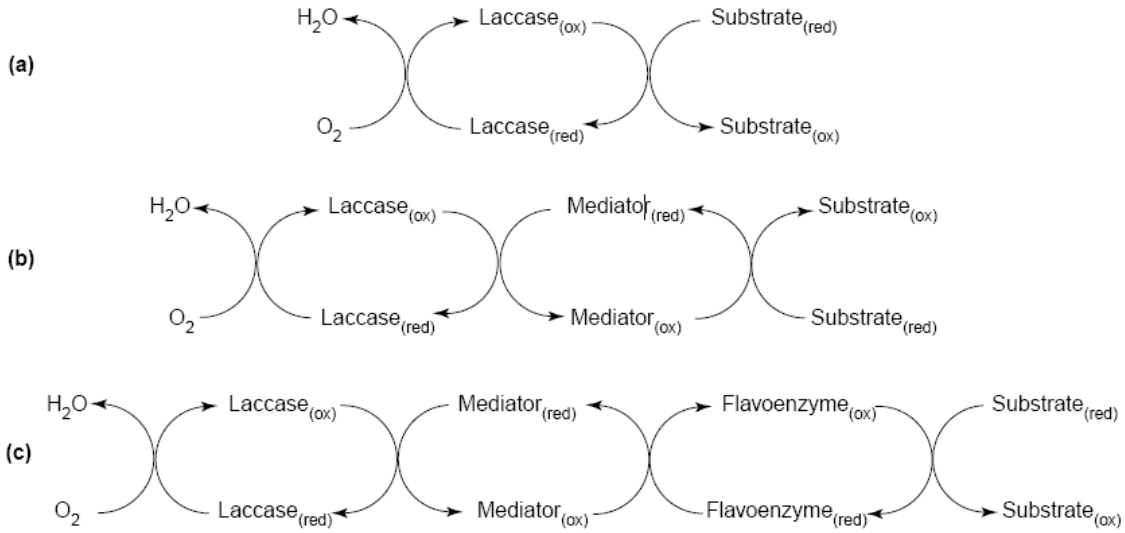


Fig. 14. *Schematic representation of laccase-catalyzed redox cycles for substrates oxidation in the absence (a) or in the presence (b and c) of chemical mediators (Riva S., 2006)*

From a mechanistic point of view, the reactions catalyzed by laccases in technological or synthetic applications can be represented by one of the schemes shown in Fig.14. The simplest case (Fig. 14a) is the one in which the substrate molecules are oxidized to the corresponding radicals by direct interaction with the copper cluster. Frequently, however, the substrates of interest cannot be oxidized directly by laccases, either because they are too large to penetrate into the enzyme active site or because they have a particularly high redox potential. By mimicking nature, it is possible to overcome this limitation with the addition of so-called 'chemical mediators', which are suitable compounds that act as intermediate substrates for the laccase, whose oxidized radical forms are able to interact with the bulky or high redox-potential substrate targets (Fig. 14b). A noteworthy example (Fig. 14c) was presented by Haltrich and co-workers, whereby the laccase-oxidized mediator, in turn, oxidizes the flavine cofactor of a dehydrogenase in a coupled enzymatic system for the in situ regeneration of FAD (Ludwig et al., 2004).
Some oxidation (Fig. 15, Fig. 16) and coupling (Fig. 17) reaction of laccase.

Fig. 15. *The oxidation of benzyl alcohols to the corresponding aldehydes (Potthast et al., 1996; Fabbrini et al., 2001; Langhals and Kunath, 1998; Barilli et al., 2004; Riva, 2006)*

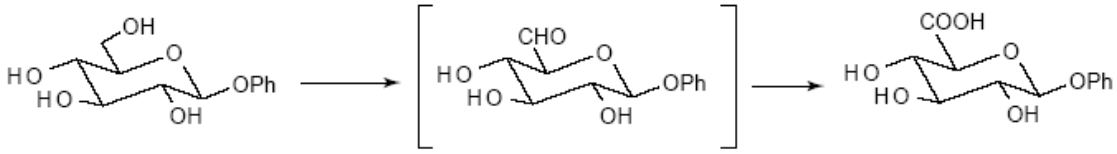


Fig. 16. *The oxidation of alkyl b-glucosides to their corresponding glucorunosides (Marzorati et al., 2005; Riva, 2006)*

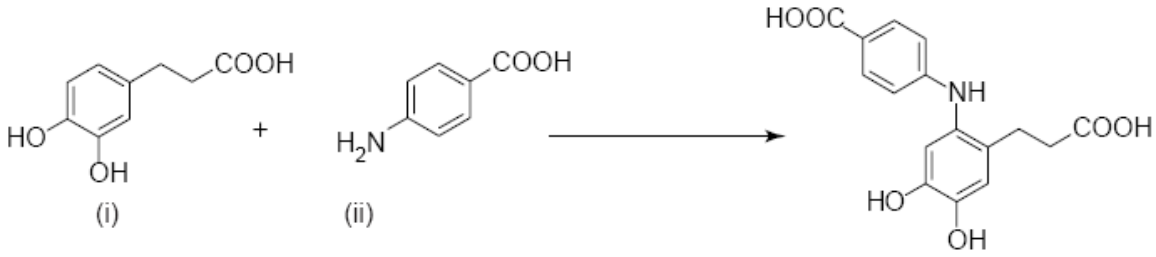


Fig. 17. *Coupling reaction product obtained from dihydrocaffeic acid (i) and 4-aminobenzoic acid (ii) (Marzorati et al., 2005).*

Possible use of laccase-generated radicals is across-coupling reaction with a different molecule, where the reactive intermediates are trapped and do not give rise to the usual family of derivatives. This approach has not been thoroughly investigated but there are some significant examples, namely the coupling of dihydrocaffeic acid and 4-aminobenzoic acid to give (3-[6-(4-carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid) (Fig. 17) as the main product at O 80% yield (Mikolasch et al., 2002) and, more recently, the coupling of p-hydroquinones and aromatic amines (Niedermeyer et al., 2005; Manda et al., 2005).

5. Aim of this work

The aim of this work is to synthesis highly phenolic functionalized chitosan microspheres for iron overload disease treatment. This functionalized chitosan microspheres for the treatment of iron overload disease would be effective, without side effects and non-toxic. The goal is create a drug (chelator), which could fully replace existing drugs with many side effects which are officially used to treat iron over load disease. Solving this problem would be able to extend and improve the quality of life to all with iron overload disease.

5.1. Objectives

- In vitro coupling of iron chelating phenolics to glucosamine.
- In vitro coupling iron chelating phenolics on to chitosan.
- Synthesis of phenolic functionalized chitosan microspheres
- Investigating the iron chelating capabilities of synthesized microspheres.

6. Materials and methods

6.1. Materials

All chemicals used were of analytical grade. The phenolic compounds (caffeic acid, 2,5-dihydroxybenzoic acid, catechol) and glucosamine were purchased from Sigma-Aldrich. Laccase from *Trametes hirsuta* was produced and purified as previously reported by Almansa et al. (Almansa et al., 2004). Chitosan samples were kindly provided by Dr. Guillermo Rocasalbas of the University of Catalunya, Spain. All other chemicals were purchased from Merck.

6.2. Method

6.2.1. Laccase activity assay

The activity of laccase was determined spectrophotometrically by monitoring the oxidation of 2,2'-azinobis-(3-ethylbenzothiazo-line)-6-sulphonate (ABTS) ($\varepsilon 436=29{,}300\ M^{-1}\ cm^{-1}$) as substrate at 436 nm in 50 mM succinate buffer at pH 4,5 and 37°C as described by Niku-Paavola et al. (Niku-Paavola et al., 1988) with some modifications. Briefly, the reaction mixture contained 30 µl laccase, 350 µl ABTS (1 mM), and 50 mM succinate buffer, pH 4,5 to make a final volume of 1,5 ml. A blank was set in the same way as the sample experiment except that the laccase was initially heat denatured at 100°C for 10 min. The spectrometric measurements were done by recording the absorbance in the time scan mode for 2 min.

6.2.2. In vitro coupling reactions of phenolic molecules onto glucosamine

To investigate if laccase was able to mediate the coupling of phenolic compounds onto chitosan, glucosamine was used as a model substrate representing monomeric unit of chitosan. The reaction mixture contained one of the phenolic compounds (catechol, caffeic acid or

2,5-dihydroxibenzoic acid) and glucosamine (200 mM) in the molar ratio of 1:1 and 13,4 nkat ml^{-1} laccase in 50 mM succinate buffer (pH 4,5). Reactions were carried out at 37°C while shaking at 650 rpm using a thermomixer (Thermomixer Comfort Eppendorf AG, Hamburg, Germany) for 24 h and the coupling products analyzed by HPLC-MS.

6.2.3. HPLC-MS analysis of reaction products

An equal volume of ice cold methanol was added to the reaction mixtures above to precipitate protein. The mixture was allowed to stand on ice for 30 min before centrifuging at 0°C for 15 min at 14,000 g and 650 µl aliquots were transferred into clean vials. HPLC analysis was performed using a system from Dionex equipped with a P580 pump, an ASI-100 autosampler, and a PDA-100 photodiode array detector while monitoring elution at 254 nm. Separation of coupling products was achieved by reversed phase HPLC RP-C18 column (Eurospher 100-5, C18, 150×4,6 mm with pre-column, Knauer GmbH, Berlin, Germany) using a linear gradient of formic acid (solvent A) and acetonitrile (solvent B) as solvent at a flow rate of 1 ml min^{-1}, an injection volume of 10 µl, and an oven temperature of 30°C. Initially, the gradient was set at 100% for 30 min, later at 50% for 20 min, then at 5% for 20 min, and finally 0% formic acid for 30 min. The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with electrospray ionization coupled to the Dionex HPLC-UVD-system described above. The coupling products were measured in positive ion mode and the electrospray voltage was set to +3500 V. Dry gas flow was set to 12 l min^{-1} with a temperature of 350°C, nebulizer to 70 psi. Maximal accumulation time was fixed to 300 ms and the loading of the trap was controlled by the instrument with an ICC of 30,000.

6.2.4. TLC - thin layer chromatography

Silica TLC *(Thin layer chromatography)* Platten were used for this analysis. TLC encompasses dividing particular constituents between advancing (mobile) phase of the solvent and solid

(stationary) phase of thin layer. Stationary phase is silikagel and mobile phase is composed of organic solvents, which in our case were optimized and eventually, their mobile phase was used for chitosan with ratio buthanol: acetic acid: water 80:30:20 and for catechol benzen: toluene: acetic acid with ratio 30:20:10. The compromise was inevitable though, and after the optimization, the most suitable ratio of buthanol: pyridine: acetic acid: water composition was 10:12:8:10. It was used and modified method from Sullivan and Sherma, 2005 and Chakraborty et. al., 1990.

The sample for this analysis was prepared same like sample for HPLC-MS. Sample was dripped on the silica plate. The plate is then inserted into the development chamber which contains a fusion of dissolving agents in certain ratio - mobile phase which immediately starts to rise by capillary action up. During its advance through silikagel, it encounters the substances in the smudge and divides them (according to the dissolvability) into mobile phase and (according to the molecular size) particular substances cease to move at different height. Thus, the reaction compound is divided into its components. Individually spots were shown by heating to 90°C of the silica plate.

6.2.5. Spectrophotometric analysis

For spectrophotometric analysis was used spectrophotometer UV-Vis (Hitachi U-2001). Wavelength scan was used for analysis in vitro coupling reactions of phenolic molecules onto glucosamine.

Was used analysis in water, but also were used extraction for better visibility of the reaction product. Glucosamine and chitosan on the contrary to catechol, 2,5-dihydroxybenzoic acid and caffeic acid are not dissolvable in organic solvents such as benzene. Therefore, benzene was used to rinse the sample to flush the molecules which did not couple.

6.2.6. FTIR spectroscopy analysis

FTIR spectroscopy analysis of reaction products FTIR spectra was carried out by using a Perkin Elmer Spectrum 2000 instrument by the attenuated total reflectance (ATR) technique. The reaction products were frozen in liquid nitrogen followed by lyophilization using Labconco Freeze Dry System/FreeZone 4,5 Liter Benchtop Model 77,500 (Vienna, Austria). The freeze drier was operated at a temperature of -48°C and at a vacuum pressure of 3×10^{-4} mbar. The lyophilized chitosan/glucosamine-phenolic reacted samples were then analyzed by FTIR. Spectra were recorded in the 4000-600 cm^{-1} range with 16 scans at a resolution of 4,0 cm^{-1} and an interval of 1,0 cm^{-1}.

6.2.7. RAMAN the method of electromagnetic spectroscopy

Raman was used for the analysis. The samples were prepared as noted earlier – like samples for FTIR analysis. This lyophilized chitosan/glucosamine-phenolic reacted samples were then analyzed by RAMAN.

Raman spectroscopy is a method of electromagnetic spectroscopy, more precisely a supplementary method to infrared spectroscopy. Intensive areas in Raman spectra are weak in infrared spectra and inversely, because the vibrations, with which the vibration is changing the polarizability have areas in Raman spectra and vibrations which alter molecule dipole have areas in infrared spectra.

6.2.8. Grafting of phenolic molecules onto chitosan

Chitosans of varying molecular weights (15, 50 and 300 kDa) were incubated with each of the phenolic compounds. The grafting of phenolic molecules onto chitosan was performed by first dissolving 1 g chitosan in 20 ml double distilled water supplemented with 2% (v/v) acetic acid. Chitosan (4 ml) solutions were then supplemented with 10 mM final concentration of the respective phenolic molecule. The reaction was then started by adding 50 µl of laccase

(20 nkat ml^{-1} in 2 ml 50 mM sodium citrate buffer at pH 4,5 and incubated for 24 h at 30°C while shaking at 150 rpm. The reaction mixture was then extensively washed (repeatedly washed five times while vigorously shaking) with deionized water in case of catechol and with ethanol in case of 2,5-dihydroxybenzoic acid, to remove any unreacted phenolic molecules. Finally, the resulting insoluble polymer was further rinsed with double distilled water to remove any ethanol.

6.2.9. Determination of the degree of coupling of different phenolic compounds onto chitosan

The ninhydrin (NHN) assay as described by Mi et al. (Mi et al., 2001) was used to determine the amount of residual free amino groups remaining in chitosan after cross-linking. The reagents were prepared in two parts. The first solution was prepared by mixing 1,05 g citric acid, 10 ml (1,0 M) NaOH, and 0,04 g $SnCl_2 \cdot 2H_2O$ and adding deionized H_2O until 25 ml. The second solution was prepared by dissolving 1 g ninhydrin in 25 ml ethylene glycolmonomethyl ether. The two solutions were then combined and stirred for 45 min before being stored in a dark bottle at 4°C. Phenolic grafted chitosans (0,5 g) dissolved in 2% (v/v) acetic acid were added to a 1 ml ninhydrin solution and heated to 100°C in water bath for 20 min. The solution was then cooled down to room temperature, diluted with 5 ml 50% isopropanol, and then the optical absorbance of the solution measured at 570 nm using a UV-Vis spectrometer (Hitachi U-2001). The change in concentration of free NH_2 groups in the chitosan samples was then determined from a standard curve of glycine concentration vs absorbance. The degree of coupling of phenolic monomers was estimated by subtracting the concentration of NH_2 groups remaining after reacting the respective phenolic monomer with chitosan/glucosamine in the presence of laccase from the concentration of NH_2 groups in the reaction containing chitosan/glucosamine and respective phenolic monomers. Laccase was used as the blank in all experiments since it also contains amino groups.

6.2.10. Preparation of hydroxyl-carboxic and catechol functionalized microspheres

Phenolic functionalized chitosan (15, 50, 300 kDa) was prepared as described before. The purified polymer was dissolved in 2% (v/v) acetic acid as described above enough to form a gel-like material. This was then introduced drop wise via a syringe pump into a solution containing NaOH-methanol bath (Agnihotri et al., 2004) while stirring solution at 100 rpm. After cross-linking, microspheres were washed three times using 96% (v/v) methanol and rinsed three times with double distilled water and a final rinse with 96% methanol and then dried for 4 h at 40°C.

6.2.11. Scanning electron microscopy (SEM) analysis of microspheres

SEM (Zeiss Ultra 55) was conducted as previously described by Mistlberger et al. (Mistlberger et al., 2010). Briefly after drying, the microspheres were fixed on a conventional SEM specimen holder with a conductive double-sided adhesive carbon tape. Sputter coating was performed, in order to apply a layer of Pt/Pd to the nonconductive samples for the avoidance of charging during SEM investigations. The functional chitosan microspheres were analyzed using a Zeiss Ultra 55 equipped with a Schottky field emitter (SFE) at a voltage of 5 kV. This scanning electron microscope is well established for the morphological characterization of particles in the submicrometer region and even of nanoparticles.

FEI ESEM Quanta 600 FEG was used for analysis of structure changing in reaction with chitosan and phenols molecules. The FEI ESEM Quanta 600 FEG is a versatile scanning electron microscope with three imaging modes. The “high vacuum mode” (HV) is a conventional SEM mode with the need of conventional specimen preparation. In the “low vacuum mode” (LV) electrically nonconductive samples can be imaged without the need of a conductive layer (e.g. carbon, gold etc.). Additionally in the “ESEM mode” (ESEM) wet samples can be investigated in their “natural” state. The thermally assisted field emission gun (FEG) delivers high brightness of the electron beam and high imaging resolution. Additionally the microscope

can be equipped with a tensile stage, a Peltier cooled specimen stage, a heating stage and an in situ ultramicrotome. Self-forming structure of chitosan compounds in response to coupling reaction in molecule (Mistlberger et al., 2010).

Sample was prepared 5 ml chitosan (1 g of chitosan 300 kDa in 200 ml 0,1% acetic acide) with 5 ml 100 mM of phenol molecule for 24 hour. Reaction was started by adding 250 µl of laccase (20 nkat ml^{-1} in 2 ml 50 mM sodium citrate buffer at pH 4,5 and incubated for 24 h at 25°C). This was dropt on to glass and dried in the air in 25°C.

6.2.12. Confocal laser microscopy

For confocal microscopy analysis was used Leica TCS SPE laser scanning confocal microscope. Sample was prepared same like samples for electron microscopy.

6.2.13. Ferrous metal ions chelating activity

The iron-chelating capacity was estimated by the method described by Dinis et al. (Dinis et al., 1994). A 25 µl solution of 2 mM $FeCl_2.6H_2O$ was added to the phenolic functionalized chitosan sample. A 5 mM ferrozine (100 µl) solution was then added and total volume adjusted to 2 ml using ethanol. The mixture was then thoroughly mixed and left to stand for 10 min at room temperature. The solution was then measured spectrophotometrically at 615 nm using a Hitachi UV-2001. The concentration of ferrozine-Fe^{2+} complex formation was calculated by subtracting the unreacted chitosan absorbance value from the grafted chitosan.

7. Results and discussion

7.1. Effect of pH optimum on laccase

Important for this work with laccase is to know their ideal optimum in which they have the highest activity. To this effect the pH optimum of laccase from *Trametes villosa* was determined spectrophotometrically using 2,2'-azinobis-(3-ethylbenzothiazo-line)-6-sulphonate (ABTS) as substrate (Niku-Paavola et al., 1988) and determined to be 4,5 (Fig. 18). These results are in good agreement with similar laccases from white rot fungi determined to be between 4 and 5 pH (Brenda Enzyme) of reaction environment is a very important factor for our coupling reaction, since chitosan is soluble in acidic environments. But always is very important to verify all information, because each enzymes can behave quite different, especially laccase.

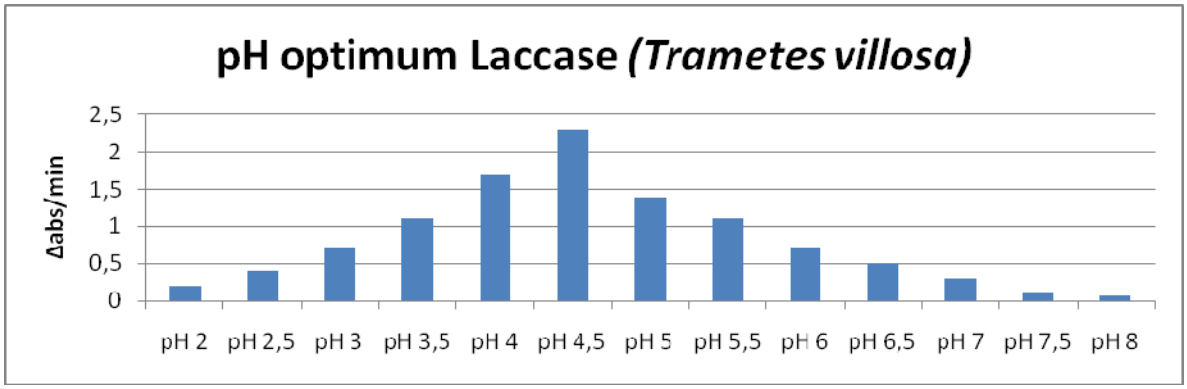


Fig. 18. *Laccase activity dependence on pH*

7.2. In vitro studies of the laccase mediated coupling of phenolics onto glucosamine

The ability of laccase to mediate the coupling of phenolic compounds onto chitosan was first assessed using glucosamine as a model substrate. Laccase oxidation of phenolic compounds can form reactive species which in turn could reacted non-enzymatically (e.g., Michaels addition) with -NH_2 groups of glucosamine (Kudanga et al., 2010). The coupling products were monitored using TLC, UV-VIS spectroscopy, HPLC-MS and FTIR.

7.2.1. TLC analysis

TLC is one of the simplest and cheapest analytical method used in analytical chemistry: To this effect we used TLC to investigate the coupling of catechol, caffeic acid, and 2,5-dihydroxybenzoic acid onto glucosamine (a model compound of chitin). As shown in Fig. 19A new coupling products were observed with catechol and 2,5-dihydrobenzoic acid and glucosamine in the presence of laccase. This shows that laccase was important to mediate the coupling. For example the mew coupling product between catechol and glucosamine was not able to move in the mobile phase, however the original substrates decreased in concentrations as evidenced by the fading spots. In the Fig. 19B the reaction of dihydroxibenzoic acid with glucosamine and laccase is shown. The optimal mobile phase for the separation of particular components of the reaction was found to be 10:12:8:10 (buthanol:pyridine:acetic acid:water). However, TLC analysis of the reaction products between caffeic acid and glucosamine did not show new peaks.

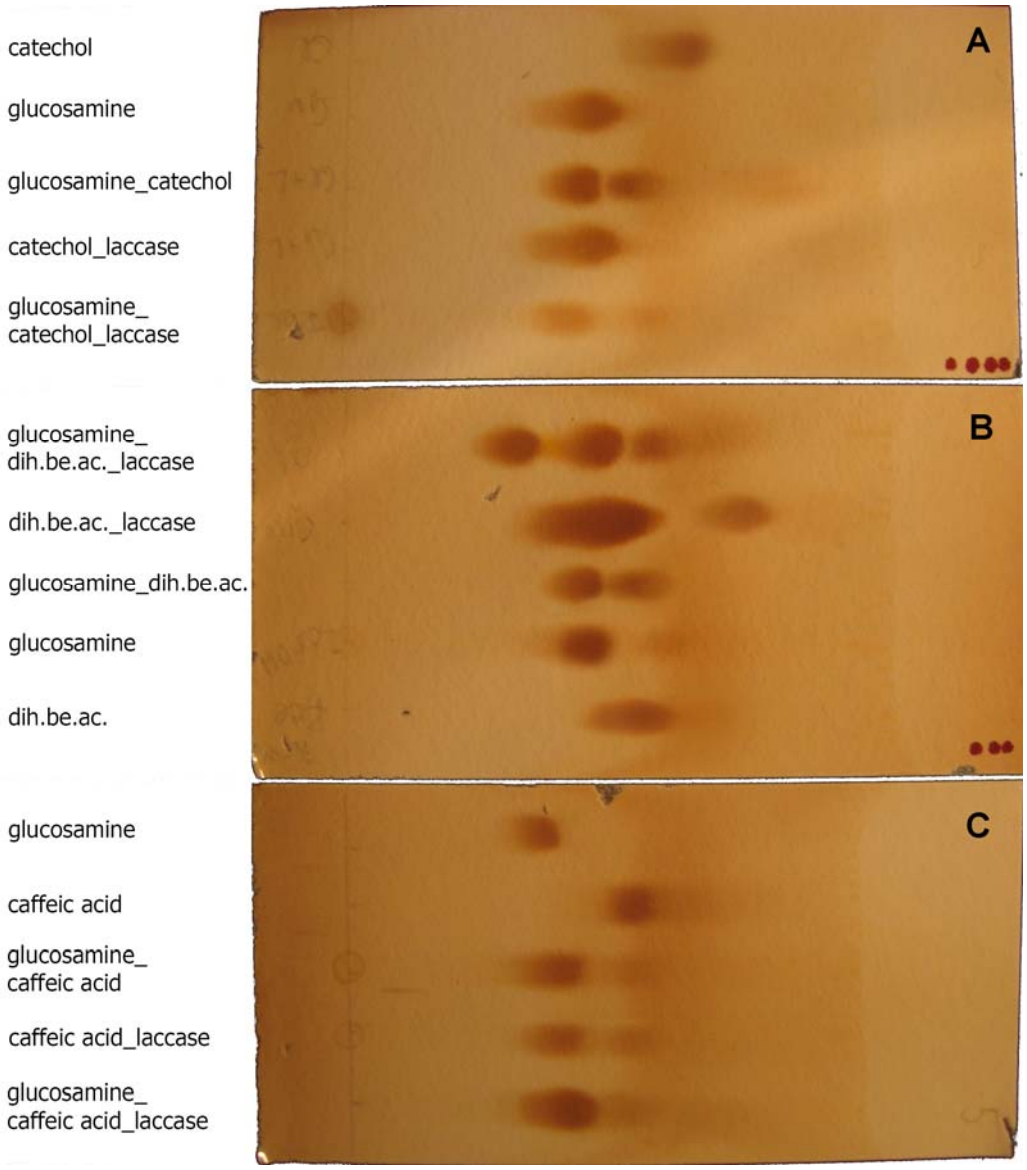


Fig. 19. *(A) coupling reaction with catechol, (B) coupling reaction with dihydroxybenzoic acid, (C) coupling reaction with caffeic acid*

7.2.2. Spectrophotometric studies of coupling of phenolics onto glucosamine

Further analysis of the laccase mediated coupling reactions onto glucosamine were monitored using UV-VIS spectroscopy.

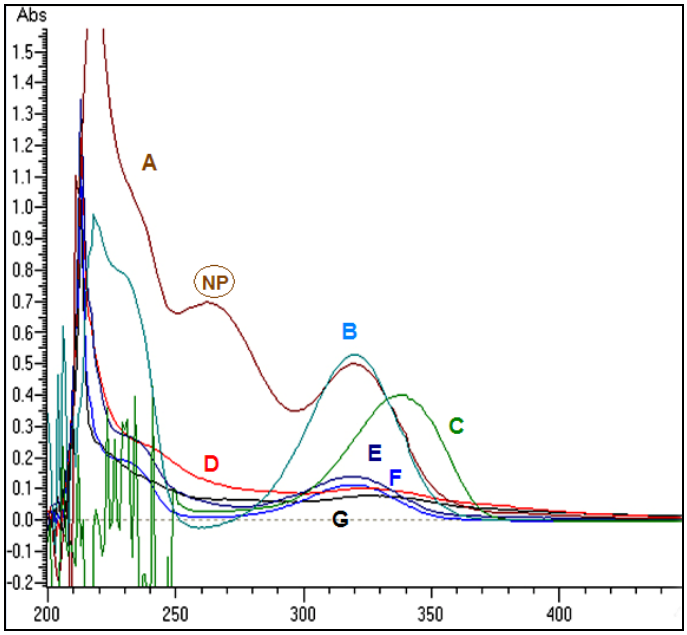


Fig. 20. *(A) glucosamine, dihydroxibenzoic acid and laccase with new product (NP);(water phase after extraction); (B) glucosamine, dihydroxibenzoic acid and laccase (water phase without extraction) before washing; (C) dihydroxibenzoic acid (just in ethylacetone); (D) glucosamine in water phase (no reaction); (E) dihydroxybenzoic acid with laccase after washing (water phase); (F) dihydroxybenzoic acid in water phase after washing (no reaction); (G) glucosamine in ethylacetone phase (no reaction)*

The phenolic 2,5-dihydroxybenzoic acid was successfully coupled onto glucosamine as evidenced by the new peak (NP). However, in order to observe this product it was necessary to wash the unbound or excess phenolics. Glucosamine and chitosan on the contrary to catechol, 2,5-dihydroxybenzoic acid and caffeic acid are not dissolvable in organic solvents such as ethylacetone. Therefore, ethylacetone was used to rinse the sample to flush the molecules which did not couple. In the Fig. 20A is displayed rinsed water phase of complete reaction (with abs 270 nm, a new product of this reaction occurs). Fig. 20E displays water phase of 2,5-dihydroxybenzoic acid with laccase and Fig. 20F shows 2,5-dihydroxybenzoic acid in water phase after the rinsing. Fig. 20C shows fresh dihydroxybenzoic acid in ethylacetone (without extraction) Fig. 20B displays complete reaction in water, concentration was for spectra analysis extensively diluted. After washing with ethylacetone we get aqueous phase with our product as seen in Fig. 20A. Fig. 20D shows glucosamine in the water phase and Fig. 20G glucosamine in

the ethylacetone phase. Although we could detect coupling products between 2,5-dihydroxybenzoic acid and glucosamine, we could find coupling products with catechol and caffeic acid. It was therefore decided to use HPLC since it is a more versatile analytical tool able to detect very low concentrations.

7.2.3. HPLC studies of coupling of phenolics onto glucosamine

HPLC chromatograms of the couplings between glucosamine with either catechol, caffeic acid or 2,5-dihydroxybenzoic acid showed new peaks as summarized in Fig. 21 indicated by red. HPLC spectra in the Fig. 21 indicate formation of new product during the reaction of catechol with glucosamine and in the Fig. 21 is displayed result of the coupling reaction of caffeic acid onto glucosamine. All newly formed peaks were further analyzed with HPLC-MS to confirm new product using molecular weight calculations of the molecules.

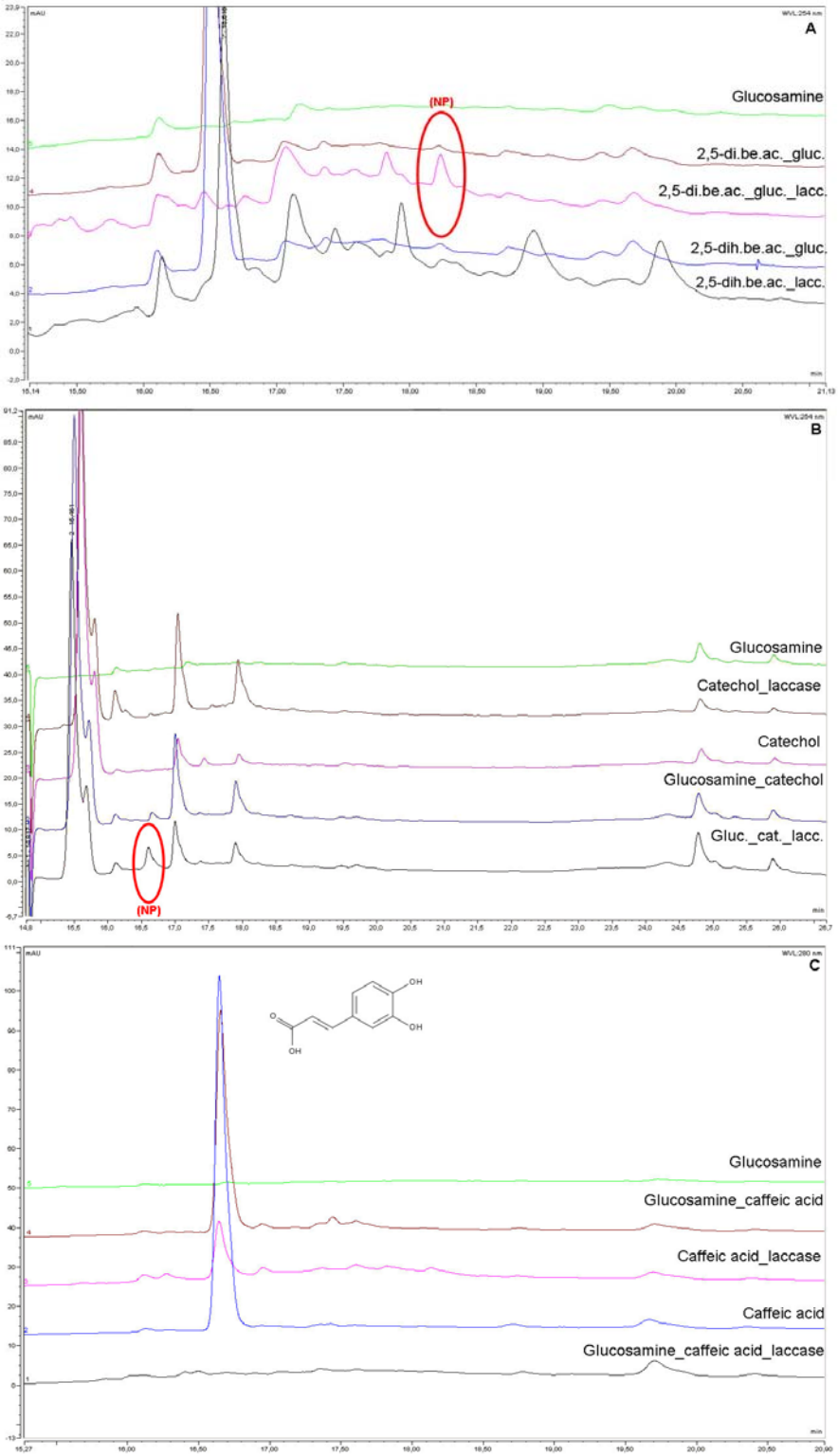


Fig. 21. *HPLC spectra of 2,5-dihydroxibenzoic acid, catechol and caffeic acid reaction with glucosamine and laccase*

7.2.4. HPLC-MS analysis of coupling of phenolic compounds onto glucosamine

Indeed, the HPLC-MS analysis indicated that both catechol moiety containing molecules (catechol and caffeic acid) and the hydroxyl-carboxic acid containing molecule (2,5-dihydroxybenzoic acid) were readily coupled onto glucosamine in a 1:1 ratio (Fig. 22). In all cases, the observed peaks correlating to the hybrid molecules showed dominant signals as compared with parent compounds (Fig. 22). For example, $[M+H]^+$ ions at m/z = 331,4 correspond to the coupling of 2,5-dihydroxybenzoic acid to glucosamine (Fig. 22) were observed. Similarly, strong signals for the $[M+H]^+$ ions at m/z = 358,1 correspond to the coupling of caffeic acid to glucosamine (Fig. 22). Coupling of catechol onto glucosamine showed strong signals for the $[M+H]^+$ ions at m/z = 288,5 together with a number of other peaks. Considering the HPLC-MS results, structures embedded in the respective MS spectra figures were proposed (Fig. 22). C-N couplings are known to occur through either Michael addition or radical coupling (Kudanga et al., 2010; Niedermeyer et al., 2005; Mikolasch et al., 2008). For example, during enzymatic coupling of chlorogenic acid onto chitosan, Kumar et al. (Kumar et al., 1999) suggested a nucleophilic attack on the chitosan $-NH_2$ on the oxidized chlorogenic acid - a mechanism expected in this study. Here, it is demonstrated for the first time using HPLC-MS the coupling of laccase oxidized phenolic substrates to the monomeric constituent of chitosan namely glucosamine.

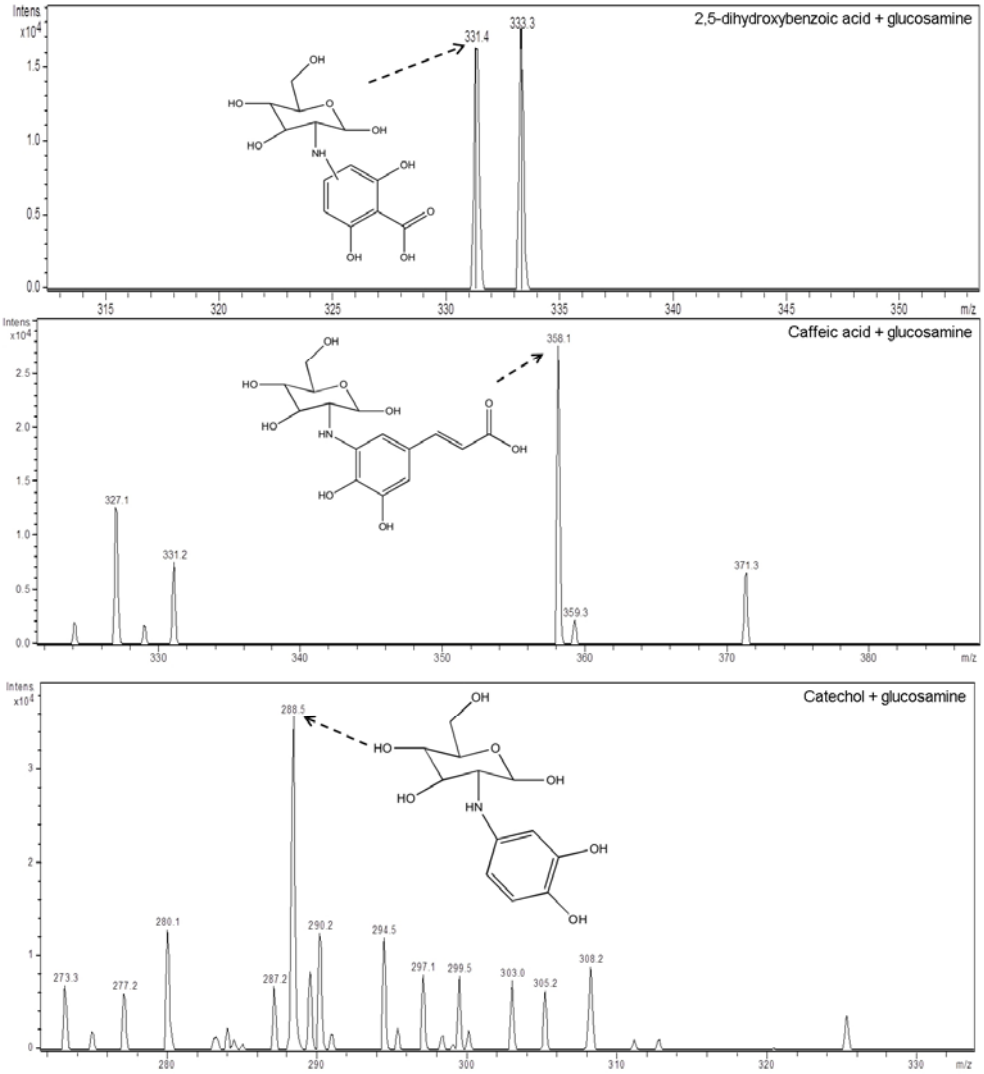


Fig. 22. *HPLC-MS chromatograms of the laccase assisted coupling of 2,5-dihydroxybenzoic acid, caffeic acid, and catechol onto glucosamine*

7.2.5. FTIR spectroscopy analysis of coupling of phenolics onto glucosamine

In previous studies, it was also demonstrated that alkylamines can be coupled onto monomeric and oligomeric forms of guaiacol and catechol once they oxidized by laccase (Kudanga et al., 2010). The fact that all phenolic compounds investigated in this study were readily coupled onto glucosamine agrees with previous observations that hydroxyl groups on the benzene ring are ortho or para directing and facilitates coupling in this way (Suparno et al., 2005;

Schultz et al., 2001, Jonas et al., 2000). Consequently, molecules with free C-5 position cross-couple through 5-5 linkages due to stability of C-C bonds (del Río et al., 2008) as speculated for catechol. FTIR was used to study the coupling of glucosamine to the different phenolic compounds (Fig. 23). The characteristic NH_2 groups were detected within the range 3000-3500 cm^{-1} wave lengths, C-O-H peak around 1060 cm^{-1}, C-O signals between 1000 and 1200 cm^{-1} as well as the strong N-H stretch at 1560-1650 cm^{-1} which is in agreement with previous observations (Burugnerotto et al., 2001; Ostrowska-Czubenko et al., 2009). Upon the enzymatic coupling of glucosamine to 2,5-dihydroxybenzoic acid (Fig. 23A), caffeic acid (Fig. 23B), and catechol (Fig. 23C), the intensity of the characteristic NH_2 groups between 3000-3500 cm^{-1} decreased in all reaction mixtures although a characteristic spectra depending on the phenolic compound used was observed. All spectra exhibited broad peaks in the range of 3450-3400 cm^{-1}. The peaks were also assigned to an OH stretching overlapping in the same region with the NH_2 group. The FTIR chromatograms provided further proof that indeed there was a decrease in $-NH_2$ group indicating reaction between the phenolic compounds with glucosamine.

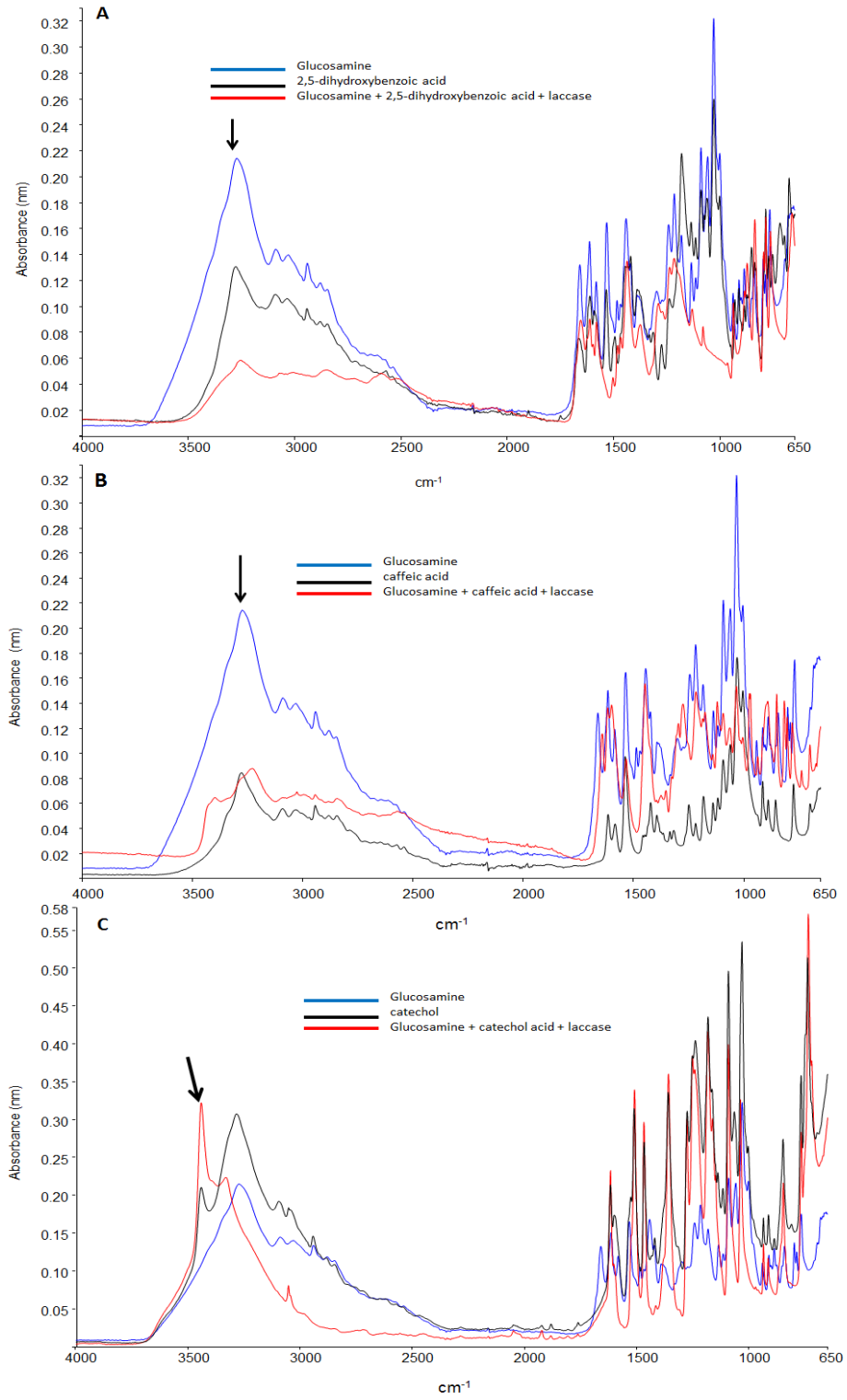


Fig. 23. *FTIR spectra of different coupling of (A) 2,5-dihydroxybenzoic acid, (B) caffeic acid, and (C) catechol onto glucosamine*

7.3. Laccase mediated grafting of phenolic molecules onto chitosan

Laccase-catalyzed oxidation of phenolic compounds and their subsequent non-enzymatic reaction with the primary amino groups of various chitosans with different molecular weights (15, 50 and 300 kDa) and glucosamine as a model substrate was investigated. Quantification of -NH_2 groups on chitosan was used to determine coupling efficiencies (Fig. 24). However, in order to determine the optimum concentration of reactants preliminary experiments were carried out with glucosamine since it has defined amount of NH_2 groups.

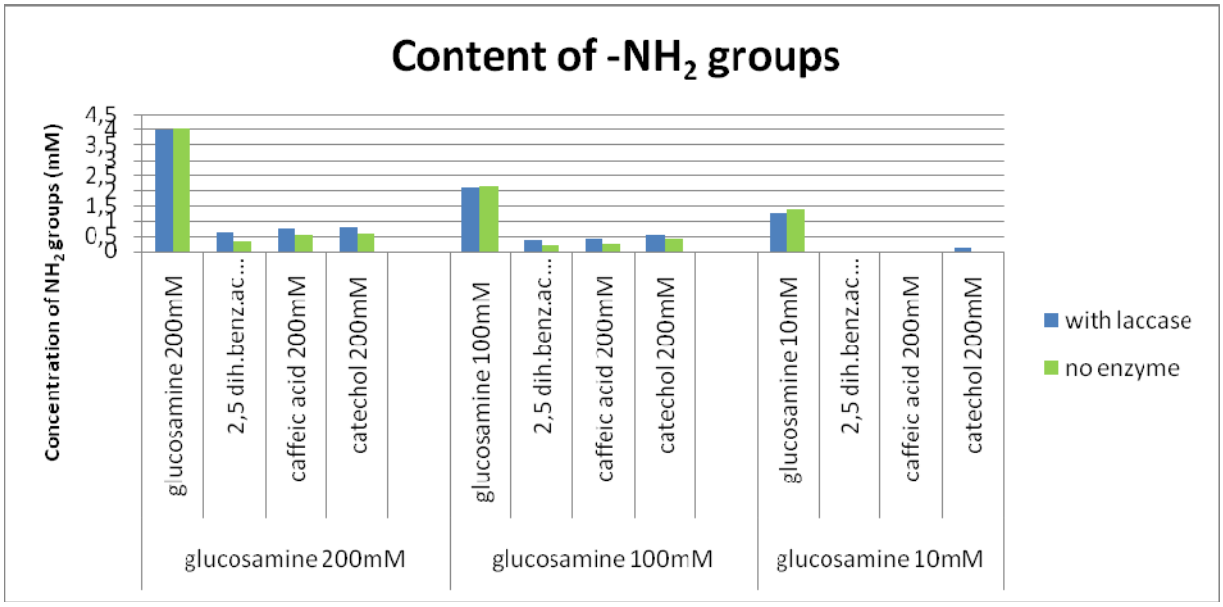


Fig. 24. *Quantification of -NH_2 groups on chitosan which determines the coupling efficiency depending on concentration of reactant*

In the first instance, the effect of pH on the consumption of NH_2 groups was investigated and their decreased assumed to represent successful coupling. Quantification of NH_2 groups on chitosan determines the coupling efficiency depending on pH and the concentration of the molecules is interesting. Changing the pH during the reaction from 2 to 4, 6 and 8 did not have visible effect on the coupling of dihydroxybenzoic acid. The coupling effect though, was more distinct with pH 2 and 4 with catechol as well as with caffeic acid. In the Fig. 25 is displayed efficiency of phenolic compound coupling effect to chitosan depending of pH and also its

dependency on laccase. The efficiency of laccase is clearly higher regarding catechol and caffeic acid. Regarding the reaction of chitosan and dihydroxibenzoic acid, no distinct change of NH_2 free groups depending on laccase is observed. Analysis of phenolic molecules itself (dihydroxybenzoic acid, caffeic acid and catechol) that do not have -NH_2 groups was used only for the results compared.

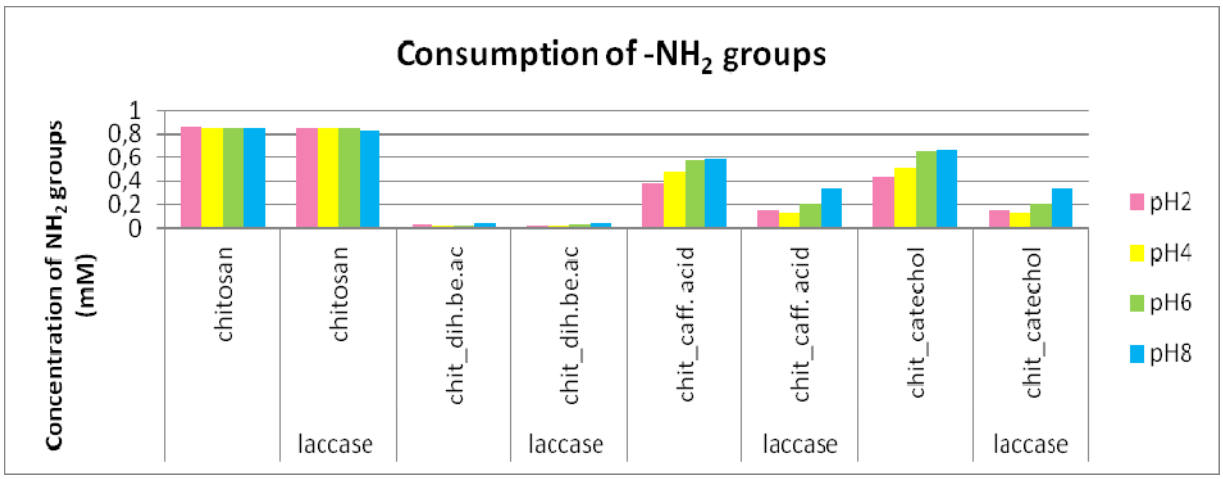


Fig. 25. *Quantification of -NH_2 groups on chitosan which determines the coupling efficiency depending on pH. Reaction with and without laccase*

Regarding the change of glucosamine and phenolic compound concentration ratio, from the Fig. 24 is visible that for the coupling of phenolic compound onto glucosamine, the ratio of 10 mM with 200 mM phenolic compound is the most suitable.

It seems that the effect of laccase is not appreciated as there is no perfect correlation between decreases of NH_2 groups in the presence of laccase. For example it seems 2,5-dihydroxybenzoic acid in the absence or presence of laccase it interacts effectively with chitosan as shown by the decrease of NH_2 groups in both cases.

Since the highest consumption of -NH_2 groups was obtained with the 15 kDa chitosan, a dose-response curve to determine the concentration of phenolic compound required to couple phenolic compound onto chitosan was investigated. As shown in Fig. 4B, 2,5-dihydroxybenzoic acid showed the most effective coupling effect to chitosan as compared with catechol and caffeic acid.

A concentration of 20 mM 2,5-dihydroxybenzoicacid was required to completely occupy all the available NH_2 groups while both catechol and caffeic acid required more than 30 mM (Fig. 26B) for the same concentration of chitosan.

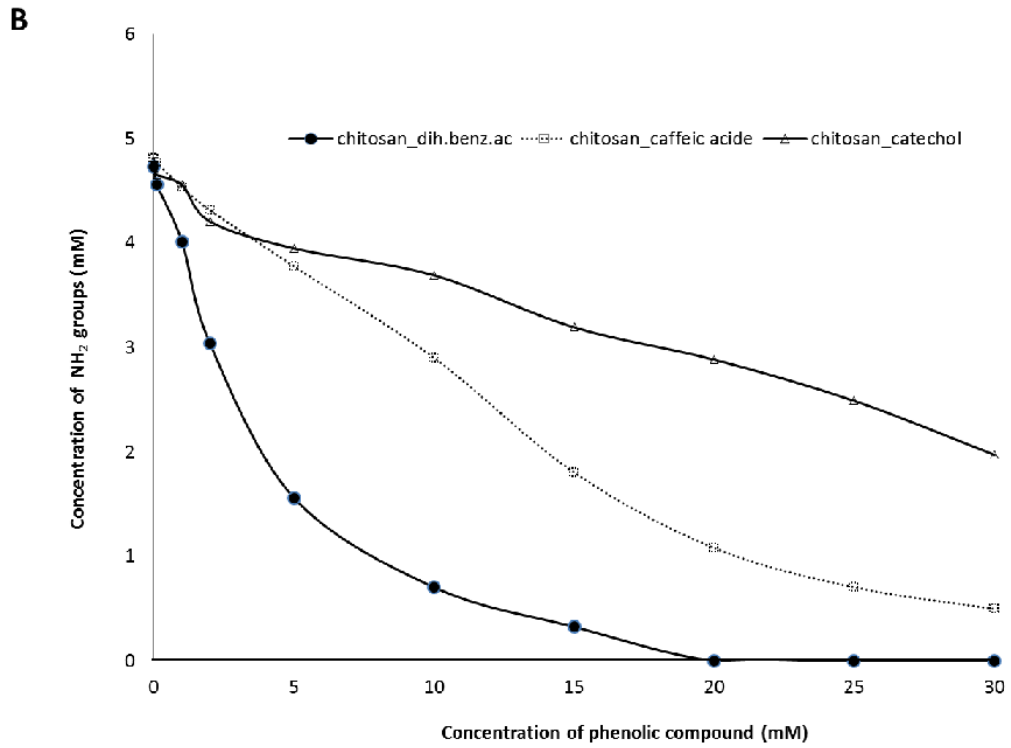


Fig. 26B. *Consumption of -NH_2 groups during enzymatic coupling of phenolic molecules to glucosamine and chitosan and the effect of the concentration of the phenolic molecules in coupling to 15 kDa chitosan*

The number of -NH_2 groups consumed during grafting decreased with increasing MW of chitosans. The ability to enzymatically graft functional molecules onto chitosan is quite interesting since current chemical based methods employ multistep organic synthesis processes (Pasanphan et al., 2008) and initiator systems (i.e., azo compounds and peroxides), which require relatively high reaction temperature (Zohuriaan-Mehr et al., 2005; Curcio et al., 2009). Therefore, the enzymatic functionalization of chitosan represents a new mild approach for grafting functional molecules onto chitosan for many biomedical applications. Some studies have previously employed tyrosinases (Muzzarelli et al., 1994; Payne et al., 1996; Chen et al., 2000; Sousaet al., 2009; Sampaio et al., 2005; Sakai et al., 2009) or peroxidases (Vachoud et al., 2001;

Pasanphan et al., 2010; Ruiz et al., 2000) for the coupling of phenolic compounds onto chitosan. However, there is hardly any study using laccase for this purpose or has shown any mechanistic insights into the coupling of phenolics onto glucosamine.

The observed general decrease in consumed NH_2 groups (Fig. 26A) with increasing chitosan molecular weight [although initial concentration of NH_2 groups was the same (4,0 mM) in all samples] may be attributed to the increasing difficulty in accessing NH_2 groups inside the chitosan polymer by the oxidized phenolics. Restricted diffusion of molecules through the polymer network has been reported by Ruiz et al. (Park et al., 2004) and may also be responsible for lowering effective functionalization of high molecular weight chitosan in this study.

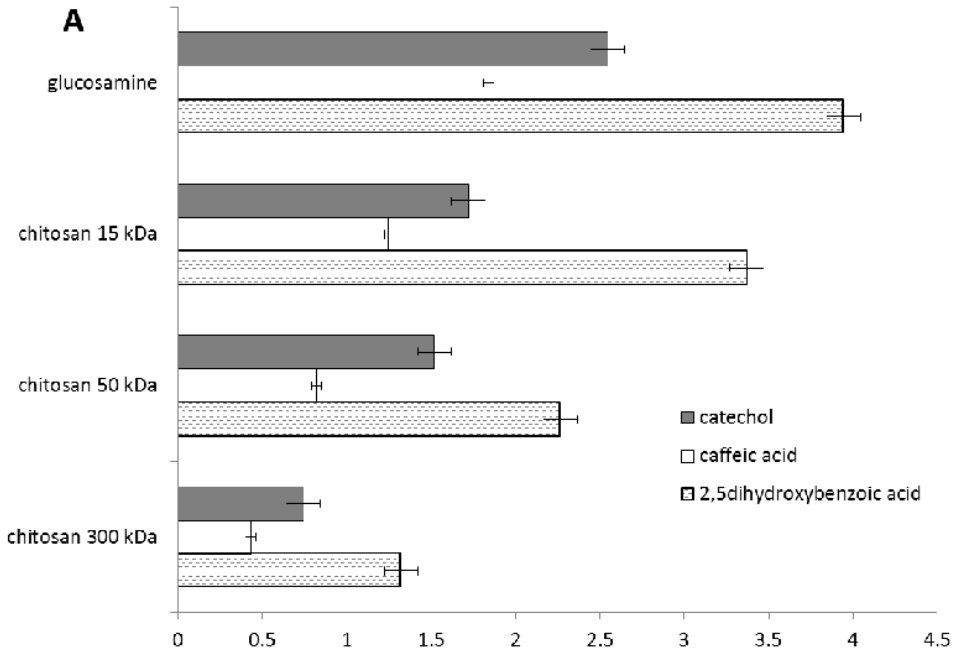


Fig. 26A. *Consumption of -NH_2 groups during enzymatic coupling of phenolic molecules to glucosamine and chitosan with different molecular weights*

Interestingly, FTIR analysis showed the decrease in signals corresponding to NH_2 group on the chitosan (Fig. 27A, Fig. 27B). This confirms the spectra obtained with glucosamine. In a similar study, Pak et al. (Andjelković et al., 2006) also demonstrated that by increasing the degree of deacetylation of chitosan also significantly increased its radical reactivity. However, this study

also shows that for successful coupling an excess of phenolic compounds is needed, probably to compensate for the intra-molecular coupling of some of the oxidized phenolic compounds.

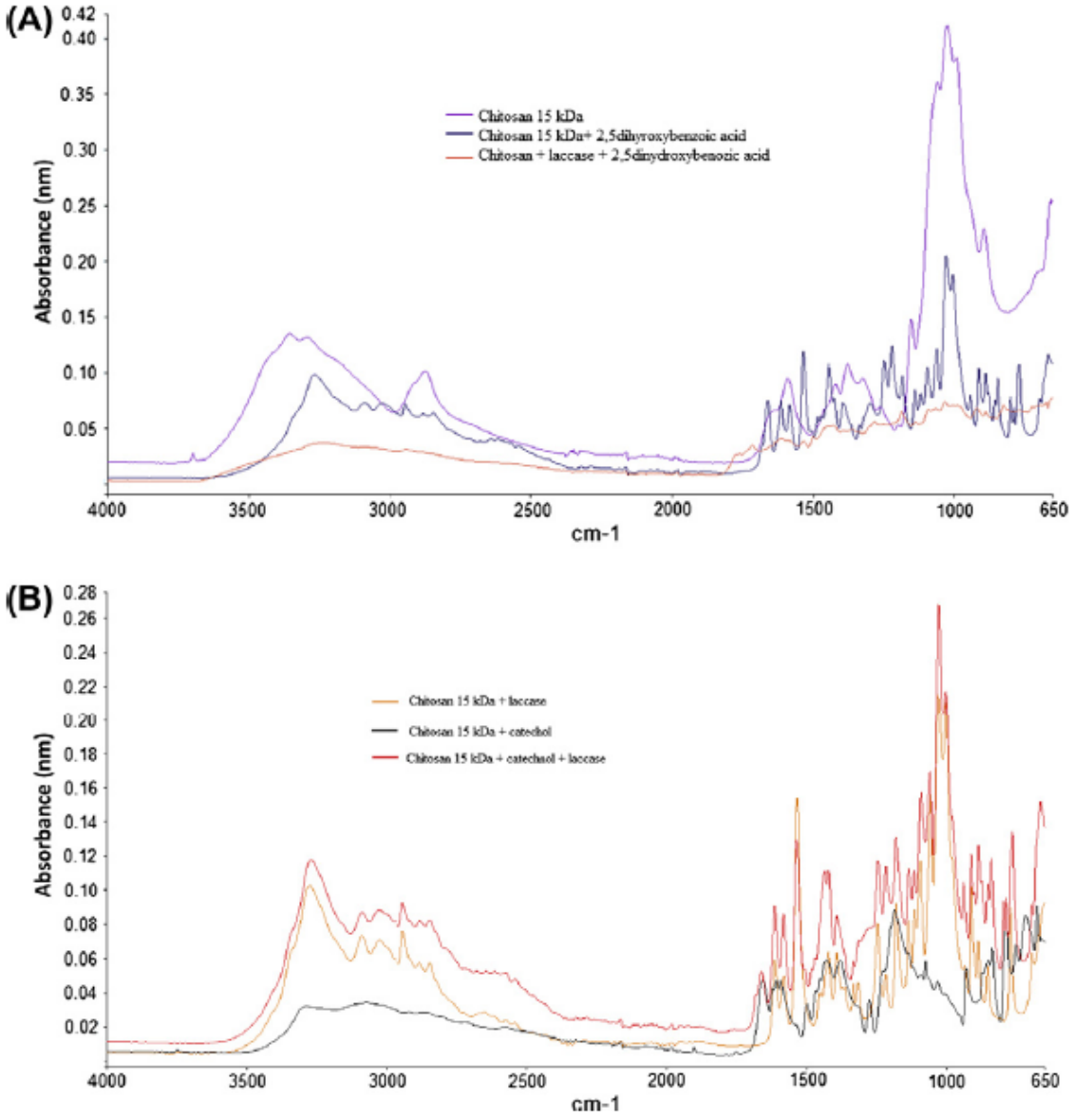


Fig. 27. *FTIR spectra of 2,5-dihydroxybenzoic acid and catechol functionalized chitosan*

7.3.1. RAMAN spectroscopy analysis

Raman spectroscopy is a method of electromagnetic spectroscopy, more precisely a supplementary method to infrared spectroscopy. Intensive areas in Raman spectra are weak in infrared spectra and inversely, because the vibrations, with which the vibration is changing the polarizability have areas in Raman spectra and vibrations which alter molecule dipole have areas in infrared spectra.

In this method laser interacts with the sample. Most of the radiation will pass through the sample and some part of the photons is absorbed - 10^{-4} photons are elastically dispersed (Rayleigh dispersion) and 10^{-8} photons is non-elastically dispersed and interact with the sample (Stokes' and anti-Stokes' lineages). This kind of non-elastic dispersion is used in Raman spectroscopy.

The samples were prepared as noted earlier (same like for FTIR analysis). Drying was done by lyophilizator. After lengthy optimization settings and changes in the sample preparation, the analysis failed. We believe, that due to the high auto fluorescence of the sample, the analytical information of the sample was too weak to be analyzed.

7.3.2. Phenolic molecules like chitosan quencher

The preparation of the samples for confocal microscopy was similar to previous procedure - modified chitosan was dropped onto a glass and dried in the air in 25°C.

Confocal fluorescent microscopy was used for auto-fluorescence analysis of sample. It was found that coupling of phenol molecules onto chitosan have quenching effect.
It was not easy to find right auto fluorescence range of spectra for these molecules. Because crystals cause various refraction and reflection of light. Then it is not easy to determine the accuracy of the fluorescence spectrum region. Nevertheless, it was found that highest auto-fluorescence activity of chitosan was measured in the range of blue light (425-500 nm). Weak

auto-fluorescent activity of chitosan was found already in the red and green range of the spectra (Fig. 28, Fig. 29, Fig. 30). By adding laccase and incubating for 24 hours, this auto fluorescence weakly decrease (Fig. 28, Fig. 29, Fig. 30).
2,5-dihydroxibenzoic acid built large crystals and the light emitted by these crystals is in the range of blue light 425-500 nm (Fig. 28). Fluorescence activity was also found in green and weakly in the red spectrum. This is probably the refraction of light at the crystal. After the incubation, 2,5-dihydroxibenzoic acid with laccase (24 hours), crystals were considerably more smaller (gentle). Fluorescent activity is in range of blue light (425-500 nm and weakly in the green section of spectrum).

Caffeic acid forms large regular and rectangular crystals which are fluorescent mostly in the green range of spectra (500-575 nm) and weakly in the red range of spectra. Caffeic acid with laccase built small slightly irregular crystals, which are fluorescent throughout the fluorescence spectrum, weakly in the green range of spectra (Fig. 30). Catechol's crystals have amorphous structure and fluorescent activity occurs in 425-500 nm (the blue light, see Fig. 29). The structure of catechol with laccase is amorphous, with fluorescent activity partially disappearing – quenching.

Coupling reaction – effect of quenching:

Chitosan with 2,5-dihydroxybenzoic acid crystals has gentle dendrite structure (Fig. 28), fluorescence is very weak and occurs in the full range of spectra (mostly in 425-500 nm). Chitosan with 2,5-dihydroxybenzoic acid cultivated for 24 hours with laccase is without regular crystalic structure, with decrease fluorescent activity (Fig. 28).
Chitosan with catechol built "dendrite" crystals (Fig. 29) with highest fluorescent activity in the wavelength range 425-500 nm (weakly in full range of spectra). The reaction of chitosan with catechol and laccase formed a biopolymer without „regular" crystalic structure and fluorescence was lost – proving of quenching effect of catechol.

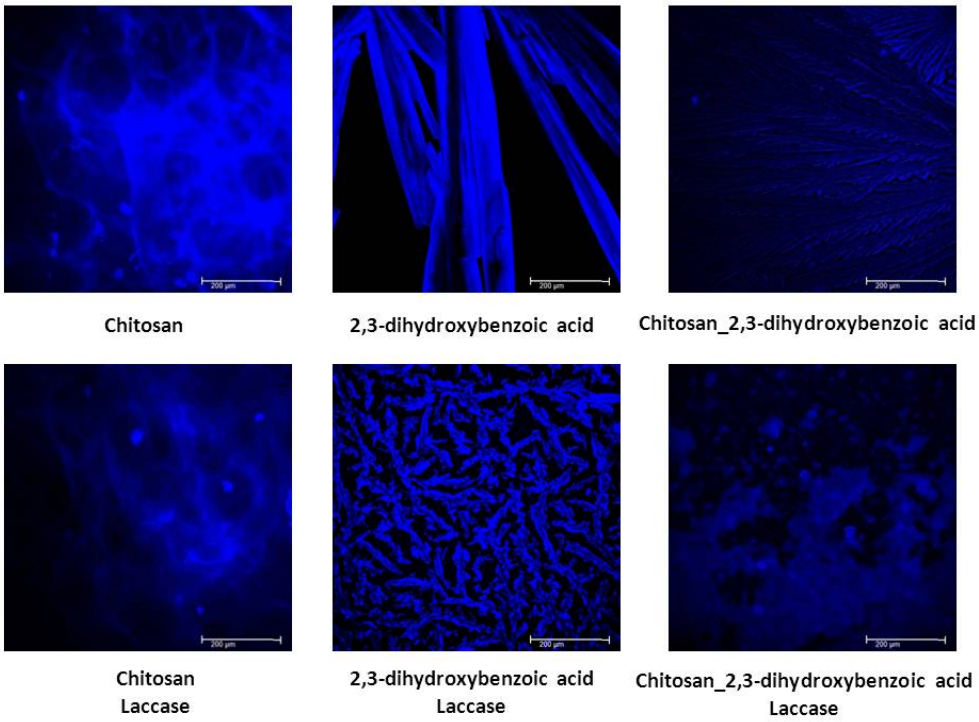


Fig. 28. *In vitro coupling reactions of 2,3-dihydroxybenzoic acid molecules onto chitosan*

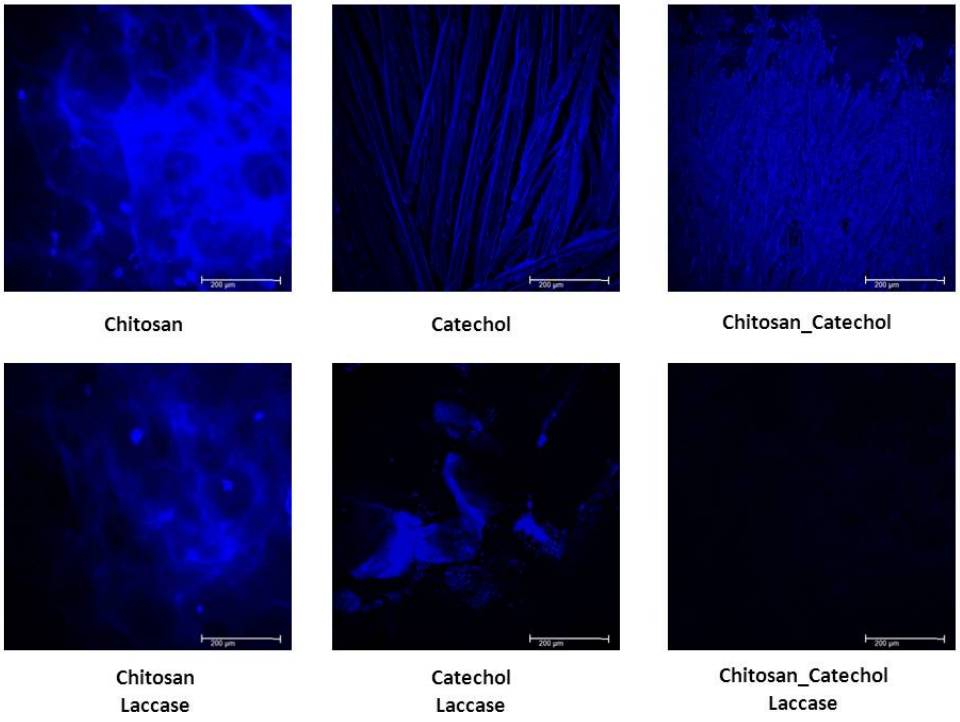


Fig. 29. *In vitro coupling reactions of catechol molecules onto chitosan*

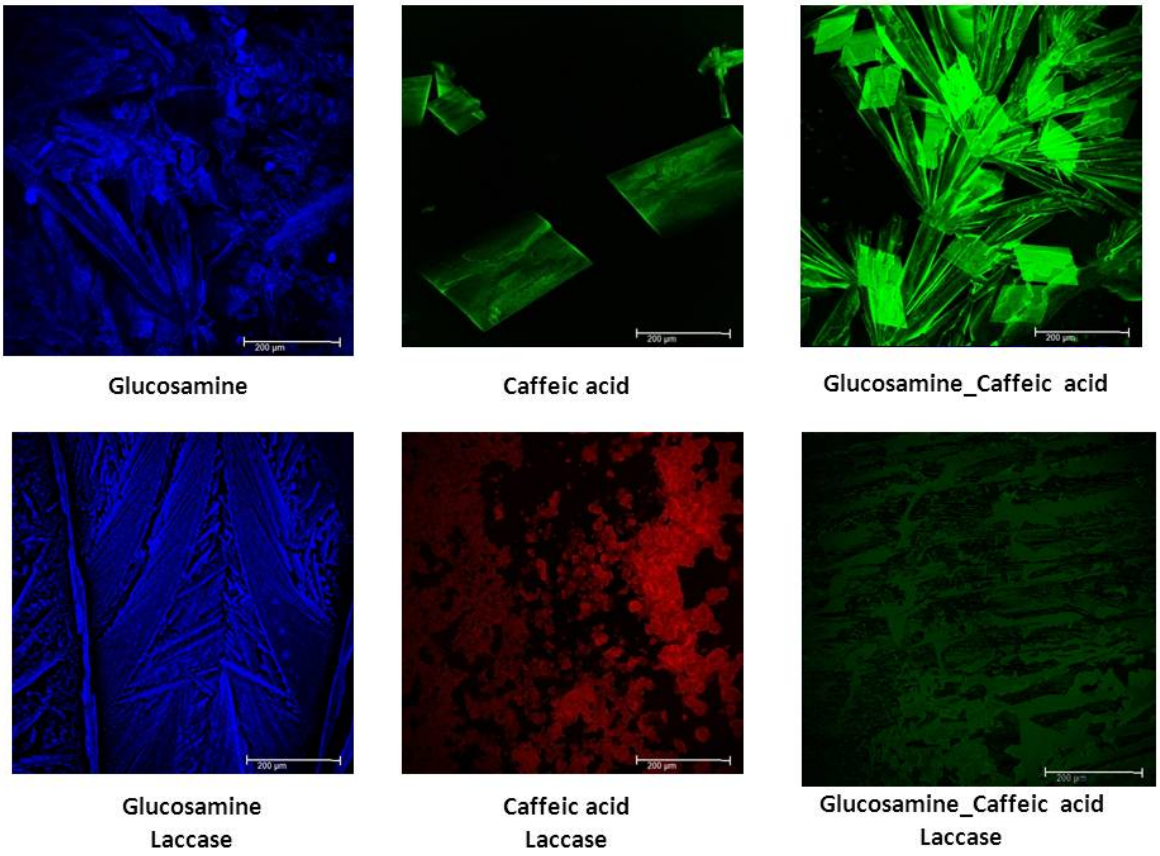


Fig. 30. *In vitro coupling reactions of caffeic acid molecules onto chitosan*

What is quenching?

Quenching is a process which reduces the intensity of fluorescence of the substance. Processes which result quenching are collision processes. Collision with another substance that will ensure non-fluorescent deexcitacion for example: O_2 (Fig. 31), I-, acrylics. Another example is the static quenching which leads to complex formation in the ground state, which have no fluorescent (but the components themselves have fluorescence). Another reason may be transfer of resonance energy transfer (FRET), charge transfer reactions, photochemical reactions which occur after excitation of the molecule, a chemical reaction. The picture Fig. 31 is described quenching of the fluorescent die by oxygen (Yinglang et. al., 2011).

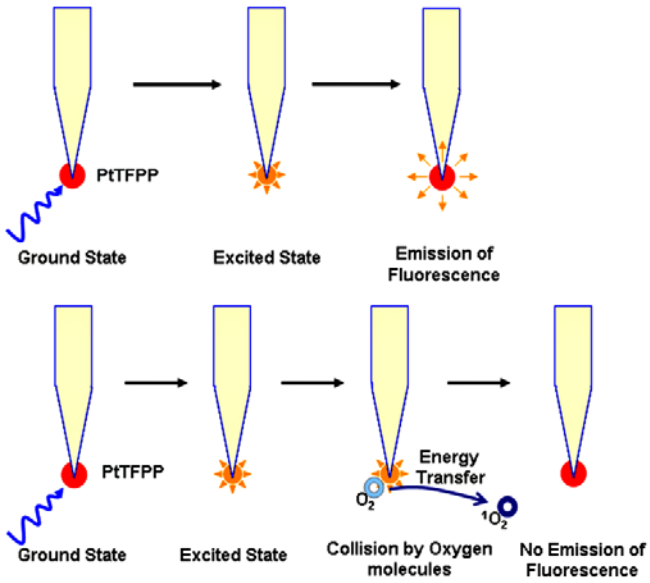


Fig. 31. *Principe of fluorescence quenching (fluorescent die by oxygen) (Yinglang et. al., 2011)*

7.3.3. Structural analysis

Electron microscopy was used for observations of the differences in the structure of the individual molecules and their mutual coupling. We found some differences between individual samples. Used samples were drops on the glass dried in the air with temperature 25°C. For microscopy were used native samples without stabilization with Pt or Au.

Structure of chitosan has spongy texture that does not change even after incubation with laccase for 24 hours (13,4 nkat ml^{-1} laccase in 50 mM succinate buffer (pH 4,5)).. Incubation of 5 ml of chitosan (1 g of chitosan 300 kDa in 200 ml 0,1% acetic acid) with 5 ml 100 mM of phenol molecule (see Fig. 32 is displayed reaction with 2,3-dihydroybenzoic acid) for 24 hours. Due to this reaction, the spongy texture of chitosan changed to the crystal form displayed in Fig. 32.
To make this structural change of chitosan, it's enough to use catechol with 50 mM concentration. One can as well use 10 mM caffeic acid, with 2,3-dihydroxibenzoic acid with concentration only 3 mM instead to provide for this structural change.

Due to incubation of phenol molecules with laccase (24h, 25°C), the crystal (and spongy) structure is lost and irregular structure is formed – see Fig. 32. The reaction of chitosan with phenols and laccase for 24 hours and at 25°C brings changes to the rope structure with many knots and irregular balls, which can be seen in Fig. 32 (concentration of laccase is for this microscope and this resolution so low, that it is not possible to see).

These results of electron microscopy show large changes in the structure depending on the degree of modification of chitosan. We can certainly say that coupling of phenol molecules onto chitosan with using laccase was successful.

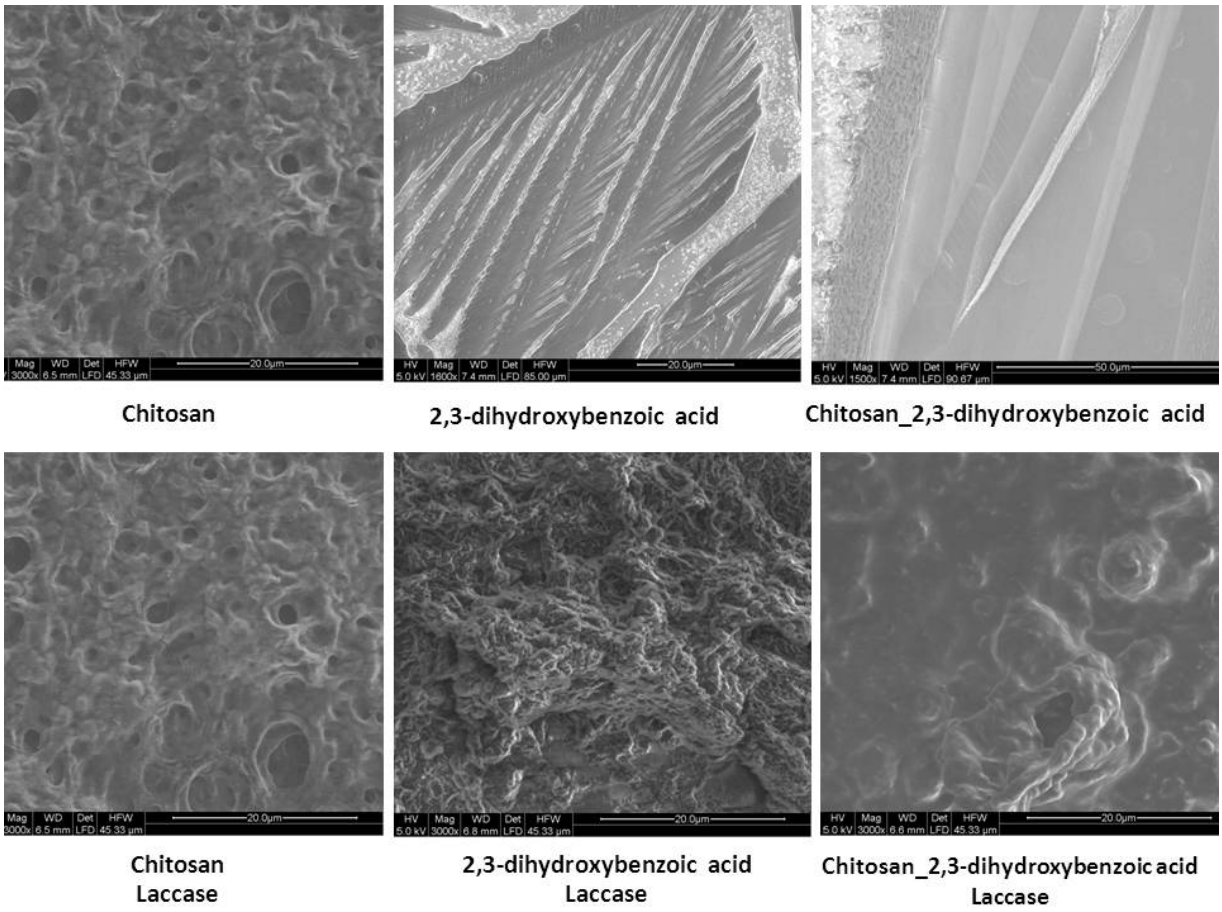


Fig. 32. *Morphological changes of chitosan upon in vitro coupling reactions of 2,3-dihydroxybenzoic acid molecules*

7.3.4. Iron-chelating ability of phenolic functionalized chitosan

The iron binding capacity of functionalized chitosan was estimated by measuring the inhibition of complex formation between ferrozine and Fe^{2+}. This reaction can be monitored based on a change in color of blue dye-chrome azurol sulphonate solution to red. The iron binding capacity of all chitosans alone was generally lower than 3% from a 5 mM solution. However, all the functionalized chitosan (15, 50 and 300 kDa) showed a remarkable increase in their respective iron sequestrating capacity to up to 70% of the iron from the solution as compared with chitosan alone (Fig. 33).

Previous iron chelation studies with phenolic compounds found varying iron binding capacity of phenolics bearing catechol and galloyl moiety and no iron binding capacity with vanillic acid, syringic acid, and ferulic acid (Andjelkovičet al., 2006). The functionalized 15 kDa chitosan carrying hydroxy and carboxyl groups showed highest iron binding capacity of 70% (Fig. 33). Although generally the iron binding capacity of chitosan functionalized with catechol was lower than that obtained with 2,5-dyhydroxybenonzoic (Fig. 33), the observed15-fold increase in the iron-chelating capacity as compared with chitosan only is still remarkable. The observed lower iron binding capacity of catechol as compared with hydroxyl-carboxylfunctionalized chitosans may also be attributed to previous observed steric obstruction of the 30, 40-catechol structure for flavonoids substituted with carbohydrate moieties (Burda et al., 2001; Van Acker, et al., 1996) and the cross-linking effects of catechol. Nevertheless, catechol containing polyphenols like catechin, an abundant catecholate-type polyphenol present in green tea, have been shown to effectively chelate iron (Elhabiri et al., 2007; Khokhar et al., 2003). Although different mechanisms implying interaction of iron with amine, carbonyl, and hydroxyl groups of chitosan shave been proposed (Rinaudo et al., 1989) which may also apply to this study, grafting of catechol and hydroxybenzoic acids functional groups onto chitosan dramatically increased iron chelation. The observed general increase in iron binding capacity for both catechol and 2,5-dihydroxybenzoic acid functionalized further demonstrates that functionalized chitosan can play a very important role in metal chelating processes. This study therefore demonstrates the ability synthesizing hydroxy-carboxy chitosan for enhanced iron chelation.

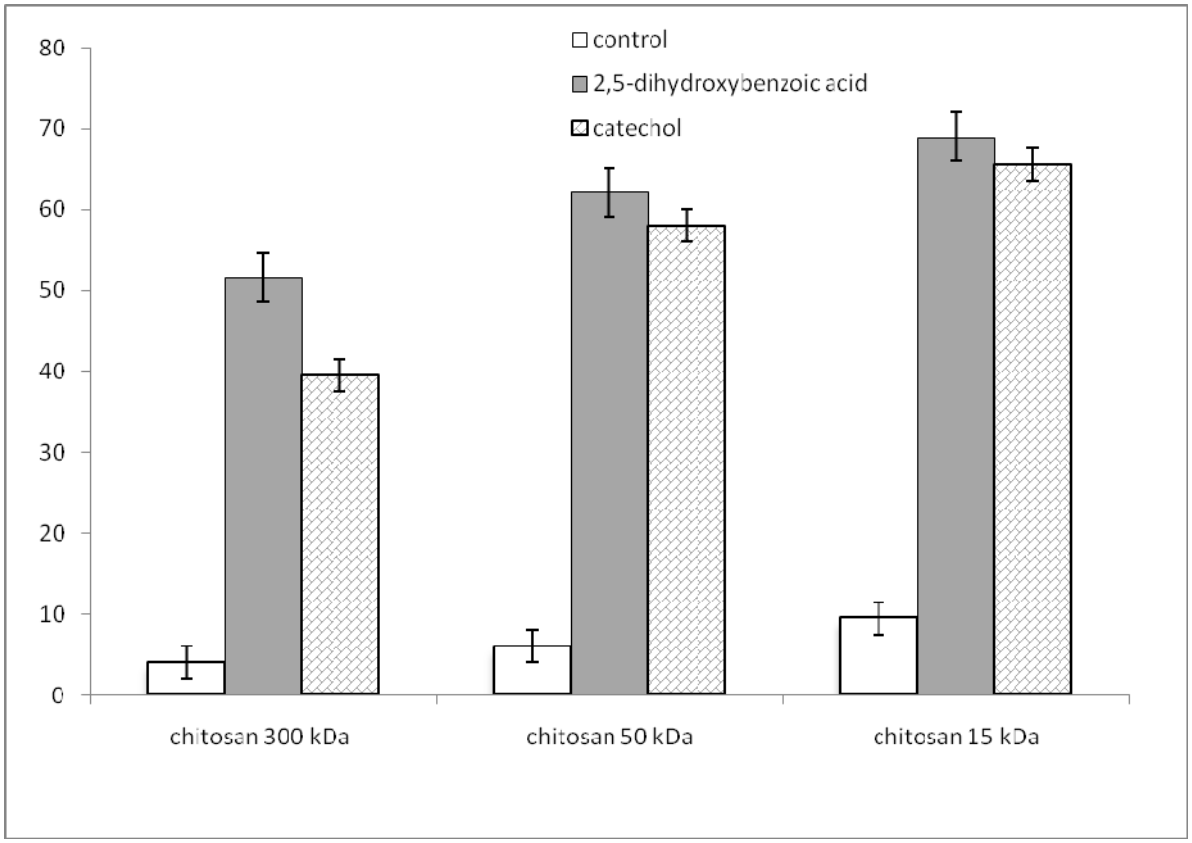


Fig. 33. *Chelation of Fe^{2+} by various chitosans and their phenolic functionalized derivatives. Data are an average of three independent reactions*

7.3.5. Synthesis of phenolic functionalized chitosan microspheres

In order to be able to roughly imagine what structure our micro particles will have, we created milli-particles, which are eye observable and therefore it is easier to have an idea about properties and behavior of these particles.

First, chitosan balls with one millimeter diameter were created as a model case. It was found out that the balls were made by solely dripping chitosan into alcalic environment. After drying in lyophylizator, non-crosslinked chitosan balls fall apart and after drying in the air they turn into a thin film. When drying in the air with room temperature, balls lose their shape completely.

Better procedure turned out to be dripping dissolved chitosan into the alcalic environment which contains catechol or dihydroxibenzoic acid. These balls keep their shape but do not achieve

solidity of balls based on chitosan which is crosslinked by catechol or by dihydroxibenzoic acid. Interestingly, catechol and hydroxy-carbox seem to act as crosslinkers producing characteristic spheres.

Mixing chitosan with catechol, dihydroxybenzois acid or caffeic acid, crosslink is made and thus the structure is reinforced. Dripping this way prepared chitosan into an alcalic environment (water with pH 10) creates spheres which after drying in the lyophilizator remain solid and drying these spheres at the room temperature they keep their shape - only partial collapse of structure will occur, comparable to partially deflated soccer ball.

Size difference of spheres is partially dependent upon molecular weight of chitosan and concentration of solution which we work with. In this case, chitosan 300 kDa and concentration of 1 g in 200 ml 0.1% acetic acid were used.

Comparison of the structure of spheres dried in lyophilizator and those dried at room temperature is shown in Fig. 34. Structure of lyophilizated spheres creates scaffolds due to which it is very spacious (it can be compared to the structure which would appear with creating nanospheres by spray dryer technique). Due to this spacious structure inside the nanospheres, their potential for transport or sorption of various molecules or substances is large. Surface of these spheres more over suggests further modification for various purposes.

The structure created by open drying (catechol, dihydroxibenzoic acid) of chitosan particles at room temperature is much more compact and appears to be fractal like (electron microscope magnification).

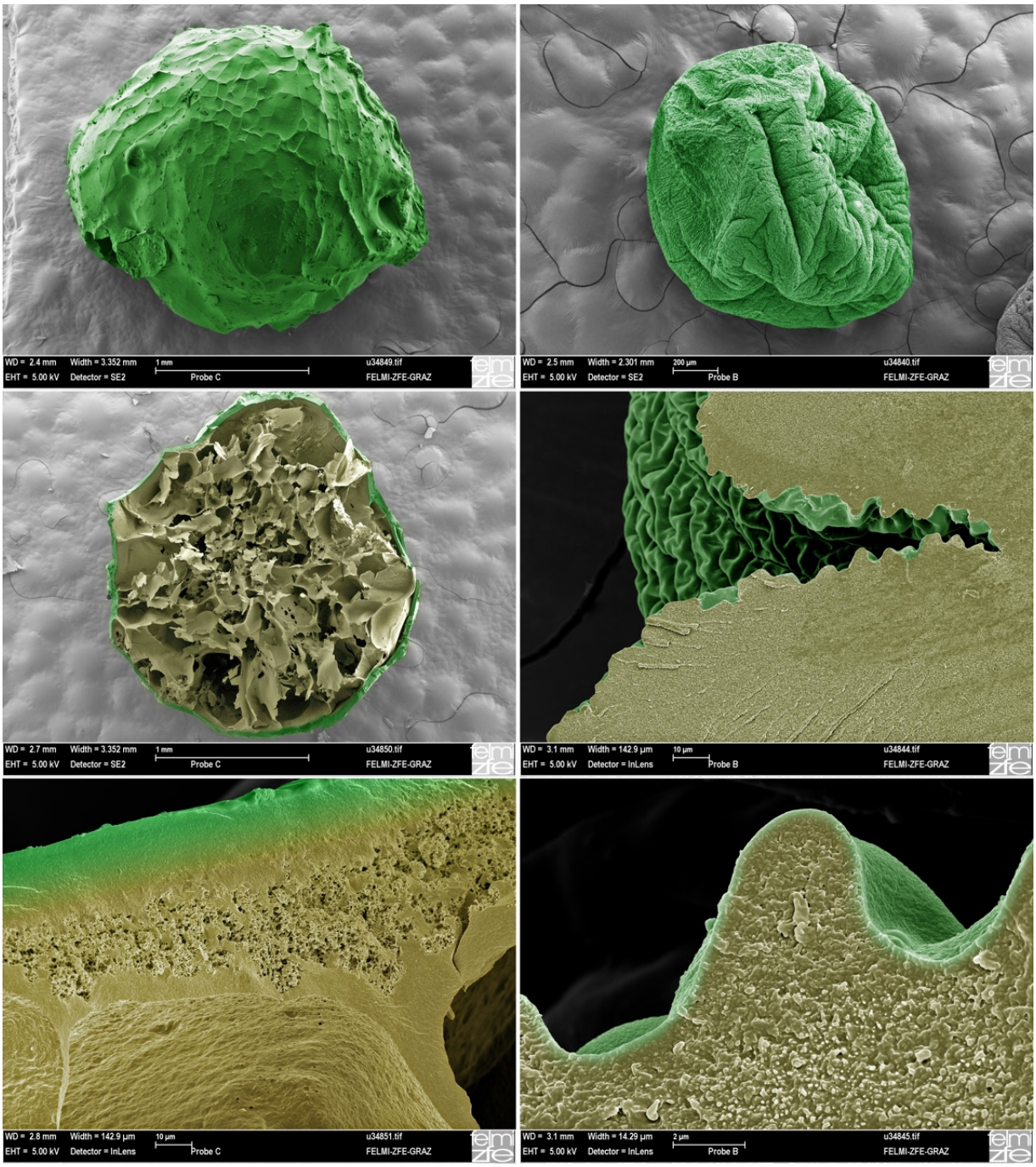


Fig. 34. *Structure of spheres dried in lyophilizator (A) and those dried at room temperature (B)*

Many different strategies for the synthesis of chitosan-based microspheres were developed as summarized by Agnihotriet et al. (Harish Prashanth, et al., 2007). Chitosan based mirco-spheres are currently being extensively investigated as medicament carrier for controlled release and focused studies of almost all the classes of bioactive molecules in medicine.

Dispersion and ultrasound were tested as methods for microspheres creation. Eventually simple drop method into alkaline solution while stirring proved to be the best and thus adopted for common use. Formation of microspheres is based on insolubility of chitosan in alkaline pH and is determined by the size of the chitosan drop.

Different characteristic structures were obtained using 15 kDa chitosan, catechol and hydroxycarboxic functionalized polymer as shown in the Fig. 35. The microspheres created had average size between 0,8 and 2 μm. Catechol and hydroxycarboxic also seem to participate as cross-liners producing characteristic spheres (Fig. 35). However, further studies are needed, e.g. we could improve the shape of micro-particles by using spray dryer.

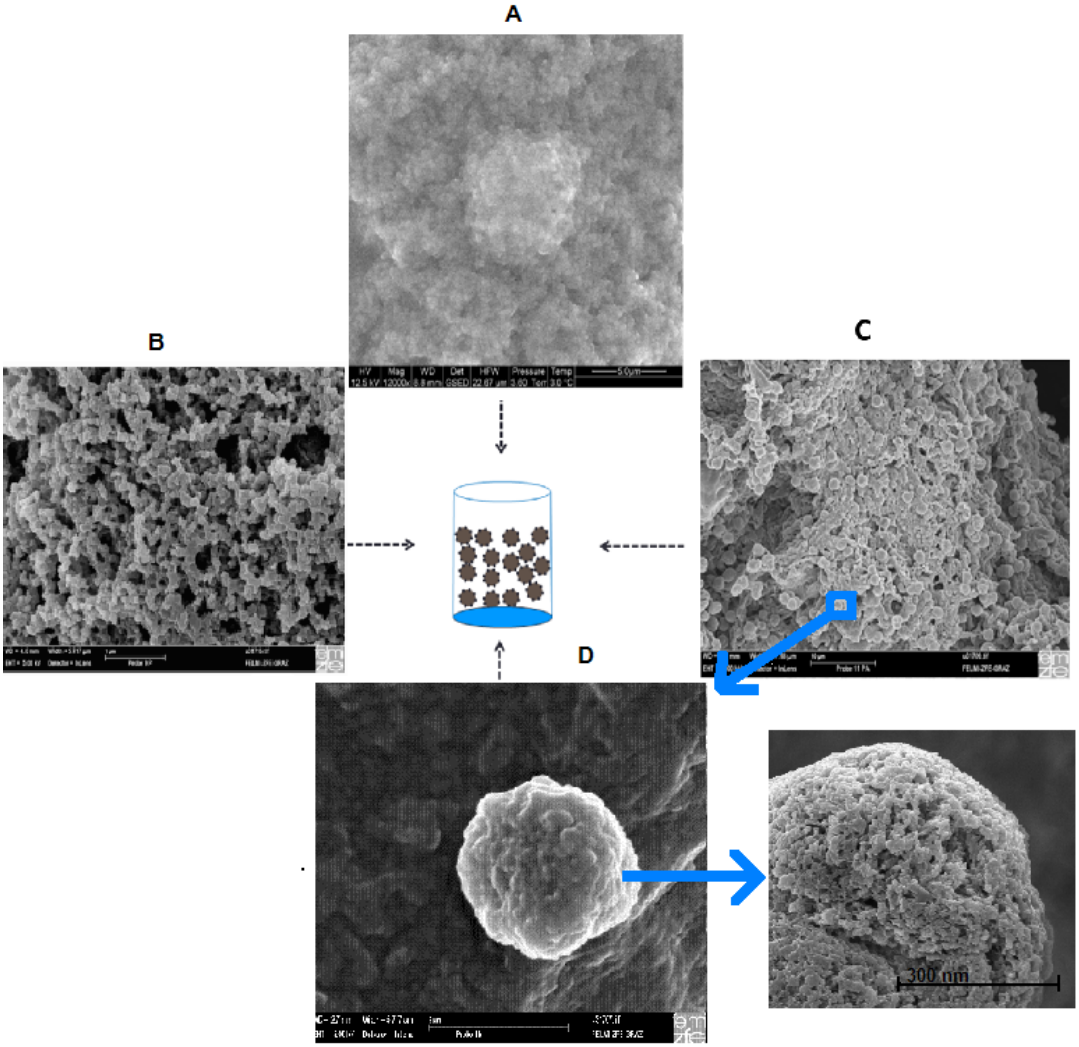


Fig. 35. *SEM of microspheres formed from 15 kDa chitosan functionalized with 2,5-dihydroxybenzoic acid (A), caffeic acid (B), and catechol (C) (micrometer bar: 2 lm for A, 10 lm for B, 1 lm for C)*

7.3.6. Iron-chelating ability of synthesized microspheres

The ability to chelate iron of catechol and 2,5-dihydroxybenzoic acid functionalized microspheres was investigated. Since the gastrointestinal pH fluctuates between 1,8 (stomach pH) and 7,4 the ability of the polymers to chelate iron was assessed in the respective pH range. Both catechol and 2,5-dihydroxybenzoic acid functionalized microspheres had a marginal loss at

pH 7,4 (<4.5%) of the 3,8 mM originally chelated iron at pH 2 (Fig. 36). This study therefore shows the great potential of using catechol and hydroxy-carboxic functionalized chitosan microspheres aimed at increasing both its iron-chelating ability and stabilizing the complex along the fluctuating gastrointestinal pH environment. This is because it is known that although ligands containing the catechol moieties possess a high affinity for iron, the binding of cations by catechol is pH sensitivity. Functionalization of chitosan with carboxylated molecules was shown to overcome its pH-sensitive (Kumar et al., 2004) which may be particularly true for these catechol and hydroxyl-carboxic functionalized chitosans.

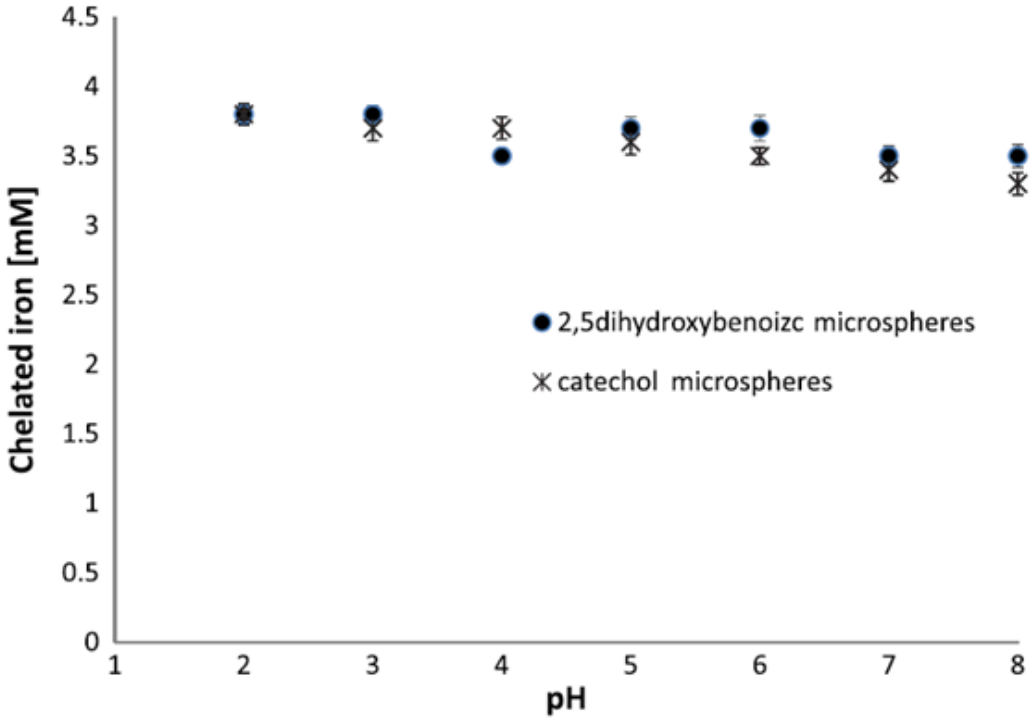


Fig. 36. *Chelation of Fe^{2+} (3,8 mM initial concentration) by 2,5-dihydroxybenzoic acid and catechol functionalized microspheres*

To show the difference between the iron binding capacity of catechol and dihydroxibenzoic acid, was measured the iron binding capacity by glucosamine-catechol and glucosamine-dihydroxibenzoic acid molecules (in concentrations 100 mM: 200 mM, incubated 24h with laccase). As seen on Fig. 37, efficiency of dihydroxibenzoic acid is nearly 50% higher than by catechol.

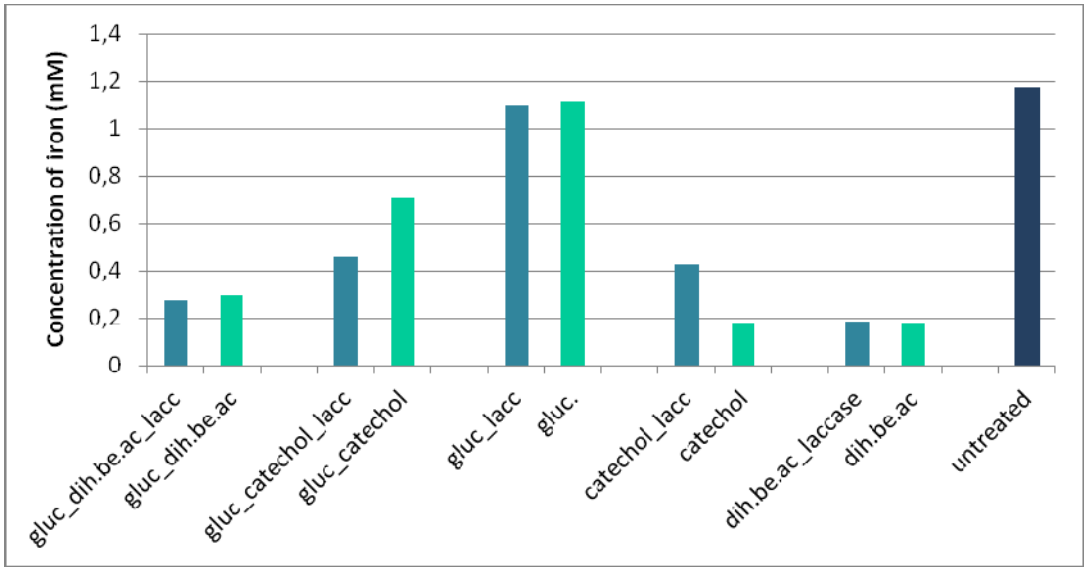


Fig. 37. *Fe^{2+} chelation by catechol and 2,5-dih.be.ac molecules grafted onto glucosamine*

Some possible reason for this result can by hide in the structure of individual molecules. In Fig. 38A, Fig. 38B it is possible to see different ways how to bind catechol and dihydroxibenzoic acid with chitosan. and various possibilities of how these molecules can bind iron. Resulting from this picture, the dihydroxybenzoic acid have possibility to build more than just one molecules of iron, however catechol have the possibility to build only one molecule of iron. Even there is strong possibility, that are used two molecules od catechol to bind one iron.

Using of functional chitosan microspheres showed that 0,1g of dihydroxybenzoic acid – chitosan microspheres can bind nearly $2{,}7\times10^{-4}$g of iron see Fig. 39.

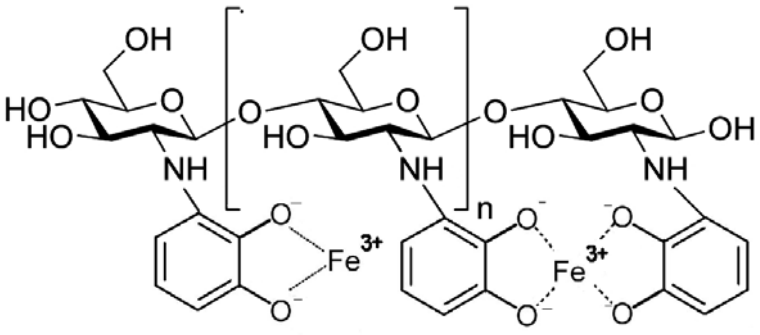


Fig. 38A. *Chitosan-catechol complex structure with iron binding possibility*

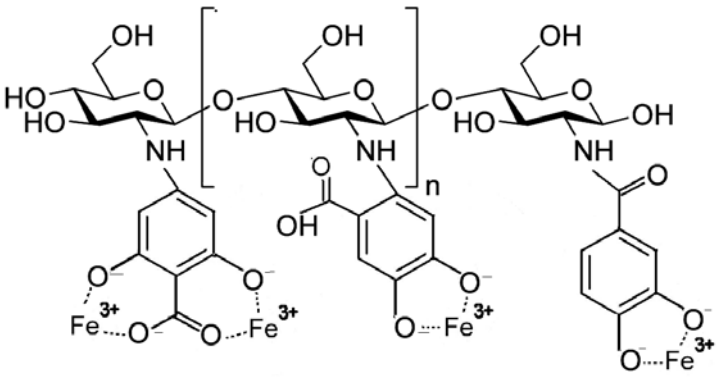


Fig.38B. *Some of the connection between X,Y-dihydroxybenzoic acid, chitosan and iron*

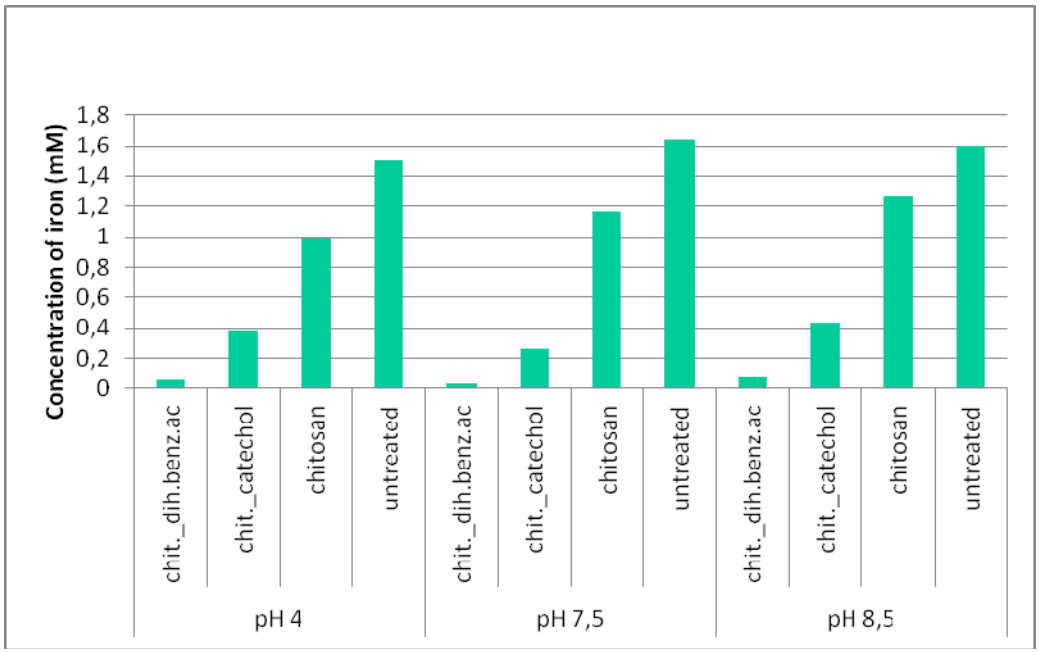


Fig 39. *Iron binding capacity of catechol and dihydroxibenzoic acid functionalized microspheres*

This study therefore shows the great potential of using catechol and hydroxy-carboxic functionalized chitosan microspheres aimed at increasing both its iron-chelating ability and stabilizing the complex along the fluctuating gastrointestinal pH environment. This is because it is known that although ligands containing the catechol moieties possess a high affinity for iron, the binding of cations by catechol is pH sensitivity. Functionalization of chitosan with carboxylated molecules was shown to overcome its pH-sensitive (Kumar et al., 2004) which may be particularly true for these catechol and hydroxyl-carboxic functionalized chitosans.

8. Conclusion

Within this work, novel strategies for enzymatic functionalization of chitosans for iron overload therapy were developed. In a first step, catechol and hydroxyl-carboxyl groups were successfully grafted onto glucosamine as model substrate for chitosan.
To verify that it was possible to couple these molecules, we first started with chitosan monomer (glucosamine). This way, we were able to carry out simpler and much more precise analysis. We have conducted in vitro coupling of catechol, 2,5-dihydroxybenzoic acid and caffeic acid onto glucosamine.

The first result from UV-spectrophotometry showed, that after the completion of the in vitro reaction by the laccase, which lasted 24 hours, new peaks appear in the UV spectra (280 nm). Based on this, further analysis was considered necessary. TLC (silica) analysis was used as a separation method due to its simplicity. After some difficulties with finding the optimal mobile phase, optimized results showed clear evidence of successful coupling, especially with 2,5-dihydroxybenzoic acid. Based on this result, we approached to HPLC-MS analysis in order to confirm this hypothesis. HLPC data were again most clear in the case of 2,5-dihydroxybenzoic acid. In all the coupling reactions though, we detected new products.

Subsequent MS analysis showed, that the newly emerged products were glucosamine-catechol, glucosamine-2,5-dihydroxybenzoic acid and glucosamine-caffeic acid coupling products (the exact point of connection of these molecules was not found; this would require NMR analysis which was not available at the time).

Based on these results we directly came to in vitro coupling reactions with chitosan (we used 15, 50 and 300 kDa chitosan). Concentration optimization (chitosan:phenols) and efficiency control of the coupling was monitored by the ninhydrin (NHN) assay through which the concentration of NH2 groups was observed. For the analysis of resultant polymer, FTIR and RAMAN methods were used (however, RAMAN method failed because of strong auto-fluorescence of the samples). This way we were pressed to use the fluorescence microscopy. It was proved that the

coupling reaction between chitosan and phenolic groups by the laccase has quenching effect on chitosan (strongest quenching was observed with chitosan-catechol-laccase reaction).

Besides fluorescence activity, also the structure of the samples was very interesting - varying depending on the reactants present. After examining this structure with electron microscope, my recommendation would be to research these further due to potential for semiconductor and nanorobotics technologies. The significant result for us was to show differences between independent reactants and final products.

The next step on the way to create functional chitosan microspheres for the treatment of iron overload was development of these microspheres. Interestingly, the use of these phenolic compounds required no additional cross-linker as they themselves appear to be participating as cross-linker, producing characteristic microspheres. The creation of nano particles was not possible without spray dryer technology, however, it was possible to create the micro-particles. These particles proved to be the effective iron chelators, where the most effective micro-particles functioned with 2,5-dihydroxybenzoic acid. This result can be employed for creation of chitosan-based chelating therapeutic polymers, which have a great potential to embody non-toxic compensation without any side effects to the therapeutics which are presently used to treat the diseases from iron overload.
This work opens new possibilities which can be further be explored for the development of a new generation of chitosan-based iron chelating therapeutic polymers.

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