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Published in:
Journal of Cleaner Production

DOI:
10.1016/j.jclepro.2021.129615

Published: 15/12/2021

Document Version
Publisher's PDF, also known as Version of record

Please cite the original version:
Lohtander, T., Durandin, N., Laaksonen, T., Arola, S., & Laaksonen, P. (2021). Stabilization of natural and synthetic indigo on nanocellulose network - Towards bioactive materials and facile dyeing processes. *Journal of Cleaner Production*, *328*, Article 129615. https://doi.org/10.1016/j.jclepro.2021.129615

Journal of Cleaner Production 328 (2021) 129615

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Stabilization of natural and synthetic indigo on nanocellulose network - Towards bioactive materials and facile dyeing processes

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ARTICLE INFO

Handling editor: Cecilia Maria Villas Bôas de Almeida

Keywords:
Fluorescence spectroscopy
Nanocellulose
Indigo
Leucoindigo
Isatis tinctoria

ABSTRACT

Synthetic dyes are vastly used for colouring numerous materials, although the adverse effects on environment are well recognized. In addition to developing the existing dyeing technologies more efficient and cleaner, the valorisation of natural dyes can enhance the sustainable development of dyeing industry. Natural indigo, derived from *Isatis tinctoria*, is a bio-based alternative for indigo produced via chemical synthesis routes. Owing to the insoluble character of indigo pigment, the dye requires conversion into soluble leucoindigo form prior to dyeing, which is often accomplished by using harsh sodium dithionite vat technique. During the processing from plant to dye attached on a fabric, indigo is transferred from the soluble leucoindigo form to the oxidized insoluble indigo and once more back to leucoindigo. Additionally, the oxidation is difficult to control and with traditional vat technique maintaining the leucoindigo through the dyeing often requires adding more reducing agent chemicals. Maintaining the soluble form throughout the process would enable lower number of processing steps and reduce the use of harmful chemical agents. In the present study, the stabilization of leucoindigo on nanocellulose matrix carrier was investigated with spectroscopic and photophysical methods. According to the results, leucoindigo was successfully stabilized on nanocellulose suspension, most likely due to the limited rate of oxygen diffusion into the viscous medium. Visual observations revealed that the leuco-form was retained even longer with natural indigo than synthetic indigo. This enhanced stability was attributed to the presence of radical scavenging species in natural indigo since the synthetic indigo did not show notable antioxidant properties. Given the promising results the paste formulation was demonstrated to be applicable for creating patterns on cotton using a screen-printing technique. Since the leucoindigo was stabilized on nanocellulose carrier, the need for re-reduction prior to dyeing was avoided and the amount of harmful reducing chemicals was reduced. These findings also show that the characteristics of natural dyes that are often considered disadvantageous compared to synthetic dyestuff, i.e. presence of co-products in the mixture, can however, create more value to the dyed material through new functionalities.

1. Introduction

The dyeing industry today relies heavily on synthetic-driven processes owing to economical, availability and durability requirements (Bechtold et al., 2003). Two thirds of the global share of synthetic dyes and pigments are used in textile industry and the annual consumption keeps increasing along the increasing consumption of clothing (Asad et al., 2007). Although the durability of synthetic colours guarantees vibrant and long-lasting colours, the good chemical resistance sets challenges to the process effluent management. Depending on the dye and process type, from 2% up to 50% of dye is not fixed on textile and is lost to effluents (O'Neill et al., 1999). The dyeing process as such is a critical point of fashion industry causing significant negative environmental impact, and approximately 20% of global industrial water pollution originates from textile dyeing and other wet processes (Kant, 2012). Due to resistant nature of the synthetic dyes, it is not

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https://doi.org/10.1016/j.jclepro.2021.129615
Received 30 June 2021; Received in revised form 28 October 2021; Accepted 5 November 2021

economically feasible to reach satisfactory levels of wastewater purity to the extent where the wastewaters entering the environment would not threaten the aquatic life (Ali, 2010; Jegatheesan et al., 2016). In addition, owing to economical reasons, the dyeing industry is localized to emerging-market countries with cheaper labor and poor environmental legislations, which causes the water pollution to accumulate in these regions (Liu et al., 2017). There have been only minor attempts to develop the existing dyeing processes towards more efficient and environmentally friendly processes. Some advancements, such as supercritical carbon dioxide dyeing (De Giorgi et al., 2000) and digital printing (Tyler, 2005), have been made to reduce the water footprint and improve dyestuff utilization. From industry point of view, changing the well-established processes is not of interest, although the increasing environmental awareness and thus stricter environmental regulations might change the course. Overcoming the environmental burden caused by textile wet processing requires revisiting and rethinking of existing processes.

Natural dyes, along with development of more efficient processes, and wastewater management for synthetic dyes, could be one solution to reduce the negative environmental impact of textile industry. The utilization of natural dyes has been diminished since the discovery of synthetic dyes. Even though the interest toward natural dyes has increased in past years, development of large scale production is still lacking (Bechtold et al., 2003). The natural dyes and pigments have often poorer colour fastness compared to synthetic dyes and they are not applicable directly to current processes operated with synthetic dyestuff (Bechtold et al., 2002). In turn, however, they are often more biodegradable and less toxic than synthetic dyes (Mirjalili et al., 2011). Additionally, the cultivation of plants has a positive effect to carbon footprint through the fixation of carbon dioxide (CO_2) (Benson and Calvin, 1950). Harnessing the properties that are usually thought as drawback in natural dyes could also open up new fields of applications for natural dye usage. Even though natural dyes cannot alone answer to the annual dyestuff demand, they could be taken into consideration especially in applications, where biodegradability is desired, such as in biodegradable textiles, plastics and cellulose-based packaging solutions.

Indigo, perhaps the most famous natural dye, was first derived from plants such as *Indigofera tinctoria* (Asia) and *Isatis tinctoria* (Europe), before discovering the chemical synthesis routes (Fantacci et al., 2010; Maugard et al., 2001). Characteristic feature of indigo is its remarkable photostability, which is not typical for natural dyes (Yamazaki et al., 2011). As other natural dyes, indigo dye obtained from plants is a mixture of compounds (Maugard et al., 2001). Even though the purity of plant derived indigo can be lower than that of the synthetically produced pure indigo, the side-products of natural indigo dye can possess functionalities not present in the synthetic indigo, such as antimicrobial and antioxidant properties (Beutner et al., 2001; Garcia-Macias and John, 2004; Kim et al., 2012). Processing of indigo from plants to blue pigment consists of multiple steps. Briefly, indoxyl molecules released from the leaves are first condensed into water-soluble leucoindigo, which is finally oxidized into water insoluble blue indigo form (Blackburn et al., 2009). As a vat dye, prior to dyeing indigo needs to be reduced back to leuco-form, which is typically done in industrial processes with sodium dithionite in the presence of alkali. The classical reducing technique, however, creates wastewater problems due to formation of sulfur-based by-products from sodium dithionite (Kulandainathan et al., 2007). Different approaches, such as traditional fermentation (Aino et al., 2010), glucose as reducing agent (Vuorema et al., 2008), and electrochemical reduction (Roessler et al., 2002), have been investigated to replace sodium dithionite in the processing, but none of them have been adapted to large-scale dyeing. The aim of this research is to investigate whether leucoindigo could be stabilized on a water dispersible matrix material and to be controllably oxidized to indigo onto the matrix material allowing the blue indigo to stay in the aqueous phase with the matrix material for direct application as printable dye solution/paste. This process would enhance the sustainability of indigo dyeing by reducing the number of chemical processing steps and use of harmful reducing agents. In the early 1900s there were multiple attempts to produce pre-reduced stable leucoindigo via various chemical syntheses but these methods faced problems with low leucoindigo yields and unsatisfactory dyeing results owing to highly coloured, unwanted side-products and impure products (Blackburn et al., 2009; Chaumat, 1908; Seidel and Wimmer, 1905; Wimmer, 1906).

Cellulose is an abundant, renewable, biodegradable, and cheap polysaccharide present in plant-based biomass. Nanocelluloses, having at least one dimension in the nanoscale, includes bacterial cellulose (BNC), cellulose nanocrystals (CNC), and cellulose nanofibrils (CNF) (Klemm et al., 2018). Through different top-down methods, the macroscopic cellulose pulp obtained from various feedstocks, including biomass from agricultural and forest residues, can be downsized to micro- and nanosized material, which lead to different properties compared to macroscopic material (Abe et al., 2007; Kamel et al., 2020; Moon et al., 2011; Yu et al., 2021). CNF has unique properties such as high aspect ratio, biocompatibility and outstanding mechanical performance, and it is a widely applied matrix for instance, in biomedical applications and polymer reinforcing (Jorfi and Foster, 2015; Moon et al., 2011). Herein, we used nanocellulose as carrier matrix for leucoindigo as it is assumed that the large surface area due to nanosized fibrils and large amount of hydroxyl groups could enable efficient adsorption of leucoindigo on cellulose nanofibrils (CNF). The adsorption of leucoindigo on cellulose is governed by physical interactions including hydrogen bonds, van der Waals and dipolar forces (Blackburn et al., 2009). In the present work, we show that leucoindigo can be stabilized by nanocellulose suspensions for time periods allowing storage and application and that indigo is oxidized on the nanocellulose matrix leading to stable indigo blue water suspensions. Furthermore, we show that the composite of nanocellulose and natural indigo has antioxidant properties, and that the purity of natural indigo from *Isatis tinctoria* in terms of indigo concentration is comparable to pure synthetic indigo. We also demonstrate a simplified green recipe for a paste of indigo and nanocellulose applicable for screen-printing patterns on cotton.

2. Experimental section

2.1. Materials

Synthetic indigo (95%), 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), tannic acid and sodium dithionite were obtained from Sigma-Aldrich. All solvents used were spectroscopic grade and water was purified with Milli-Q filtering unit. Nanocellulose (CNF) suspension of 1.6 wt-% solid content was mechanically disintegrated from never-dried bleach birch kraft pulp according to method reported earlier (Kangas et al., 2014). Briefly, the pulp was first washed to sodium form and then mechanically disintegrated first using a Masuco grinder (Supermasscolloider MKZA10-15J, Masuko Sangyo Co., Japan) and then a microfluidizer (M-110P, Microfluidics, USA) by six passes. For stability measurements and fluorescence studies, the nanocellulose suspension was diluted with MQ-grade water and stirred for at least 1 h. Aqueous solution of natural indigo produced from *Isatis tinctoria* (Dyer's Woad) was obtained from Natural Indigo Finland Oy. A small batch of aqueous solution was dried at 70°C overnight to be used in chromatographic analysis. In all other measurements, the natural indigo specimens were prepared from freeze-dried powder.

2.2. Preparation of leucoindigo nanocellulose suspensions

The stability of aqueous leucoindigo in nanocellulose suspension over time was investigated by varying the nanocellulose concentration from 0.1wt-% to 1.0wt-%. The reduction was performed using following sodium dithionite vat method: the aqueous indigo solution (2 mg/mL) was first heated to 50 °C, then sodium carbonate was added until reaching pH 10 and finally reduced for 30 min using sodium dithionite (2 mg/mL). Reduced leucoindigo solution was added to nanocellulose suspension (volumetric ratio 1:2.5) and mixed with a magnetic stirrer in open vials.

2.3. *Photophysical characterization*

The spectroscopic and photophysical properties of indigo were investigated in terms of fluorescence and absorption. The excitation and emission spectral properties of oxidized indigo and reduced leucoindigo were measured in aqueous nanocellulose suspensions. First, the absorption spectra of pure indigo (DMF, 0.05 mg/mL) and leucoindigo (aq, 0.05 mg/mL) were recorded with a UV–Vis spectrophotometer (Shimadzu UV-2550, Japan) to obtain excitation wavelengths for both samples. The fluorescence spectra of aqueous nanocellulose leucoindigo suspensions were then recorded by using fluorescence spectrometer (Perkin Elmer LS45, UK) immediately after mixing leucoindigo (2 mg/mL) to 1.0 -wt% nanocellulose and next day after oxidation to indigo form. Both fluorescence and absorption spectra were normalized relative to maxima.

For time-resolved fluorescence spectroscopy, leucoindigo was prepared according to method described elsewhere (Rondão et al., 2012) using synthetic indigo for simplicity. Briefly, indigo was dissolved in dioxane (0.03 mg/mL), reduced by adding 2–3 drops of sodium dithionite/NaOH mixture and bubbled with N_2 through rubber sealant septum to remove oxygen. The indigo in its neutral form was measured in dimethylformamide (DMF, 0.03 mg/mL). With nanocellulose suspension (1.0 wt-%), aqueous leucoindigo (2 mg/mL) was prepared using vat dye reduction method described earlier here. Fluorescence intensity decay curves of leucoindigo, indigo and nanocellulose stabilized leucoindigo were measured by using time-correlated single photon counting (TCSPC) system (PicoQuant, GmBH) equipped with PicoHarp 300 controller and a PDL 800-B driver. LDH-P-C-405 laser head was utilized to excite the sample at 405 nm with the time resolution of ~100 ps. Microchannel plate photomultiplier tube (Hamamatsu R2809U) was used for the detection of the signal. Cutoff filter of transmission >450 nm was applied to reduce an impact of the excitation light scattering. Fluorescence decays were monitored at 495 nm for leuco-form and 625 nm for keto form, respectively. Instrumental response function (IRF) was measured separately at monitoring wavelength 405 nm and used for deconvolution analysis of the fluorescence decays followed by their fitting by sum of exponents (eq. (1))

$$I(t,\lambda) = \sum_i \alpha_i e^{-t/\tau_{fi}} \tag{1}$$

where τ_{fi} is the fluorescence lifetime and α_i is the amplitude (pre-exponential factor).

2.4. *Antioxidant activity*

The antioxidant activity of indigo was evaluated using ABTS assay reported elsewhere (Re et al., 1999) by monitoring the change in absorption intensity at 734 nm using UV–Vis spectrophotometer (Shimadzu UV-2550, Japan). Briefly, ABTS$^{\bullet+}$ radical cation solution was diluted until reaching absorbance of 0.7. The radical scavenging activity was investigated by adding powder samples of pure synthetic indigo and natural indigo (0.5 mg/mL), or circular films (d = 6 mm) prepared from aqueous nanocellulose indigo suspension (indigo 10% of CNF dry weight) into 2 mL of diluted ABTS solution. The samples were mixed for 30 min before recording absorption spectra. Tannic acid (TA) was used as calibration standard and the calibration curve was prepared from five different concentrations (0.2–2.5 μg/mL) (Fig. S1). The ABTS radical scavenging activity was determined as the decrease in absorbance at the wavelength 734 nm of the samples versus ABTS radical solution. The results are reported as mean radical scavenging activity percentage (%) and as tannic acid (TA) equivalents (mg/mL), which were calculated with tannic acid calibration curve.

2.5. *Composition of indigo*

Identification of compounds was performed using information from chromatographic separation, absorption spectral data and mass spectra, which were compared to information reported in literature. The composition of pure synthetic indigo, freeze-dried natural indigo and natural indigo dried at elevated temperature were analysed using an Agilent 1260 High performance liquid chromatography (HPLC) coupled with a Diode array detector (DAD) and a quadrupole-time-of-flight mass spectrometer (QTOF MS, 6350, Agilent Technologies, USA). Acquired data was handled using MassHunter software version 7.0 (Agilent Technologies, USA). Powder samples were dissolved in 1:1 DMSO: MeOH (0.5 mg/mL), sonicated for 15 min at 21 °C and filtered with 0.22 μm syringe filter. The chromatographic separation was done using Phenomenex® Kinetex Biphenyl column (100 mm × 2.1 mm, 2.6 μm, 100 Å) with flow rate of 0.250 mL/min and 5 μL sample injection volume. The following gradient was used (A water: B methanol): 0–2 min 85%:15%, 2–4 min 75%:25%, 4–14 min 30%:70%, 14–19 min 85%:15%. The conditions in ESI-QTOF-MS were as follows: positive ionization, gas temperature 350 °C, gas flow 11 L/min, nebulizer 40 psi, capillary voltage 3500 V, fragmentor 150 V, skimmer voltage 65 V and mass range *m/z* 100–1100. The comparison of indigo and indirubin amount was done by calculating the integrated surface area of corresponding peaks in chromatogram at 285 nm.

2.6. *Surface morphology and elemental composition*

The structure of pure synthetic indigo and films from pure nanocellulose and nanocellulose-natural indigo were imaged in planar direction using a field emission scanning electron microscopy (FE-SEM, Zeiss Merlin, Germany) at 5 kV accelerating voltage in secondary electron mode. The sample material was fixed on carbon tape and sputtered with a 2 nm thick Au/Pd coating. The composition and elemental mapping of specimens were carried out using X-ray energy dispersive spectrometer (EDS) equipped with Ultra Dry Silicon Drift Detector (Thermo Scientific, USA).

3. Results and discussion

Pre-reduced leucoindigo from synthetic indigo was mixed with nanocellulose suspension for characterization of photophysical properties. The time-resolved fluorescence spectroscopy was used for investigating whether the indigo in the nanocellulose matrix was in reduced leuco or in oxidized keto form. The decay rates, or fluorescence lifetimes, describe the time a certain molecule remains in the excited state and it depends on the structural characteristics of molecules and thus each molecule has its characteristic decay rate. Fluorescence lifetime decay graphs in Fig. 1 show that the leucoindigo remains in leuco-form in the presence of nanocellulose. The leucoindigo embedded into nanocellulose suspension had a decay rate (1.99 ns) comparable to pure leucoindigo (1.95 ns). The small deviation of decay rate is likely due to the different environments of the molecules since the pure leucoindigo presented in Fig. 1b) is in dioxane solvent while in Fig. 1c) the leucoindigo is in nanocellulose suspension. The observed fluorescence lifetime of oxidized indigo was similar to the values reported in the literature, however, the decay of leucoindigo was slightly different from values seen in earlier study despite of similar solvent environment (Rondão et al., 2010; Seixas De Melo et al., 2004). All analysed specimens showed two decay components, although pure indigo was used in the determinations. The purity of used indigo was 95%, which was in good agreement with the contribution of major decay component. The origin of second decay component was unclear. The good agreement of

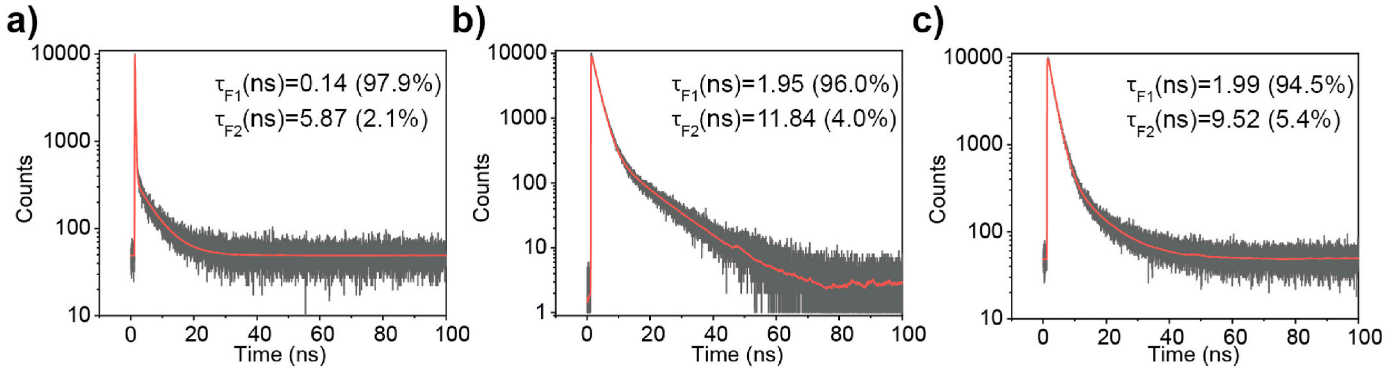


Fig. 1. The fluorescence lifetime decay graphs of a) indigo in dimethylformamide (DMF) solvent, b) leucoindigo in dioxane solvent, and c) leucoindigo stabilized in nanocellulose suspension. Synthetic indigo with a purity of 95% was used in the experiment. τ_{F1} and τ_{F2} are the decay rates of the main component, i.e. indigo, and secondary component, respectively.

the indigo purity with the contribution of major decay component, suggest that the second component could be attributed to a by-product of indigo synthesis. Nowadays, the chemical synthesis of indigo is done via few different synthesis routes depending on the manufacturer. All processes, however, use either N-phenylglycine or N-phenylglycine nitrile as a starting material (Steingruber, 2004). Indirubin, a red isomer of indigo, is produced as by-product both in processing of natural indigo and chemical synthesis from fossil-based raw materials (Maugard et al., 2001; Sánchez-Viesca et al., 2016). The fluorescence lifetime of second decay component seen here does not match to the lifetime reported for indirubin (Seixas De Melo et al., 2004), suggesting that the component originates from some other compound. Other by-product traces in synthetic indigo include aniline, N-methylaniline and anthranilic acid (Steingruber, 2004) and based on lifetime values reported in earlier studies, the second decay component could arise from these by-products. For both aniline derivatives and anthranilic acid, the used solvent system has been shown to influence the fluorescence lifetime (Abou-Zied et al., 2014; Oshima et al., 2006), which was observed also here as lifetime of the second component varied from 5.87 up to 11.84 ns in different environments. Another indigo derivative showing similar fluorescent lifetimes is cibalackrot, which, however, is unlikely present here as its formation would require condensation reaction of indigo and phenylacetyl chloride (Seixas De Melo et al., 2006). Although, the origin of the second component was unclear, the results of time-resolved fluorescence spectroscopy verified that the leucoindigo remained in leuco form in the nanocellulose suspension.

Indigo and leucoindigo has considerably different photophysical properties as can be observed from their fluorescence lifetimes and absorption characteristics (Fig. S2). The short fluorescence lifetime of indigo (0.14 ns) indicates that the excited state of fluorescent molecule dissipates quickly, whereas for leuco-form the decay is considerably longer (1.99 ns). The fluorescence lifetimes are related to the structural differences between oxidized indigo form and reduced leucoindigo form. In indigo form, the intra- and intermolecular hydrogen bonds are responsible for the high photostability and fast deactivation of excited state through proton transfer (Seixas De Melo et al., 2006). Owing to the loss of two hydrogen atoms upon reduction, the stabilizing hydrogen bonds are no longer present in leucoindigo and photo-isomerization from *trans* to *cis* configuration can occur (Moreno et al., 2013; Rondão et al., 2012; Seixas De Melo et al., 2004).

Fig. 2 presents the fluorescence spectral differences of leucoindigo and oxidized indigo forms in the presence of nanocellulose suspension along a stability experiment and printing demonstration. The excitation and emission maxima of leucoindigo in nanocellulose were at around 340 and 430 nm, whereas the corresponding maxima of oxidized indigo in nanocellulose were at the wavelengths 620 and 640 nm (Fig. 2a and b). The difference in the wavelength of fluorescence emission and excitation maxima suggests that in the beginning of the stability

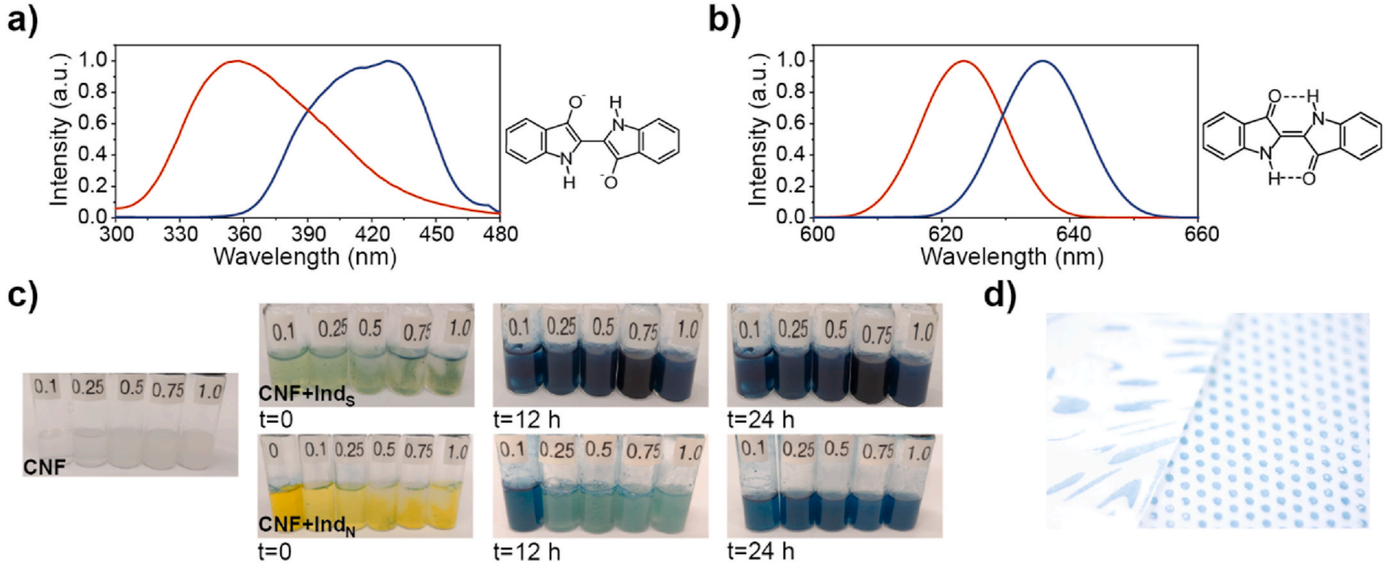


Fig. 2. The excitation (red) and emission (blue) spectra of nanocellulose suspension with a) leucoindigo after mixing and b) oxidized indigo on the next day. Synthetic indigo was used in fluorescence spectra measurements. c) The evolution of indigo oxidation in different nanocellulose concentrations (0.1–1.0wt-%) with synthetic indigo (CNF + Ind_S) and natural indigo (CNF + Ind_N). d) The patterns screen-printed with leucoindigo nanocellulose suspension on cotton fabric.

experiment the indigo is in leuco-form but on the next day after exposing to atmospheric oxygen, the leucoindigo has been oxidized to indigo form. A similar shift from shorter to longer wavelengths was observed in absorption spectra of pure leucoindigo and indigo in the absence of nanocellulose (Fig. S2). For indigo form, the excitation and emission bands were mirror images to each other and the difference between maxima of excitation and emission, i.e. Stokes shift, was small, which indicate that indigo molecule remains in the same conformation in both excited- and ground states. For leucoindigo, due to absence of internal hydrogen bonding, twisting around the central C–C bond is possible and therefore the shape of excitation and emission bands were not identical and Stokes shift was larger than for oxidized indigo (Seixas De Melo et al., 2004).

The mechanism of the nanocellulose stabilizing effect is most likely related to the lower rate of oxygen diffusion in the suspension. The diffusion of solutes in polymer solutions and gels is known to be lower than in liquids and it depends on the concentration and swelling of polymers (Masaro and Zhu, 1999). The blueish tint at the air-water interface at the beginning of stability experiment shows that the leucoindigo is oxidized immediately to indigo form at the very surface where the oxygen transfer is not hindered and the local concentration of oxygen is higher than in the bulk. The evolution of leucoindigo oxidation in Fig. 2c was followed only with nanocellulose suspension and not in an aqueous environment since the leucoindigo in water in open vials oxidizes quickly. The different nanocellulose suspension concentrations seem to have small effect to the stability. The natural leucoindigo embedded into lowest nanocellulose concentration was already in oxidized form after 12 h, whereas other concentrations retained indigo in leuco-form. The leuco-form of natural indigo seemed to remain in the reduced form longer than pure synthetic indigo (Fig. 2c). In addition, the visual inspection revealed that the yellow colour of reduced form is notably different from greenish colour of synthetic indigo despite the similar reduction conditions. The natural indigo can contain also other components, which contribute to the colour (Maugard et al., 2001). Wide variety of different compounds, such as alkaloids, phenolic compounds, polysaccharides, glucosinolates, carotenoids, volatile constituents, and fatty acids, have been found from the leaves of *I. tinctoria* (Speranza et al., 2020). The additional side-products, coloured or colourless, can have an influence on the colour and the observed difference in stability of leuco-form in natural indigo compared to synthetic indigo.

In Fig. 2d we show the paste-like material of nanocellulose and natural indigo can be used to create patterns on cotton fabric using screen-printing technique. Plenty of different plant-based dyes have been utilized to colour mainly textiles, food, and cosmetics (Costa and Santos, 2017; Frick, 2003; Shahid et al., 2013). In research related to dyes, nanocellulose materials have been utilized to create composite films with bioactive properties originating from natural dyes to be used for instance in food packaging (Bastante et al., 2021; Lohtander et al., 2021; Razavi et al., 2020). Nanocellulose has also been applied as adsorbent material for dye removal from effluents (Voisin et al., 2017). This proof of concept test showed that during printing step and drying at ambient conditions, the leucoindigo is oxidized to blue indigo form. However, the wash fastness of patterns was not determined as the paste did not contain binding agents essential for achieving high fastness levels. The basic ingredients of textile printing pastes include the colourant, thickener for modifying the rheology and binding agent for fixing the colourant permanently (Ibrahim et al., 2013). The binding agents are often curable polymers, such as formaldehydes, in volatile organic solvents, although some more eco-friendly binders have been investigated as well (El-Molla and Schneider, 2006). Cellulose interacts strongly with it-self via hydrogen bonding and theoretically the CNF material could act as a binder in addition to its role as a rheology modifier (Klemm et al., 2005).

Natural indigo obtained from *I.tinctoria* showed high antioxidant activity compared to pure synthetic indigo (Fig. 3a). The observed radical scavenging (%) activity of natural indigo was nearly 80% whereas pure synthetic indigo scavenged less than 5% of ABTS radical cations in the same time period. The bioactive properties of *I.tinctoria* are a well-known feature of the plant. Besides indigo dye production, *I. tinctoria* has traditionally been utilized as a medicinal plant owing to its anti-inflammatory, anti-tumor, antimicrobial and antioxidant activity (Speranza et al., 2020). For instance, hydrophilic phenolic compounds present in the water extract can act as radical scavengers and contribute to the bioactive properties of natural indigo (Taviano et al., 2018). The radical scavenging mechanism of phenolics is attributed to the hydrogen donating hydroxyl groups (Soobrattee et al., 2005). This observed antioxidant activity can also explain the longer stability of leucoindigo from natural indigo compared to pure synthetic indigo in the nanocellulose matrix seen in Fig. 2c. Besides the slower diffusion of oxygen to viscous suspension, the radical scavenging species can have an active role in stabilization mechanism of leucoindigo. Our results suggested that the antioxidant species of natural indigo acted as sacrificial compounds that were oxidized at the first phase by the dissolved oxygen and therefore leucoindigo molecules were not oxidized until the antioxidant activity possessing species were consumed (Fig. 3c). The oxidation of the synthetic dye was restricted only by the oxygen transfer rate in the viscous suspension and thus proceeded faster than the oxidation of the natural dye.

The antioxidant activity was preserved also when natural indigo was incorporated in the nanocellulose matrix, however, the rate of radical scavenging (%) was slower than for free natural indigo (Fig. 3b). With free natural indigo nearly all ABTS radical cations were scavenged after 1 h but when antioxidant species were immobilized onto the nanocellulose film, after 3 h approximately only 80% of ABTS radicals were scavenged. The radical scavenging activity of synthetic indigo as a function of time was not determined as its antioxidant activity was almost negligible (Fig. 3a). The more prolonged and controllable antioxidant activity of nanocellulose natural indigo composite could be interesting for creating naturally dyed functional textiles and for packaging applications.

The composition of natural indigo and pure synthetic indigo were compared using HPLC-DAD-MS analysis. The chromatograms and UV spectra (Fig. 4a and b) show that the major constituents in all three analysed samples were indigo at 13.9 min (*m/z* 263.08 (MH^+); λ_{max} 615, 285 nm) and indirubin at 14.8 min (m/z 263.08 (MH^+); λ_{max} 550, 290 nm) (Maugard et al., 2001). In addition to indigo and indirubin, the pure synthetic indigo contained two other compounds. The MS information and UV spectral data of compounds observed at 9.6 min (*m/z* 263.08 (MH^+), λ_{max} 280, 365 nm) and at 12.1 min (*m/z* 263.08 (MH^+); λ_{max} 285, 410 nm) suggested that they were structural isomers of indigo showing yellow-greenish colour instead of characteristic indigo blue. The chromatograms of lyophilized natural indigo and natural indigo dried at elevated temperatures were similar to each other, except the first peak that eluted at 8.0 min, which present only in lyophilized indigo. The compound at 8.0 min (*m/z* 148.04 (MH^+); λ_{max} 240, 300 nm) corresponded to values reported for isatin (Maugard et al., 2001). Indirubin is formed by condensation of isatin and indoxyl molecules (Kokubun et al., 1998; Maugard et al., 2001). The absence of isatin and higher indirubin amount, i.e. the surface area of peak, in natural indigo dried at elevated temperatures (Fig. 4a, c) suggested that the isatin had been converted to indirubin. Previously it has been demonstrated that oxygen is a significant factor in increased formation of indirubin (Kokubun et al., 1998). Results shown here suggest that the drying method to obtain dye powder influenced the indigo-indirubin ratio. The purity in terms of indigo amount was in similar order of magnitude for both pure indigo and lyophilized natural indigo from Woad (Fig. 4c). In addition to indigo and indirubin, plant-derived indigo can contain other compounds as well, such as isomers of indigotin and indirubin, brown-coloured isoindigo, indigo gluten, indigo yellow, and traces of flavonoids (Maugard et al., 2001). The structures of compounds at 9.8 min (*m/z* 241.20 (MH^+); UV λ_{max} 295, 550 nm) and 10.8 min (*m/z* 261.10 (MH^+); UV λ_{max} 285, 500 nm) remain unclear, but the UV

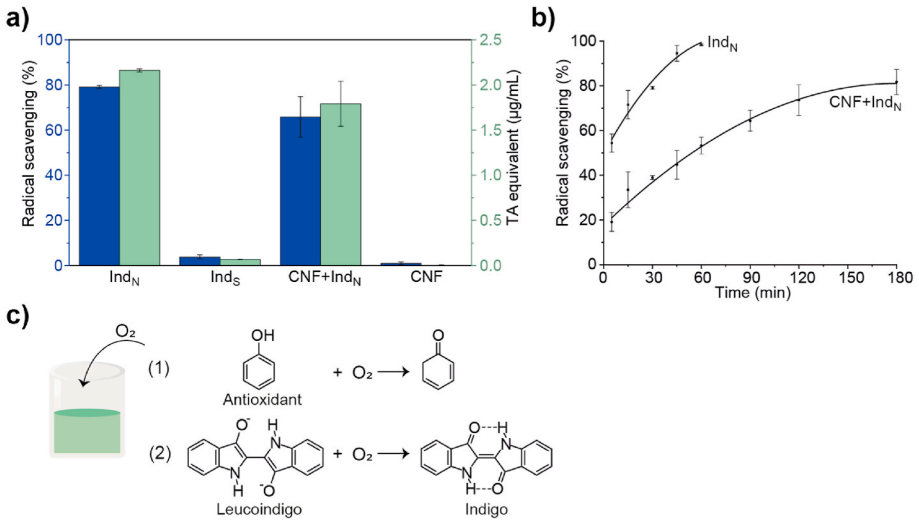


Fig. 3. a) The radical scavenging (%) activity and tannic acid (TA) equivalents of natural indigo (Ind$_N$, 0.5 mg/mL), pure synthetic indigo (Ind$_S$, 0.5 mg/mL)), natural indigo nanocellulose composite film (CNF + Ind$_N$, indigo 10% of CNF dry weight) and pure nanocellulose film (CNF). b) The radical scavenging (%) activity as a function of time for natural indigo and natural indigo nanocellulose composite film. c) Schematic illustration of proposed stabilization mechanism of natural leucoindigo. Antioxidant species are oxidized in the beginning of exposure to aerial oxygen (1) before leucoindigo molecules are oxidized to indigo (2). Phenol is a representative of antioxidant compounds present in natural indigo.

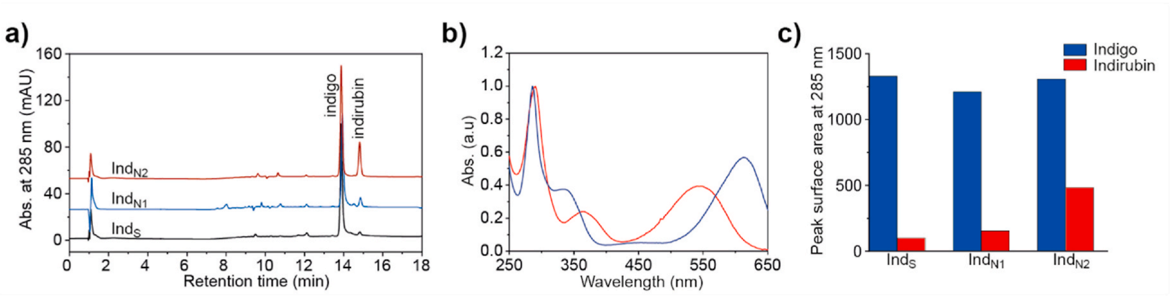


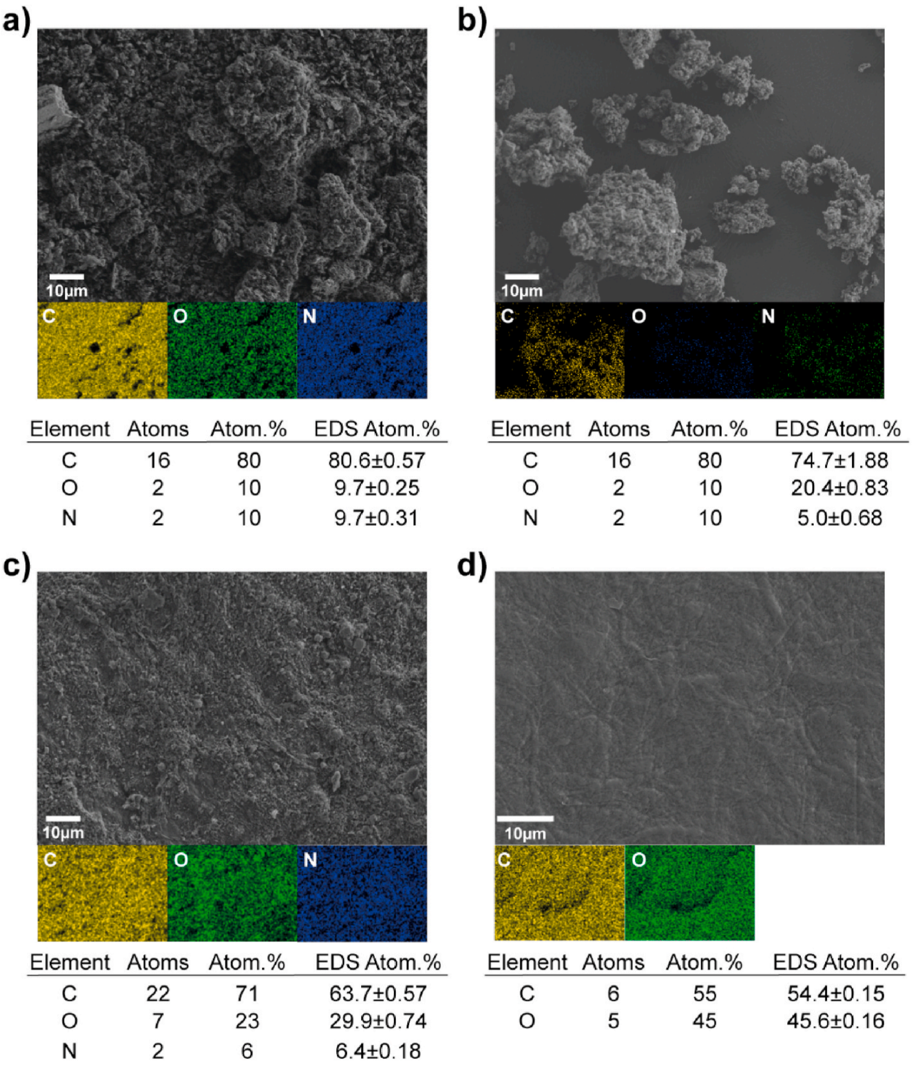
Fig. 4. a) The chromatograms of synthetic indigo (Ind$_S$), lyophilized natural indigo (Ind$_{N1}$) and natural indigo dried at elevated temperature (Ind$_{N2}$), b) the absorbance spectra of indigo (blue) and indirubin (red), and c) surface area of indigo and indirubin peaks detected at 285 nm for corresponding samples.

absorbance maxima indicated that both components showed colour in violet to red region and thus contributed to the resulting colour of natural indigo. In addition to these, a colourless compound at 12.1 min (m/z 282.28 (MH^+); UV λ_{max} 285, 340 nm) was observed, which could possibly be attributed to a phenolic constituent. The presence of a phenolic compound is supported by our antioxidant activity results (Fig. 3a and b) for the natural indigo.

In order to study the localization of indigo on the nanocellulose material we used scanning electron microscopy (SEM). The SEM images in Fig. 5 showed that the natural indigo pigment particles were evenly embedded in the nanocellulose film (Fig. 5c). The presence of indigo, however, increased the roughness of the film compared to the pure nanocellulose film, which could indicate that indigo deposits onto the cellulose fibril surface as very small nano/micro particles. Pure synthetic and natural indigo powder (Fig. 5a and b respectively) consisted of fine, tens to hundreds of microns in size, particulates and it formed a rough surface. Similarly, the surface of nanocellulose films containing natural indigo (Fig. 5c) formed a surface with particles, although the particles were much smaller (a few microns and bellow) compared to pure indigo alone. In the nanocellulose sample containing indigo, the particles were aligned along the fibrillary matrix typical for pure nanocellulose film (Fig. 5d). This alignment also indicates that the indigo particles were formed onto the nanocellulose fibril surface. The elemental mapping did not show significant differences in the distribution of C, N and O in any sample. The distribution of nitrogen is even in the sample containing nanocellulose and indigo indicating a uniform distribution of indigo on the surface of nanocellulose. For natural indigo pigment, the distribution is not as uniform as for other samples since the background of the specimen holder is visible. The atomic weights of pure nanocellulose and synthetic indigo observed with EDS were in good agreement with molecular formula of corresponding compounds. The calculated and observed elemental atomic weight percentages of natural indigo pigment and nanocellulose film containing natural indigo does not match, suggesting that the specimen contains also other compounds, which is in line with findings from HPLC-MS analysis.

4. Conclusions

In the present study, the stabilization of leucoindigo on a nanocellulose carrier matrix was investigated with spectroscopic and photophysical methods. The fluorescence spectra and lifetime decays confirmed that the leucoindigo form was retained longer when indigo was embedded on to the nanocellulose fibril matrix. The stabilizing effect of nanocellulose matrix was attributed to the limited rate of oxygen diffusion. The leucoindigo form was retained even longer when indigo from natural origin (*Isatis tinctoria*) was used. The longevity of leucoform of natural indigo compared to pure synthetic indigo was assigned to the antioxidant species present in natural indigo. According to antioxidant studies, natural indigo had a significantly higher radical scavenging capacity compared to synthetic indigo and the rate of antioxidant activity was adjustable when natural indigo was deposited on to the nanocellulose matrix. Our findings demonstrated that it is possible to stabilize leucoindigo on to nanocellulose matrix carrier to avoid further reducing steps with chemical agents making the use of the dye more environmentally friendly. The stabilization step could be combined to the extraction process of natural indigo and the use of harmful reducing agent could be avoided as the leucoindigo would be trapped and stabilized on the carrier material before oxidation to the pigment form. The proposed process would enhance the sustainability of indigo dyeing and make the natural indigo more attractive than artificial indigo.



a)

Element	Atoms	Atom.%	EDS Atom.%
C	16	80	80.6±0.57
O	2	10	9.7±0.25
N	2	10	9.7±0.31

b)

Element	Atoms	Atom.%	EDS Atom.%
C	16	80	74.7±1.88
O	2	10	20.4±0.83
N	2	10	5.0±0.68

c)

Element	Atoms	Atom.%	EDS Atom.%
C	22	71	63.7±0.57
O	7	23	29.9±0.74
N	2	6	6.4±0.18

d)

Element	Atoms	Atom.%	EDS Atom.%
C	6	55	54.4±0.15
O	5	45	45.6±0.16

Fig. 5. The SEM micrographs, EDS elemental maps and atomic weight percentages (%) of a) pure synthetic indigo pigment, b) natural indigo pigment, c) natural indigo embedded in nanocellulose film, and d) pure nanocellulose film. In EDS elemental maps the location of corresponding elements (C, O, N) on the specimen is indicated with the coloured dots. Atomic weight percentages (%) describe the theoretical composition of indigo or nanocellulose based on the molecular formula and the observed composition with EDS.

Furthermore, this paste-like material can be used for printing and creating patterns on for example cotton and the co-products present in the natural dye, which often are considered as a drawback, could bring additional functionalities besides the colouration.

CRediT authorship contribution statement

Tia Lohtander: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Nikita Durandin:** Methodology, and, Formal analysis, Writing – review & editing. **Timo Laaksonen:** Writing – review & editing. **Suvi Arola:** Conceptualization, Project administration, Writing – review & editing. **Päivi Laaksonen:** Conceptualization, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was part of Crops4Luxury project funded by The Finnish Innovation Fund Sitra and supported by the Academy of Finland's project #311608/326262 and 327178/327195. We are grateful for the support by the FinnCERES Materials Bioeconomy Ecosystem and the Bioinfra on the Otaniemi campus.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2021.129615.

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