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Localization and dynamics of amylose–lipophilic molecules inclusion complex formation in starch granules†

Marianna Manca,[a] Albert J. J. Woortman,[a] Andrea Mura,[ab] Katja Loos*[a] and Maria Antonietta Loi*[a]

Inclusion complex formation between lipophilic dye molecules and amylose polymers in starch granules is investigated using laser spectroscopy and microscopy. By combining confocal laser scanning microscopy (CLSM) with spatial resolved photoluminescence (PL) spectroscopy, we are able to discriminate the presence of amylose in the peripheral region of regular and waxy granules from potato and corn starch, associating a clear optical fingerprint with the interaction between starch granules and lipophilic dye molecules. We show in particular that in the case of regular starch the polar head of the lipophilic dye molecules remains outside the amylose helix experiencing a water-based environment. The measurements performed on samples that have been extensively washed provide a strong proof of the specific interaction between lipid dye molecules and amylose chains in regular starch. These measurements also confirm the tendency of longer amylopectin chains, located in the hilum of waxy starch granules, to form inclusion complexes with ligands. Through real-time recording of CLSM micrographs, within a time frame of tens of seconds, we measured the dynamics of occurrence of the inclusion process between lipids and amylose located at the periphery of starch granules.

Introduction

Starch is the main source of carbohydrates for humans and many animal species, and consequently is one of the main ingredients of food.[1–3] Starches from different botanical sources are found in the form of granular structures, ranging in size between ∼1 and over 100 μm,[4] which are characterized by variable contents of amylose and amylopectin. They consist typically of 10–30% amylose and 90–70% amylopectin, but high-amylose starches with a content up to 85% exist, and the so-called waxy starches are constituted of nearly 100% amylopectin.[2,5]

Several models have been proposed in order to explain the architecture of these semi-crystalline structures.[6] A cluster-like arrangement of the amylopectin polymers generates alternating stacks of amorphous and crystalline lamellae. The amorphous regions consist of branching zones of the amylopectin chains, while the crystalline lamellae are constructed by an ordered packing of parallel linear glucan chains.[7–9] Amylose chains

appear generally interspersed among the amylopectin clusters,[10] although it has been commonly assumed that amylose resides mainly in the amorphous regions.[11,12] Jane and co-workers demonstrated that amylose bundles are more concentrated near the surface of the granule,[13] which in native starch and according to Lineback's model[14] ("hairy billiard ball" model) and later experiments (by Stark and Lynn)[15] has been characterized by the terminals of the amylose chains protruding from amylopectin clusters.

The tendency of amylose chains to generate inclusion complexes in the presence of an appropriate guest molecule has been the object of wide interest.[10,16,17] The amylose helix presents a hydrophobic cavity that shows high affinity for apolar guest molecules.[18] In the process of inclusion complex formation, amylose coils undergo a conformational change, assuming the structure of a left-handed helix that allows inclusion of guest molecules such as iodine,[19] dimethyl sulfoxide (DMSO)[20] or potassium hydroxide.[21] Among others, inclusion complexes between amylose and lipids such as fatty acids and phospholipids have been widely investigated.[22–24] It has been demonstrated that amylose–lipid inclusion complexes affect the staleness,[25] digestibility,[10] and rheological properties of food.[26] It is therefore important to understand the nature of the process and its dynamics.

The most accepted model describes the aliphatic chain of the fatty acid molecule hosted inside the hydrophobic cavity of the amylose helix,[27] while the polar group, including the carboxylic

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acid, remains outside the helix due to electrostatic interactions.[27–30] Several studies have been carried out in order to validate the models. Karkalas and co-workers, for example, determined the thermal properties of the inclusion complexes, investigating their dissociation temperature and dissociation enthalpies.[31] Lately, we have shown by Confocal Laser Scanning Microscopy the formation of specific inclusion complexes between lipophilic molecules with regular and waxy starch granules from different botanical sources.[32] Importantly, and in contrast to what is commonly believed, we demonstrated that the inclusion also occurs at temperatures below the gelatinization point for very low lipid concentrations.

Although there is great interest in the inclusion complex formation, very limited data are available concerning the dynamics of the process. Recently, Cao and co-workers presented a study on the dynamics of complex formation involving well-defined amylose brushes and fatty acids characterized by different lengths of the aliphatic chain (C8 and C14), showing that octanoic acid (C8) is included more efficiently than longer fatty acids.[33]

The aim of this work is to associate an optical fingerprint with the interaction between starch granules and lipids, thus determining the role of the amylose chains present in the peripheral region of the granules. Combining Confocal Laser Scanning Microscopy (CLSM) with spatial resolved photoluminescence (PL) spectroscopy, we established that the presence of amylose in the starch granules modifies the PL signature of the lipid–dye, which is used as a nano-probe to detect its interaction with the surroundings. We demonstrate in this way that in regular starch, as opposed to waxy starch, the hydrophobic head remains outside the helix. Furthermore, measurements performed on samples that have been extensively washed provide a strong proof of the specific interaction between lipid dye molecules and amylose chains in regular starch. These measurements also confirmed the tendency of longer amylopectin chains, located in the hilum of waxy starch granules, to form inclusion complexes with ligands.

Finally, the study conducted in real time of the dynamics of inclusion of lipophilic molecules in the peripheral starch of the granules, demonstrated that the process is completed in about 10 seconds, demonstrating again the efficiency and specificity of the process of inclusion complex formation between the amylose in the periphery of the starch granules and lipids.

Results and discussion

Confocal Laser Scanning Microscopy (CLSM) combined with spatially resolved photoluminescence (PL) spectroscopy is an ideal tool to investigate the nature and the properties of the inclusion complex formation between starch granules from different botanical sources and lipids. The samples were prepared by suspending starch granules in water and exposing them to 5-hexanodecanoylaminofluorescein, a lipophilic fluorescent molecule (lipid–dye) characterized by an aliphatic chain of 15 carbon atoms and a polar head based on a carboxylic acid and a fluorescein molecule. The exposure of the starch granules

to the lipid–dye molecules, as well as the CLSM measurements, were performed at room temperature, well below the gelatinization temperature of starch, using lipid–dye concentrations as low as 0.02% with respect to starch. The amylose content in starch has been estimated to be roughly 30%.[34,35] Therefore the amount of complex formed in our case is limited by the lipid–dye concentration we have used. It is therefore evident that techniques such as DSC and X-ray analysis are not sensitive enough to detect such a low percentage of complexes. While photoluminescence based techniques are the only ones which may allow this level of sensitivity.

Fig. 1 shows the CLSM images of starch granules in water-based suspension after addition of 0.02% (based on starch) lipid–dye molecules at room temperature. The starch granules are from regular potato (Fig. 1a), waxy potato (Fig. 1b), regular corn (Fig. 1c) and waxy corn (Fig. 1d). The CLSM images were recorded after rotating the starch–lipid–dye suspensions overnight in a dark environment at room temperature.

The micrographs reveal a bright rim area around the granules from both the regular potato and regular corn starch (Fig. 1a and c), which highlights the typical shape and structure of the starch granules.[36] Potato starch granules appear to be in oval shape with smooth surfaces with dimensions ranging between 10 and 100 μm. Corn starch granules present a truncated shape with dimensions ranging between 5 and 30 μm.[37]

The sharpness and the thickness of the rim (dimension smaller than 1 μm) indicate the formation of inclusion complexes between the amylose chains present in the outer region of the granules and the lipid–dye molecules. In agreement with the above-mentioned theories, the aliphatic chain of the lipid–dye can be
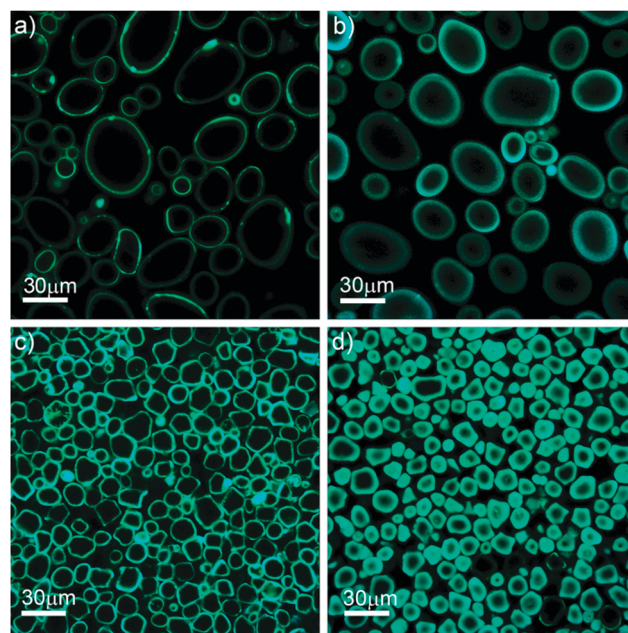

Fig. 1 CLSM micrographs of potato (a) and corn (c) regular starch granules; potato (b) and corn (d) waxy starch granules, in suspension after addition of lipid–dye molecules to starch in a ratio of 0.02%. The suspensions were rotated overnight at room temperature in a dark environment.

included in the amylose polymer, while the fluorescent polar head remains outside. In both regular corn and potato starches the background remains dark, suggesting that all lipid–dye molecules are involved in the inclusion complex process leaving no molecules in the water-based solution.

The micrographs of the waxy potato and waxy corn starches (Fig. 1b and d) show a contour area around the granules which is not as defined as in the case of the regular starch granules. The rims in these cases appear much broader, with a variable thickness up to 5μm after one day of incubation, which is the same incubation-time as the non-waxy granules in Fig. 1a and c. The diffuse emission might be considered a sign that the lipid–dye molecules are involved only in weak interactions with the components of the starch granules.

In a recent work[32] we presented an investigation of the interaction of the lipid–dye molecules (5-hexanodecanoylaminofluorescein) with potato starch granules in their regular and waxy forms, demonstrating that the absence of amylose in the waxy starch granules affects the size and shape characteristics of the luminescent rim, due to the weak interactions of the lipid–dye with the components of the waxy starch. Here, our goal is to find a quantitative fingerprint characteristic of the interaction of the lipid–dye with amylose and amylopectin by recording point by point the emission spectra in different samples.

Fig. 2 shows the CLSM images with a 50 μm field-of-view of regular potato (Fig. 2a) and waxy potato (Fig. 2b) starch granules in water-based suspensions after addition of the lipid–dye solution. Fig. 2c shows the spatially resolved PL spectra recorded in the regions highlighted by white circles in Fig. 2a and b and the spectrum of the lipid–dye molecules in DMF–water solution (20 μl of 0.02% lipid–dye in DMF solution in 1 ml of water-based solution). The regions are specifically selected focusing the laser at these points after image acquisition. The spectrum recorded exciting the regular potato starch sample in region A (Fig. 2a) shows the emission of 5-hexadecanoylaminofluorescein molecules, with the main emission peak at ∼530 nm.

The spectrum associated with region B in the rim of waxy potato starch granules (Fig. 2b) also presents the main photoluminescence peak at 530 nm, without a spectral shift with respect to the spectrum recorded exciting the regular potato starch; in this case, however, the emission appears narrower (the difference in full width at half maximum (FWHM) is ∼10 nm between spectra A and B). The emission resulting from the free lipid–dye molecules in DMF–water solution shows the same spectral characteristics of emission recorded exciting regular starch granules.

In Fig. 3 we present the CLSM micrographs with a field-of-view of ∼30 μm of regular (Fig. 3a) and waxy corn (Fig. 3b)
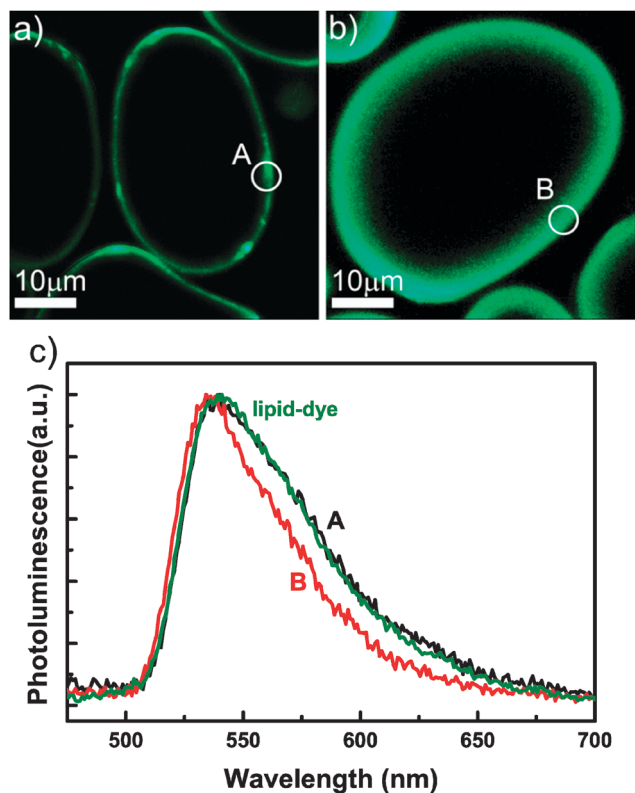
Fig. 2 CLSM images of regular potato (a) and waxy potato (b) starch granules after addition of 5-hexadecanoylaminofluorescein molecules. PL spectra (c) recorded in the regular potato rim area (circle A) and in the waxy potato rim area (circle B). The emission of the reference lipid–dye molecules in solution is also shown. Excitation wavelength 488 nm. The lipid–dye to starch ratio used is 0.02%.

Fig. 3 CLSM images of regular corn (a) and waxy corn (b) starch granules in suspension after addition of 5-hexadecanoylaminofluorescein molecules. PL spectra (c) recorded in the regular corn rim area (circle A) and in the waxy corn rim area (circle B). The emission of the reference lipid–dye molecules in DMF–water solution is also shown. Excitation wavelength 488 nm. The lipid–dye to starch ratio used is 0.02%.

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starch granules, in water-based suspension after exposure to 5-hexadecanoylaminofluorescein molecules in solution. As in the case of all samples reported, exposure to the lipid–dye was performed at room temperature, well below the gelatinization temperature of starch. Fig. 3c shows the spatially resolved photoluminescence spectra recorded in the two regions indicated by a white circle in the CLSM micrographs and the spectrum recorded exciting the lipid–dye molecule in DMF–water solution as a reference. In this case we also found that a narrower emission spectrum is associated with the fluorescent rim of the waxy starch granules and a superposition of the spectra obtained from regular starch and free lipid–dye molecules.

The consistent finding of different spectra of the same dye in regular and waxy forms of starch suggests that the presence of amylose chains in the peripheral regions of the granules affects the nature of the interaction and consequently the photoluminescence properties of the complex formed between the lipid–dye molecules and the starch.

The difference between the spectra associated with the regular and waxy starch granules can be explained by the different environment experimented with the lipid–dye, depending on the chemical composition of the granule. In the case of the regular potato starch, the aliphatic chains of the lipid is included inside the amylose chains and the polar groups stay outside due to the hydrophobic nature of the interaction, while in the case of the waxy form the lipid–dye molecule (both the aliphatic chain and the polar group) enters the inner structure of the granule giving rise to a broader and less-defined rim.[32] The embedding of the lipid–dye molecule in the granule structure causes the suppression of vibrational modes of the molecule, for example, C–H stretching and bending vibrations, and C–C and C–H bending, which are reflected in a narrower and less structured emission spectrum compared to the spectrum of the lipid–dye molecule complexed with the regular starch granules.

The emission fingerprint of the free lipid–dye molecules in DMF–water solution coincides with that of the regular potato starch, indicating in both cases that the chromophore is in contact with water. These kinds of chromophores are extremely sensitive to the medium in which they are embedded (dielectric constant); when the polar group of the lipid–dye molecule is in water and free to move, the emission spectra results are broader and more structured than those generated by an embedded lipid–dye molecule, as in the case of waxy starch.

To confirm that there is an actual difference in strength and specificity between the interactions in the case of regular and waxy starches, we performed a "washing" process on regular and waxy potato granules, and regular and waxy corn starch granules, using different concentrations of DMF in water (50, 80, 90 and 100% v/v). Because the lipid–dye molecules are poorly water-soluble but very well soluble in DMF, it may be expected that washing with a high DMF concentration might remove the lipid–dye molecules from the starch granules in the case of non-specific interactions with the starch granules.

When the exposure time of the starch granules with the lipid–dye solution is increased the luminescent rim around the

granule becomes broader, especially in the case of the waxy starch granules. We therefore increased the exposure time with the lipid–dye solution from 1 day to 7 days.

Fig. 4a, c, e, and g show the CLSM micrographs of regular potato, waxy potato, regular corn and waxy corn starch granules, respectively, acquired after 7 days of exposure to the lipid–dye solution.

The regular starch granules (Fig. 4a and e) present a bright rim, which is identical in dimension and shape to the rim imaged after one day of exposure to lipid–dye molecules as
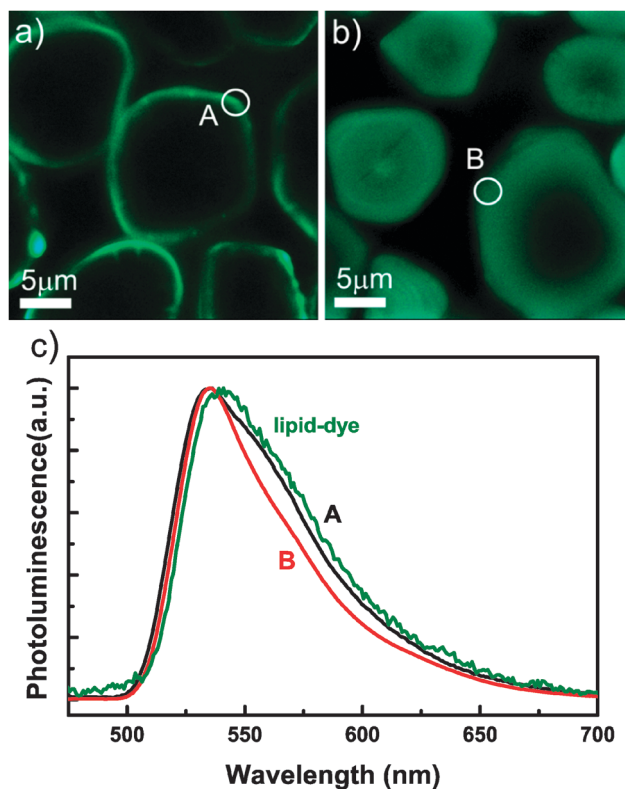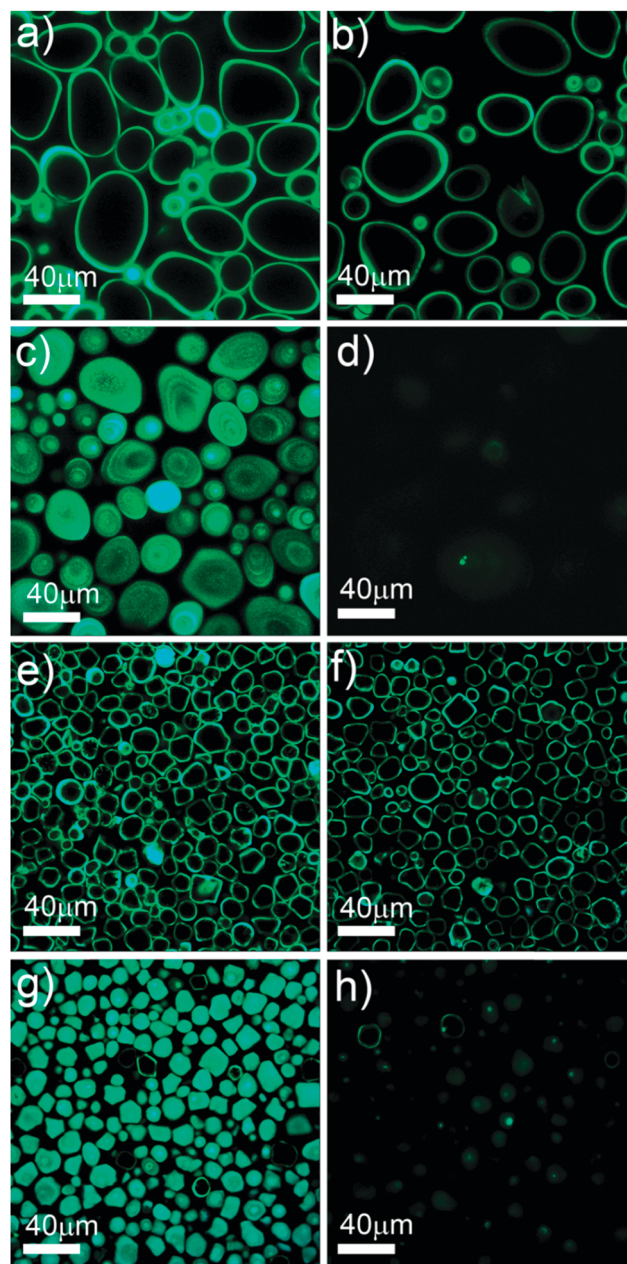

Fig. 4 CLSM images of regular potato (a), waxy potato (c), regular corn (e) and waxy corn (g) starch granules in suspension 7 days after the addition of 5-hexadecanoylaminofluorescein molecules. The images (b), (d), (f), and (h) refer to the same starch species acquired after the "washing" procedure with 80% DMF. The lipid–dye to starch ratio used is 0.02%.

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reported in Fig. 1a and c. The longer incubation time clearly increased the absorbance of the lipid–dye molecules in the waxy potato and waxy corn starch granules. Fig. 4c and g evidence that the starch granules are completely stained. Comparing these with Fig. 1b and d it is clear that the lipid–dye goes through a time-dependent diffusion process inside the waxy starch granules. We can also observe (Fig. 4c and g) that the hilum of the waxy starch granules is brighter compared to the rest of the granule, which is most probably due to the complex formation between the lipid–dye molecules and the longer amylopectin chains located in the inner part of the granules.

Fig. 4b, d, f, and h show the CLSM images of, in the order, regular potato, waxy potato, regular corn and waxy corn starch granules washed with 80% DMF in water (v/v) solution, after 7 days of exposure to lipid–dye molecules. While the regular starch granules (Fig. 4b and f) do not present a substantial difference with respect to the unwashed granules (Fig. 4a and e), the micrographs in Fig. 4d and e reveal, very interestingly, that the washing process with the DMF-based mixture removes dramatically the lipid–dye molecules from the granules.

This finding confirms that the presence of amylose in the regular starch granules generates specific interactions between the host and the ligand. The nature of such bonds has been extensively debated, the general conclusion being that the hydrophobic effect seems to be the main force responsible for the specific interaction.[30] Conversely, we can infer that in the case of waxy starch the lipid–dye molecules do not seem to undergo a specific interaction with amylopectin, and weaker interactions explain the experiments in Fig. 4c and g. However, after washing, some photoluminescence emission from the lipid–dye molecules appears to persist only in the hilum of the waxy starch granules (see Fig. 4h and Fig. S1e, ESI†). This finding confirms our previous hypothesis of the interaction between the lipid–dye molecules and the longer amylopectin chains present in the hilum.[32] However, the fact that a very small amount of amylose chains is still present in the hilum of waxy starches cannot be excluded.[11]

Washing procedures with 50% and 90% DMF were also performed (see ESI,† Fig. S1), revealing that the increasing percentage of DMF has a proportionally stronger capacity to remove the lipid–dye molecules from the waxy starch compared to the regular starch granules. Washing with 90% and 100% DMF solutions also resulted in a gradual loss of staining in the case of regular starch granules.

In order to investigate the time scale of the inclusion complex formation at room temperature, we performed real-time CLSM measurements of the process. The experiment was performed exposing potato and corn starch granules in the regular and waxy forms to the lipid–dye molecules at room temperature.

The inclusion complex formation could be observed from the initial stage by adding a small amount of the lipid–dye solution (3 μl, see the Experimental section) directly to the starch suspension already present on the sample stage of the confocal microscope. The effect of the addition of the lipid–dye
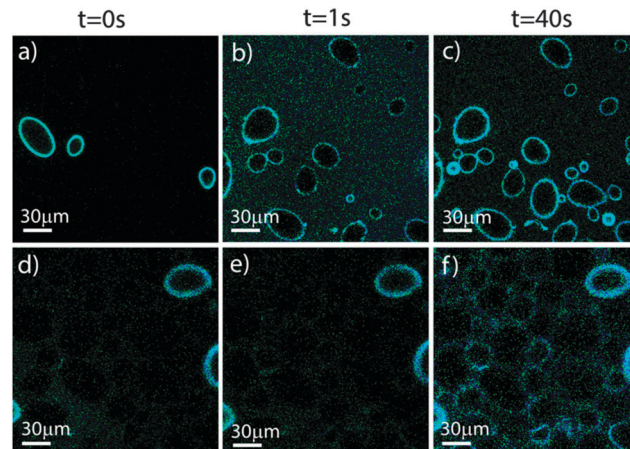

Fig. 5 Photograms extracted from CLSM videos recorded on regular potato starch (a–c) and waxy potato starch (d–f), (a and d) before the addition of the lipid–dye solution (t = 0), (b and e) recorded at 1 s after the addition of the lipid–dye solution and (c and f) at 40 s after the addition of lipid–dye solution. The lipid–dye to starch ratio used for the experiment is 0.08%.

solution was monitored in real time without stirring or heating. Here it is important to notice that the ratio between the concentration of the lipid–dye and the starch used for the dynamics is 4 times higher (0.08%) than what has been used for the previously reported measurements (0.02%).

A small amount (0.05%) of overnight complexed starch granules was added to the starch suspensions as reference starch granules, in order to facilitate the focusing operations in the microscope stage.

In Fig. 5 we present a sequence of micrographs of potato starch granules in the regular and waxy forms, extracted from CLSM live recordings. Fig. 5a and d present the initial situation at t = 0 s, when the lipid–dye molecules are added to the water-based suspension of the starch granules of regular potato and waxy potato located on the microscope stage, respectively. These micrographs (Fig. 5a and d) are characterized by a dark background except for a few reference granules, which were inserted into the sample composed of not-complexed granules with the aim of finding the focal plane. In Fig. 5d the presence of the starch granules is very faintly visible before the addition of the lipid–dye. This phenomenon can be explained by the fact that the waxy starch interaction with the lipid–dye in this case is not specific, and that the bright rim is due to adsorption and diffusion of the lipid–dye molecules.[32] The lipid–dye molecules in this case can easily be released in the solution and be re-adsorbed by other granules in water-based suspension.

Fig. 5b presents the micrograph recorded 1 s after exposure of the regular starch granules to the lipid–dye molecules. The background appears luminescent, indicating that the fluorescent lipid–dye molecules are homogeneously distributed in the water based sample solution. At this time a weak luminescent rim starts appearing, revealing the presence of several starch granules. At 40 s after the exposure of the granules to the lipid–dye molecules (Fig. 5c) the rim around the granules appears brighter and more defined, while the background appears

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darker, indicating that most of the added lipid–dye molecules are involved in the complex formation with the amylose chains present in the outer region of the starch granules. The different behaviours of regular and waxy starch within the interaction with the ligand molecules are underlined by the comparison of the photograms related to the two different species. Fig. 5e and f show the waxy potato starch granules at 1 s and at 40 s, respectively, after the exposure to the lipid–dye molecules. In Fig. 5e the background luminescence is substantially the same, compared to that shown in the photogram at t = 0 s, suggesting that the interaction taking place between waxy starch and lipid–dye molecules is slower than the interaction with the regular starch. After 40 seconds the granules are highlighted by a blur rim (much less defined compared to those in the regular starch). These findings confirm that in the case of the waxy form of potato starch, a different and slower process occurs due to the absence of amylose.[32]

Fig. 6 presents a sequence of photograms extracted from live CLSM recordings on regular and waxy corn starch granules before and during the exposure to the lipid–dye molecule. Also in this case the photograms show different phases of the process initialized by the addition of the lipid–dye to the solution.

Fig. 6a and d present the situation at t = 0 s, when the addition of lipid–dye solution to the solution takes place. Also in this case a few granules already complexed with lipid–dye molecules were added before to the not-complexed suspension of starch granules in order to facilitate the localization of the unstained starch granules.

At t = 5 s after the exposure of the not complexed granules to the lipid–dye molecules, a defined and bright rim around a multitude of regular corn starch granules appears (Fig. 6b). At 40 s after the exposure (Fig. 6c), a large assembly of fully complexed granules appears. The background is very dark, as are the inner regions of the granules where the lipid–dye is not present. This observation suggests that most of the added lipid–dye molecules are involved in the inclusion complex formation.

As expected, the waxy corn starch shows a totally different behaviour. At t = 5 s after the exposure (Fig. 6e), the profile of the granules becomes highlighted by the presence of the lipophilic fluorescent molecules in a rim which is less defined and fuzzier than the one which characterizes the regular starch granules, also confirming in this case that the presence of amylose chains at the peripheral regions of the granules is a discriminant for the inclusion complex formation. Fig. 6f shows the waxy corn starch granules 40 s after the addition of the lipid–dye molecules. In this case the whole starch granules look more or less stained by the lipid–dye, indicating that the lipid–dye molecules are able to penetrate the whole granule, confirming again that the presence of the amylose chains affects the interaction of the starch granules with the lipid–dye molecules, and that it is the necessary element for the inclusion complex formation in the peripheral region of the granules at room temperature. Furthermore, a diffuse luminescence is present in all samples, suggesting that a significant amount of lipid–dye molecules is still not adsorbed within the waxy corn starch granules at this time scale.

The experiments carried out on potato and corn starch demonstrate that at a very low lipid–dye concentration inclusion complex formation occurs at room temperature in a quantity detectable only by CLSM within tens of seconds.

Experimental

Materials and methods

Corn starch, waxy corn starch, potato starch, and Lugol solution for microscopy were purchased from Sigma-Aldrich. Waxy potato starch (Eliane 100) was supplied by Avebe Food. 5-Hexadecanoylaminofluorescein ($C_{36}H_{43}NO_6$) with a M_w of 585.74 g mol^{-1} was purchased from Life Technologies. Dimethylformamide (DMF) extra pure was purchased from Acros-Organics.

Starch suspensions for Confocal Laser Scanning Microscopy (CLSM) were prepared at a concentration of 2% in distilled water with 0.02% sodium azide as a preservative. 1 ml of the as-prepared suspension was stained with additional 20 μl of a 0.02% solution of the fluorescence dye 5-hexadecanoylaminofluorescein in DMF, by rotating overnight at room temperature in the dark (stock suspension).

For CLSM measurements 40 μl of the stained samples were transferred to an object glass.

Washed starch suspensions for CLSM were prepared at a concentration of 2% in distilled water with 0.02% sodium azide as a preservative. 10 ml of the as-prepared suspensions was stained with 200 μl of 0.02% DMF solution of the fluorescent dye 5-hexadecanoylaminofluorescein, by rotating the preparation for 7 days at room temperature in the dark. The suspensions were centrifuged for 5 min at 2000 rpm in a Heraeus Labofuge 400R with a swing-out rotor. The supernatants were decanted and an equal amount of distilled water with 0.02% sodium azide was added. 1 ml of the suspension was taken as a reference unwashed sample. For the washing procedure, several other aliquots
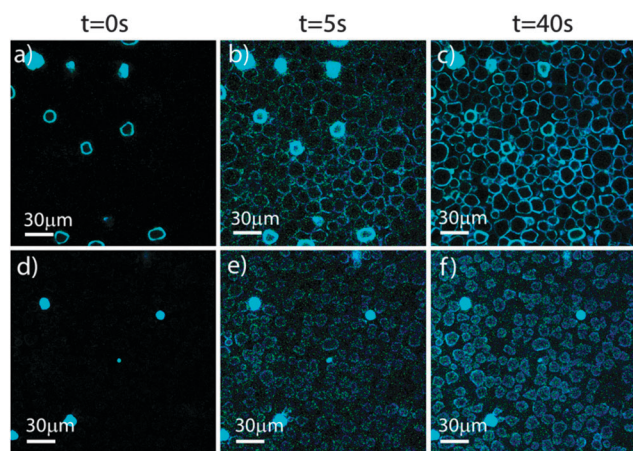

Fig. 6 Photograms extracted from CLSM recordings performed on regular corn starch (a–c) and waxy corn starch (d–f), during (a and d) the addition of the lipid–dye solution (t = 0), at 5 s after the addition of lipid–dye solution (b and e) and at 40 s after the addition of lipid–dye solution (c and f). The lipid–dye to starch ratio used for the experiment is 0.08%.

of 1 ml of the suspension were transferred into separate microtubes and centrifuged for 5 min at 2000 rpm. The supernatant was decanted and 1 ml of 50%, 80%, 90% or 100% DMF was added, followed by rotation for 1 hour in the dark. The suspensions were centrifuged for 5 min at 2000 rpm and the residues were washed two additional times under similar conditions. The residues washed with different percentages of DMF were suspended in 1 ml of distilled water with 0.02% sodium azide. For CLSM imaging 40 μl of the washed and unwashed samples were transferred to an object glass.

For real time observations, 10 μl (potato starches) and 5 μl (corn starches) of the stock suspensions (see above) were added to 200 μl of the starch suspensions without lipid–dye. From the mixed solutions a drop (37 μl) was placed on the microscope object glass and 3 μl of lipid–dye solution (molecular probe 0.02% 5-hexadecanoylaminofluorescein in DMF) was added during the on-going CLSM measurements.

The reference measurement for the lipid–dye emission was obtained by diluting 20μl of DMF solution in 1 ml of water.

Photoluminescence measurements

Confocal laser scanning microscopy and spatially resolved photoluminescence measurements were performed with an experimental set up based on an inverted Nikon Eclipse Ti microscope with a 60 times magnification oil immersion objective characterized by NA = 1.4. The excitation source was an Ar$^+$ laser (wavelength 488 nm).

The sample images were recorded using a set of photomultiplier tubes (PMT) covering a spectral detection range of ∼460–750 nm. The spatial resolution achievable, depending on the excitation wavelength, was according to the equation:

$$d = 0.46\lambda/\text{NA},$$

where λ is the excitation wavelength and NA is the numerical aperture of the microscope objective.

The real time CLSM measurements were performed acquiring image frames with 1002 ms time intervals and dwell time-line of 1.68 μs.

Spatially resolved PL spectroscopy was performed collecting the photoluminescence signal of the sample using a monochromator coupled with an Image EM CCD camera from Hamamatsu. After raster scanning and imaging of the sample, the laser beam was focused on a selected region (dimensions smaller than 1 μm^2) of the sample, and for each position the signal was measured using the Image EM CCD camera.

Photoluminescence (PL) measurements were performed exciting the samples at 488 nm and recording the spectra using a monochromator coupled with an Image EM CCD camera from Hamamatsu. The spectra were corrected for the spectral response of the set up.

Conclusions

Our work investigated the role of amylose chains in the inclusion complex formation between starch granules and lipophilic fluorescent molecules. By performing Confocal Laser Scanning Microscopy we explored the inclusion complex formation involving regular and waxy forms of starch obtained from two different botanical sources. In particular, by correlating Confocal Laser Scanning Microscopy and spatially resolved spectroscopy we were able to discriminate the presence of amylose polymers in starch granules using the photoluminescence spectra of the dye as a nano-probe of the interaction experienced by the lipid–dye molecule with the surroundings, demonstrating that in regular starch the hydrophobic head remains outside of the amylose chain.

By performing a ''washing'' process, we proved the specific interaction of the lipid–dye with amylose while the adsorbed lipid–dye in the waxy starches was easily reversed by washing. Only in the hilum do traces of complexation remain with the longer amylopectin chains or with eventual traces of amylose.

Finally, real time monitoring of the inclusion process allowed us to estimate within tens of seconds the dynamics of the inclusion complex formation, visualizing in a relatively simple way the first stage of the interaction between amylose in the outer part of starch granules and the lipid–dye ligand below the gelatinization temperature of the starch.

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