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Ultrasensitive and fast detection of denaturation of milk by Coherent backscattering of light

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In this work, Coherence backscattering (CBS) of light has been used to detect the onset of denaturation of milk. The CBS cone shape and its enhancement factor are found to be highly sensitive to the physical state of the milk particles. The onset of denaturing of milk not visible to the naked eye, can be easily detected from changes in the CBS cone shape. The onset of denaturation is confirmed by spectral changes in Raman spectra from these milk samples. Further, the possibility to estimate the dilution of milk by water as an adulterant is demonstrated. The method reported has a broad scope in industry for making an inline ultrafast cost effective sensor for milk quality monitoring during production and before consumption.

Natural milk, an important ingredient of food, many a times undergoes undetected for denaturation causing health concerns and economic loss. Denaturation is a process in which proteins (i.e. amino acid polymers)/nucleic acids lose their bioactivity due to external stress like elevated temperatures, exposure to extreme pH, and various non-physiological conditions. During denaturation proteins or, nucleic acids lose their native secondary, tertiary and quaternary (either or mixed) structures partially or, completely. Milk on denaturation exhibits characteristics in the form of loss of solubility and 'communal aggregation' due to formation of aggregation of the hydrophobic proteins. This leads bonding between them so as to reduce the total exposed area to water.

With recent technological advancement in optoelectronics, light scattering measurements have turned as important tools for medical diagnostics[1–3]. Additionally, scattering measurements find huge applications in the field of quantum optics[4–9], nonlinear optical materials and photon localization[10–12] etc. Light scattering is caused by variation in physical property (like refractive index, density etc.) of the particles of the accompanying medium in which light travels and it depends strongly on the particle size. After scattering, the direction of the wave is changed but its frequency may or may not change depending upon nature of scattering, whether it is inelastic or elastic scattering. A limiting case of inelastic scattering may also exist where the frequency change upon scattering is small compared to the incident energy known as "quasi-elastic scattering (QELS)" regime. QELS is often used as a particle size characterization tool[13].

Coherent backscattering (CBS) of light is an interference effect which occurs due to superposition of the multiple scattered lights that have traced identical paths inside the sample but in reverse direction[14–16]. It leads to an enhanced scattered intensity in the region along the exact backscattered direction, forming a CBS cone. CBS has been used as a tool to monitor the food quality[17]. In our study, we have tried to explore the potential application of the CBS of light for detection of denaturation of milk.

We have presented results from CBS measurements of milk samples containing different amount of fat, commercially called 'Full cream milk' and 'Toned milk' containing milk with fat and without fat respectively. We have shown that the shape of the CBS cone starts changing as soon as the milk starts denaturing, which is nearly three hours prior to show any visible change through naked eye in terms of coagulation at room temperature. Further, the CBS cone line profile of milk is found sensitive to dilution with water. Here we have demonstrated a faster method to monitor the quality of the milk, opening up the possibility to check before consumption.

Results

To observe CBS from milk samples, a vertically polarized coherent beam was steered on it as shown in Fig. 1. The backscattered profile was collected through a lens L_1 (focal length ~25 cm) by a CCD camera. Measurement was

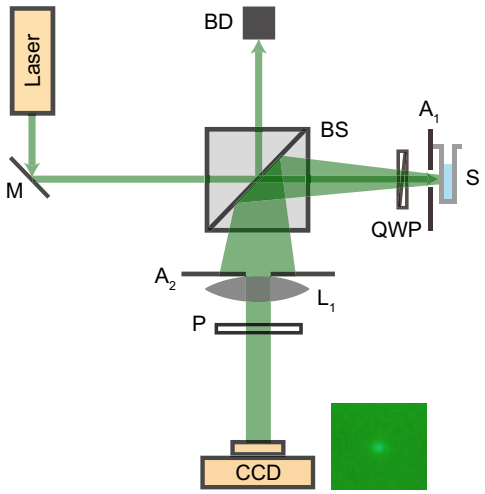

Figure 1 | Schematics of coherent backscattering (CBS) setup. A bright spot in the image (inset) shows typical intensity enhancement in the exact backscattered direction due to CBS. The contrast of the image is enhanced for better viewing. The abbreviations represents: M-mirror; BD-beam dump; QWP-quarter wave plate; P-polarizer; BS-beam splitter; A_1,A_2-variable circular aperture; L-converging lens; S-sample; CCD-Detector.

done at different intervals of time at room temperature. The variation in CBS cone line shape with time for full cream milk and toned milk are shown in Fig. 2(a) and 2(b) respectively. Experimentally observed cone profile was fitted to obtain the cone profile parameters, typically shown for initial Toned milk sample in Fig. 2(c). For Full-cream milk sample, CBS cones do not show any significant line shape change either in terms of peak width or, peak height with time (see supplementary Information, Table S2) till the milk begins to denature upto 13.5 hours. After 13.5 hours, the observed centrally bright spot (CBS cone) disappears and is replaced by speckle pattern, indicating onset of denaturation of milk. Through naked eyes, after 16 hours the coagulated milk protein granules were visible on careful look. After 20 hours, the denatured milk was easily distinguishable through naked eyes due to visible large size formed protein granules. CBS measurements can detect denaturation of milk at least 2.5 hours before any visible change is seen through naked eyes. For toned milk sample, the coherent cone disappears 5 hours later than the Full-cream milk sample. The disappearance of coherent bright spot at center and appearance of speckle pattern can be used as a technique to detect denaturation of milk.

The onset of denaturation of milk was confirmed through Raman Spectroscopy. Raman spectroscopy is sensitive to molecular atomic arrangements and any modification to it. Figure 3, shows Raman spectra of Full-cream milk samples collected at different time intervals 0, 13.5, 14, 15, 16 and 20 hours. Raman spectra collected for extended range from 100 to 3000 cm^{-1} is shown in three different parts for clarity in Fig. 3 (a), (b) and (c). During denaturation, the milk protein changes their native chemical structures. Structural changes are expected to show signatures in Raman spectroscopy. Figure 3(a) shows Raman spectra in the region 100 to 1000 cm^{-1}. To monitor the spectral parameter changes with time, it was deconvoluted as shown by dotted curve for initial (0 hours) sample. Lorentzian peaks were observed at 191.5, 263.3, 354.5, 881.6 and 925.2 cm^{-1} with an exponential background. The peak profile with increasing time is summarized in Table-S1 (Supplementary Information). The peak intensity and peak width of 191.0 cm^{-1} peak (CH_2-CH_2 torsional band) monotonically increases with time, while the peak shifts towards lower Raman shift with time. The broad Lorentzian peak observed at 263.6 cm^{-1} (CH_3-CH_2 torsional) shows monotonic decrease in peak intensity after 13.5 hours and systematically upshifts with denaturation time. The intensity of the peak

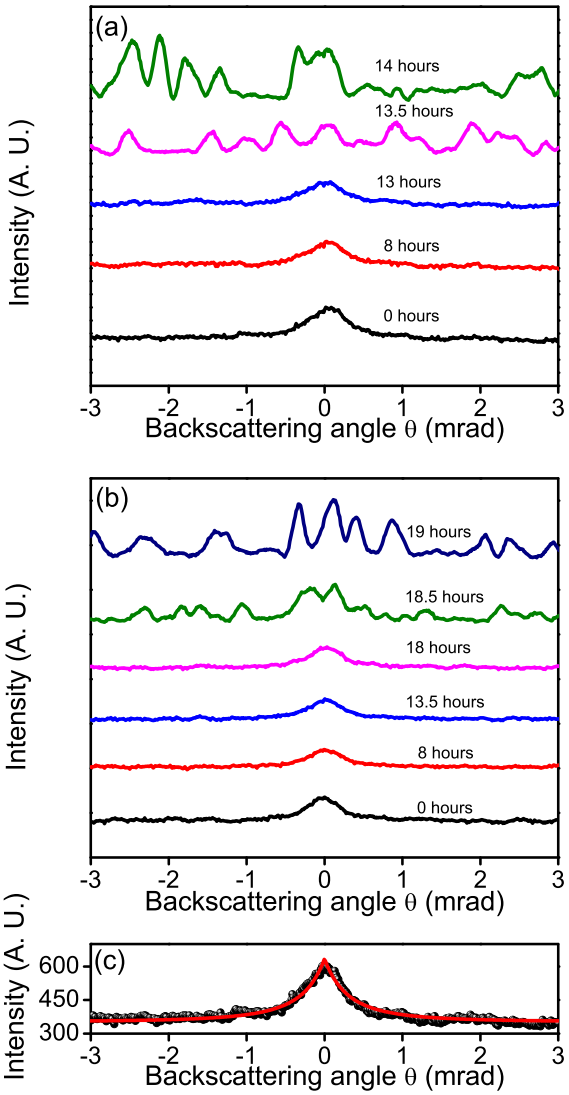

Figure 2 | Angular distribution of backscattered intensity at different spans of time (a) for Full-cream milk (b) for Toned milk. Time t=0 hours represents the beginning of the experiment. (c) shows theoretical fit to the experimentally observed CBS cone profile using Eq. (1) for Full cream milk at time t=0 hours.

observed at 881.6 cm^{-1} (C-C stretching) decreases with denaturation, while another weaker component observed at 925.2 cm^{-1} does not show any systematic change.

Similarly, Fig. 3(b) shows the Raman spectra in the range 1000 to 2250 cm^{-1} which contains most of the characteristic lines of milk fat and proteins[18]. The Raman peaks in the range 1000 to 2250 cm^{-1} were sitting over the Gaussian fluorescence background. To identify the peak position and change in spectral profile with denaturation time, Raman spectra in the range 1000 to 2250 cm^{-1} required four broad Gaussian peaks and three sharp Lorentzian peaks as shown by thinner black curve in Fig. 3(b). The fit to the initial sample experimental data (0 hour) is shown by thin curve, while the experimental curve is shown by thicker solid line. The variation in the spectral peak profiles in the Raman shift range 1000–2250 cm^{-1} with time are summarized in Table- SII (Supplementary information). The broad Gaussian peak was observed at 1148.6, 1331.1, 1439.1 and 1749.6 cm^{-1}. The characteristic Raman peaks (Lorentzian) were sitting over the broad Gaussian luminescence background at 891.8, 1445.3, 1660.1 and at 1749.6 cm^{-1}. The peak at 1445.3 cm^{-1} arises due to δ (C-H) scissoring of –CH2 and 1660.1 cm^{-1} peak is assigned

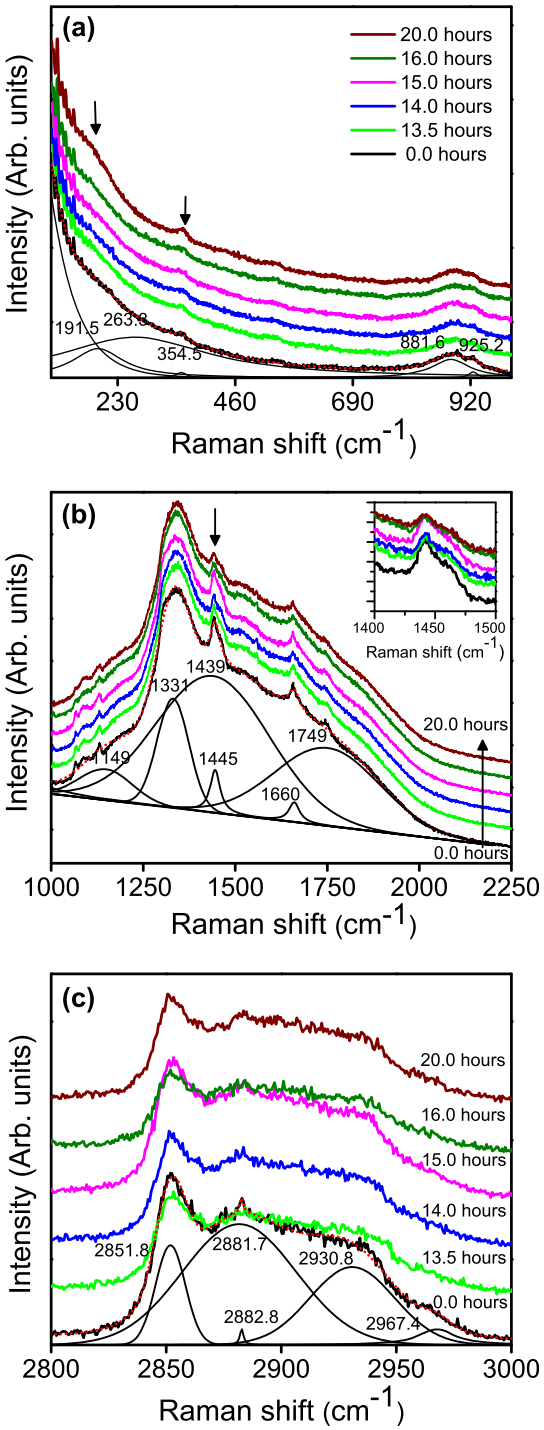

Figure 3 | Raman spectra of Toned milk samples collected at different intervals of time. Raman spectrum is shown in three different parts for clarity. (a) 100–1000 cm^{-1} (b) 1000–2250 cm^{-1} (c) 2850–2940 cm^{-1}. For t=0 hour samples, the fitting is shown by dotted red curve and deconvoluted peaks are shown by thin lines (black).

to ν (C=C) cis double bond stretching of RHC = CHR[18]. A relatively weak peak not resolved in fitting ~ 1747 cm^{-1} ν (C=O) stretching of RC=OOR is observed sitting over the broad fluorescence background. The intensity of sharp peak at 1445.3 cm^{-1} monotonically decreases with increasing time i.e with denaturation of the milk indicating modification of C-H bonds with time. The C=C peak (1660.1 cm^{-1}) upshifts by 4 cm^{-1} upon denaturation due to compressive strained C=C bond with time. The peak width of this peak monotonically increases with increasing time, i.e with denaturation. These spectral changes indicates that after 13.5 hours the denaturation of milk is onset with modification of C-H bonds and strained C= C structures of proteins. Additionally, the broad Gaussian peak observed at 1439 cm^{-1} and 1749 cm^{-1} shows upshift upon denaturation. The width and amplitude of these broad Gaussian components observed at 1439.1 and 1749 cm^{-1} decrease with time. Reduction in the peak intensity of C-H bond and simultaneous increase in the intensity of C=C peak, indicates that with time the milk proteins are denatured with significant increase in the double bond structure and decrease in C-H bonds. The decreased C-H bonds indicate formation of aggregation of hydrophobic proteins leading to formation of aggregation on denaturation. These spectral changes confirm that with time structural modification of milk proteins leads to disappearance of coherent backscattered signal.

Fig. 3(c) shows the change in Raman spectra of milk in the high wave number range (2850–2940 cm^{-1}). Peaks in this range observed at 2850 and 2940 cm^{-1} are characteristic of the symmetric and asymmetric ν (C-H) stretching vibrations of the CH_2 and CH_3 groups. The peaks were Gaussian in nature in this spectral range (Spectral profile summary Table-SIII, Supplementary Information). The deconvoluted spectra consist of peaks at 2851.8, 2881.7, 2930.8 and 2967.5 cm^{-1} with peak width 15.2, 58.2, 43.3 and 18.9 cm^{-1} respectively. All the peaks in this spectral range show decrease in intensity with increasing time. Decrease in the peak intensity with time shows modification of C-H bonds in milk proteins and fats with time. Such changes lead to coagulation, detected by CBS measurements.

Further, we monitored the effect of dilution on CBS profile for both samples. Fig. 4 shows the changes in CBS cone profile with increasing dilution % (i.e. water volume fraction) of Full-cream milk with water. The CBS cone width (w) and its enhancement factor (F) changes monotonically with increasing dilution % as shown in Fig. 5. Along with the changes in coherent peak, the incoherent backscattered background decreases with increasing dilution %. The experimental cone profile was fitted to estimate the transport mean free path (l^*) and enhancement factor (F). Fig. 5(a) and (b) shows change in l^* *and* F *with* increasing dilution % for Full-cream milk and Toned milk respectively. For both samples, l^* increases linearly with increasing dilution % following $l^* = l_0 + m^* dil\%$, while the F decreases exponentially mathematically expressed as $F = a - b*\exp\left(\frac{dil.\%}{\tau}\right)$. Water added to milk for dilution does not give any CBS contribution. The wide angle background goes down on dilution since the number of scattering centers decreases upon dilution as can be seen from Fig. 4. If the scattering centers are less, there will be less

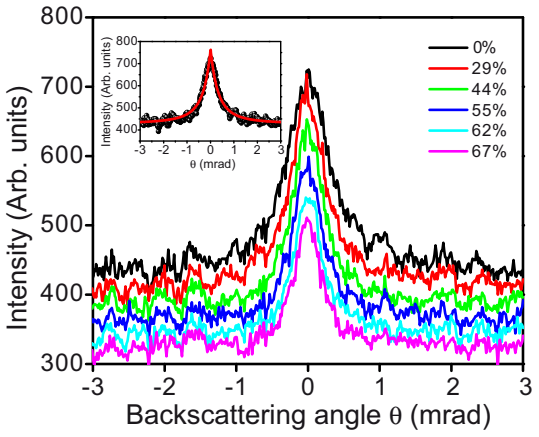

Figure 4 | Shows angular distribution of backscattered intensity from Full-cream milk sample with increasing dilution with water. Curve in the inset show the backscattered intensity distribution for 0% dilution and the fitted curve (red).

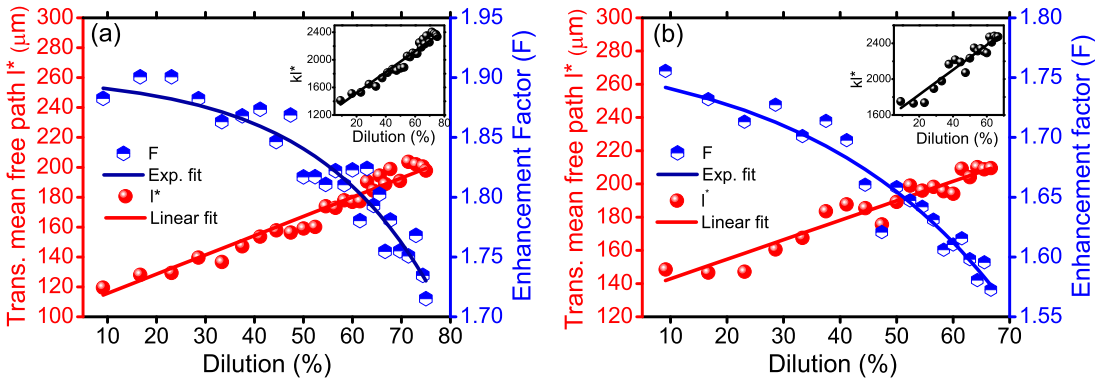

Figure 5 | (a) and (b) Shows variation of transport mean free path *l** and enhancement factor *F* for Full-cream milk and Toned milk samples respectively on dilution with water. Inset shows increase in *kl** with dilution (*kl**≫1 shows weak localization).

scattering in the backscattering direction too. The enhancement factor (F) decrease with high dilution due to increasing refractive index mismatch between wall of cuvette and diluted sample.

For Full-cream milk, $l_0 = 102.8$, $m = 1.287$, $a = 1.903$, $b = 0.008152$ and $\tau = 24.55$. For Toned milk $l_0 = 131.2$, $m = 1.173$, $a = 1.780$, $b = 0.029310$ and $\tau = 34.39$. This shows that the scattered cone profile depends on the fat content of the milk. The inset of Fig. 5(a) and (b) shows variation in *kl* with changing dilution %, where k is the propagation vector*. The product *kl** represents strength of scattering within the sample. The value of $kl^* < 1$ indicates very strong scattering while $kl^* > 1$ indicates weak scattering[19]. The insets in Fig. 5(a) and (b) shows the variation of *kl** (i.e. the amount of scattering) with dilution. The value of $kl^* \gg 1$ in our experiments indicates milk as very weak scattering medium.

Discussion

Natural milk contains various types of proteins and fat globules molecules which act as scattering sites for incident lights. In milk samples, we get centrally bright coherent spot in backscattered direction from constructive interference of multiple scattered lights which has traced identical paths inside the sample but in reverse direction. Milk being a homogeneously dispersed stable colloidal suspension of proteins chains and fat globules, acts as a medium for CBS of light. The width of the backscattered cone represents distribution of path length of the backscattered light before interference. The CBS peak disappears upon denaturation due to changed molecular length of proteins and modification of its structural conformations leading to coagulation. Coagulation due to hydrophobic nature of denatured proteins results into increased size of protein globules leading to seizure of Brownian motion giving rise to a speckle pattern in the back scattered direction and CBS peak disappears. A closer look to the CBS cone profile indicates rounding of cusp due to optical absorption of scattered light within sample. Absorption of scattered light inside sample limits the path length of incident beam within it, resulting into rounded cusp due to lesser contribution of longer scattering path lengths[16].

The disappearance of CBS peak and appearance of speckle pattern works as perfect indicator of denaturation of milk proteins. Here, we have shown disappearance of CBS peak on denaturation for two type of milks and it would be equally applicable to all forms of milks, the pasteurized milk and unpasteurized milk both. Further, the technique of CBS can be used to directly to scan the packed milk since the transparent polyethylene does not affect the CBS cone produced by milk. Since the CBS cone profile depends on material properties like particle size, density and dielectric constant of the medium, it was found to vary with the dilution %. Due to the differences in the material properties of the initial samples, the estimated parameters *l** and *F* were different. The nature of change of these two parameters were similar for both the samples (*l** linear and *F* exponential). For a calibrated standard milk sample, dilution % of other sample of similar milk can be estimated.

Further, milk samples of different ages were subjected to Raman spectroscopy. Raman spectroscopy is uniquely ultrasensitive to molecular changes and is found sensitive to modification in the atomic arrangements. Raman studies shows modification of C-H bonds and strained C=C upon denaturation. Denaturation of milk is lead through increase of double bonded carbon structures and decrease of C-H bonded structures. Decreasing concentration of C-H bonded structures leads to hydrophobicity causing coagulation of milk proteins. Coagulated protein structures leads to seized Brownian motion causing appearance of speckle pattern and disappearance of backscattered cone. Although, Raman spectroscopy provides quantitative estimation of molecular changes, but it requires sophisticated instrumentation, making it difficult to be used as handy monitoring tool. While, CBS is a very handy, low cost and faster technique (since no scans are required). Using CBS a clear distinction can be made between good milk and denatured milk in terms of disappearance of CBS peak. While, it is difficult to conclude the onset of denaturation of milk from Raman spectroscopy measurements due to absence of any prescribed parameters of denaturation in terms of extent of molecular structural changes. Additionally, use of CBS for detection of denaturation do not require any preknowledge of spectral positions and post measurement data analysis for information extraction.

To conclude, the Coherent backscattering (CBS) cone profile disappears sensitively with changes in the molecular structure of milk proteins upon onset of denaturation process. The CBS measurement senses denaturation of milk couple of hours prior to any visible change observed through naked eyes. Our experiment establishes CBS as a new tool capable of early ultrasensitive detection for spoilage of milk. Additionally, the transport mean free path (*l**) and the enhancement factor (*F*) varies monotonically upon dilution of milk with water, acting as a potential laser based lactometer. This establishes CBS measurements as an inline monitoring technique for various stages of processing and consumption of milk. The method is non-destructive, cost-effective and faster, making it superior to the other existing techniques for milk quality monitoring and assessment. CBS equipment can be realized in the form of a compact tool like a laser barcode-reader. The technique of CBS can be further explored to analyze and detect different analyte and external contaminants in biological systems.

Methods

Experimental details. The vertically polarized pulsed laser beam of 532 nm (Expla PL2210) with Rep. rate ~1 KHz, beam diameter~3 mm and with beam divergence ~1.6 mrad was used for experiment. The scattered field, was collected through a lens L_1 (focal length~25 cm) using CCD camera (micropublisher 5.0 RTV, pixel size ~3.4

micron). The incident beam was split into two parts by a 50-50 non-polarizing beam splitter BS. One part of the beam was made circularly polarized by a quarter wave plate QWP and was steered on the sample S contained in a quartz cuvette. The other part of the beam was disposed in a beam dump BD. The cuvette and CCD camera were placed at the back and front focal planes of the lens L_1 respectively. The cuvette was placed sufficiently closer to the beam splitter to ensure that collected backscattered cone on CCD is not limited by finite size of beam splitter. Size of aperture A_1 was just greater than the beam diameter. The QWP and a polarizer P were used to collect scattered field in the helicity conserving channel to reject any contribution from single scatterings as it did not contribute to the intensity enhancement in the back scattering direction[16,20]. Aperture A_2 was placed before lens L_1 to limit the field of view of the scattered field. Antireflection coated optics was used in the experiment. To avoid any direct reflection from the quartz surface into the CCD camera the cuvette was tilted by a small angle. Raman spectra of the milk samples were recorded with 785 nm excitation from diode laser and a single monochromator micro-Raman spectrometer with 5× objective using commercial system (Renishaw inVia Raman Microscope).

Estimation of CBS parameters. The angular distribution of the backscattered light from milk samples is mathematically[21–23] expressed as

$$\gamma_0 = \frac{1}{(1+2z_0)} \times \frac{1}{\{(\alpha+v)^2+u^2\}} \times \left\{\frac{1}{v} + \frac{(1-e^{-2\alpha z_0})}{\alpha}\right\} \quad (1)$$

where, $u = kl^*(1-\cos\theta)$, $v = (1 + \sec\theta)/2$ and $\alpha = kl^*|\sin\theta|$. The FWHM of the CBS cone is given by $\sim 0.7/kl^*$ and z_0 is the location of the trapping plane used in diffusion model. The backscattering cone profile parameters F, l* were estimated by fitting the experimental data with above equation.

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Author contributions

D.K.S. had the original idea. M.V. designed the experiment. M.V. and D.K.S. performed experiment and analyzed the data. M.V., D.K.S., P.S., J.J. and H.C.K. wrote the paper. All the authors discussed the results and commented on the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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