FULL PAPER

Novel All-Natural Microcapsules from Gelatin and Shellac for Biorelated Applications

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The generation of novel all-natural biopolymeric microcapsules fabricated using natural biopolymers, protein (gelatin) and resin (shellac), is reported. These novel microcapsules are generated using a simple extrusion method wherein the gelatin-shellac mixture is dropped in an acidic medium resulting in an instantaneous solidification of aqueous drops into solid spherical microcapsules that retain their shape on air-drying. The formation of the microcapsules is basically due to the strong interactions between two oppositely charged polymers (as confirmed from isothermal titration calorimetry and infrared spectroscopy) and the instant precipitation of acid-resistant shellac. These novel microcapsules prepared without the help of any cross-linkers or harsh solvent are extensively characterized and several biorelated applications for pharmaceuticals (encapsulation and release of bioactive molecules), foods (loading of colorants and flavors), sensors (encapsulation of pH sensitive dye), and biotechnology (enzyme immobilization) fields are further demonstrated.

1. Introduction

Novel colloidal structures such as microcapsules generated from natural materials hold great potential for applications in biorelated fields including pharmaceuticals (encapsulation of drugs), diagnostics (bio imaging and sensors), foods (stabilization and delivery of functional ingredients such as flavors and probiotics) and biotechnology (cell and enzyme immobilization)[1–3] Microcapsules represent a class of colloidal structures ranging in diameters from few micrometers to millimeters.[4] Though the attributes for ideal microcapsules vary significantly with the type of desired application, at least all or most of the following requirements should be met when generated for biorelated applications: a) use of biocompatible and biodegradable materials; b) preferably avoiding the use of harsh organic solvents and chemical cross linkers; c) use of edible and GRAS (generally recognized as safe) approved materials (especially for food and nutraceutical applications); d) desired strength to withstand various mechanical insults during processing; and last but not the least e) use of cheap, environmentally safe and sustainable raw materials for future commercial prospects.[4,5]

Gelatin (protein) and shellac (resin) are two GRAS approved, edible and natural materials used widely in pharmaceutical and food industries. Specifically in foods, gelatin (E 441) and shellac (E 904) are primarily used as gelling and glazing agents respectively.[6,7] Due to the biodegradable nature of gelatin, it has been used as preferred material to generate microcapsules for bio-applications related to microencapsulation and delivery of drugs.[8–10] However, gelatin is hydrophilic and hence, cross linking of gelatin is essential to modulate the general characteristics of microcapsules such as the strength and rigidity. Next to the technological complications, the safety issues of biomedical applications have been a major concern in recent years and accordingly increased efforts are being made to reduce or eliminate the use of chemical cross linkers such as formaldehyde.[11,12]

Shellac is a natural, biodegradable resin from insect origin (Kerria lacca). It is a complex mixture of polar and non-polar components consisting of polyhydroxy polycarboxylic esters, lactones and anhydrides with the main acid components being aleuritic and terpenic acids.[13,14] Shellac, like other polymers with carboxylic groups, is practically insoluble in acidic to neutral aqueous medium at pH < 7.[15–17] In the present work, the acid resistant nature of shellac was exploited to identify a novel approach for fabricating all-natural microcapsules by simple precipitation of associated gelatin-shellac mixtures in acidic pH resulting in an instantaneous formation of rigid, solid microcapsules that retained their spherical shape on air drying. A range of characterization techniques were employed for the morphological evaluation (optical and electron microscopy), mechanical strength testing (fracture force), spectral and calorimetric analysis (Infra-red spectroscopy, differential scanning calorimetry and isothermal titration calorimetry). Further, to demonstrate versatile applications of these novel microcapsules, we successfully evaluated a range of biorelated applications including the

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loading and release of bioactives (silibinin and epigallocatechin gallate), encapsulation and temperature dependent release of flavor (d-Limonene), loading of food-grade colors (curcumin, indigocarmine and purpurin) and pH sensitive dye (alizarin) and enzyme (α-amylase) immobilization.

2. Results and Discussion

2.1. Fabrication and Characterization of Microcapsules

Shellac is known to interact with hydrophilic polymers such as xanthan gum, pectin and cellulose derivatives[18,19] based on non-covalent interactions which is attributed to the main component of shellac (hydroxy aliphatic fatty acid-aleuritic acid). Owing to the presence of a large number of carboxylic and hydroxyl groups and a strong negative charge, aleuritic acid participates in hydrogen bonding and electrostatic interactions.[20] On the other hand, gelatin behaves like an amphoteric electrolyte in solution (carrying a net positive charge below its isoeletric pH) and is also known to interact via non-covalent interactions with other hydrocolloids including alginate, gellan, carrageenan and konjac glucomannan.[21–23] Gelatin, type A has a IEP of 8–9 and accordingly in the pH range studied here, gelatin had a positive charge (ζ-potential = 3.7 mV) whereas, shellac had a net negative charge (ζ-potential = –32.7 mV). The ζ-potential values of gelatin:shellac (GL:SL) mixtures (1:3 to 1:6 wt/wt) were in the range of –7.6 to –12.1 mV indicating the neutralization of charge on shellac due to the electrostatic interactions between gelatin and shellac. The existence of molecular interactions between gelatin and shellac was also recently reported.[15,24] We used isothermal titration calorimetry (ITC) to further obtain interaction parameters between these two molecules. **Figure 1**A shows the raw data plot of heat flow against time for the titration of 0.1 mM shellac into 2.7 μM gelatin at 20 °C. As seen from the figure, the interaction of shellac with gelatin was exothermic (i.e., heat is released) suggesting a strong binding of shellac with gelatin. The corresponding plot of the molar enthalpy change (ΔH) against the molar ratio of shellac:gelatin (SL:GL) is presented as Figure 1B. From the plot, the stoichiometry (n) of the binding was calculated to be 2.06 ± 0.03 suggesting that approximately two molecules of shellac were bound to one molecule of gelatin. The molecular interactions between two molecules were quite strong with a binding constant (K_a = 5.98 × 10^8 M^{-1}) in the range of high affinity binding.[25–28] The endothermic binding enthalpy (ΔH = –110.37 ± 4.14 kJ mol^{-1}) along with the value of binding entropy (ΔS = –414.03 J K^{-1} mol^{-1}) suggests that the interaction is entropy driven as is the case with inter-polyelectrolyte electrostatic interactions.[29,30] The binding enthalpy (ΔH) can be correlated to the combined effect of non-covalent intreactions (hydrogen bonding, electrostatic and hydrophobic interaction) and conformations changes,[25–28,31,32] which can be caused by electrostatic interactions and hydrogen bonds between amino acid groups with charged carboxyl or hyroxyl groups or hydrophobic interaction of amino acid groups in gelatin with non-polar groups of shellac. The molecular interactions of shellac with gelatin resulted in the formation of a gel-like structure which precipitated in acidic medium. This phenomenon was exploited to generate novel microcapsules by simply extruding this mixture (using a syringe and needle) into water acidified with 0.1 N HCl (pH 1.0). Usually, an extrusion method is commonly employed to generate microcapsules via surface cross linking which results in the formation of capsules with a shell and a hollow core. However, there is no cross linking involved in case of the capsules described in our manuscript. The mechanism of capsule formation is based completely on the acid-resistant characteristics of shellac. Solid microcapsules were formed immediately due to the solidification induced by the precipitation of shellac-gelatin combination at acidic pH. The formation of microcapsules with a spherical shape and a desired rigidity was dependent on the amount of gelatin and shellac used in the preparation. Concentrations lower than 2 wt% of gelatin resulted in a complete loss of structure leading to the formation of aggregated lumps whereas, at concentration higher than 2 wt%, addition of shellac resulted in the formation of a rigid gel which was difficult to extrude through the needle. Hence, microcapsules were prepared by keeping the gelatin concentration constant at 2 wt% and the concentration of shellac was varied from 6 to 12 wt% to obtain microcapsules at varying GL:SL ratios (i.e., 1:3, 1:4, 1:5 and 1:6 wt/wt). The resulting spherical microcapsules solidified immediately

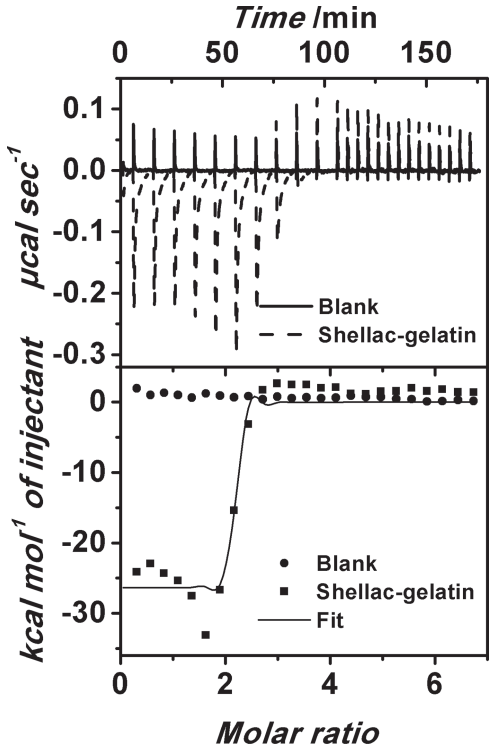

Figure 1. A) Raw data plot of heat flow against time for titration of (0.1 mM) shellac into (2.7 μM) gelatin at 20 °C. Each peak indicates the heat change associated with injection of (10 μL) aliquots of shellac solution into calorimeter cell containing the gelatin solution. A negative heat flow represents exothermic change and a positive peak represents endothermic change. B) Corresponding plot after integration of peak areas and normalization with respect to injectant concentration to yield a plot of molar enthalpy change (ΔH) against molar ratio of shellac:gelatin.

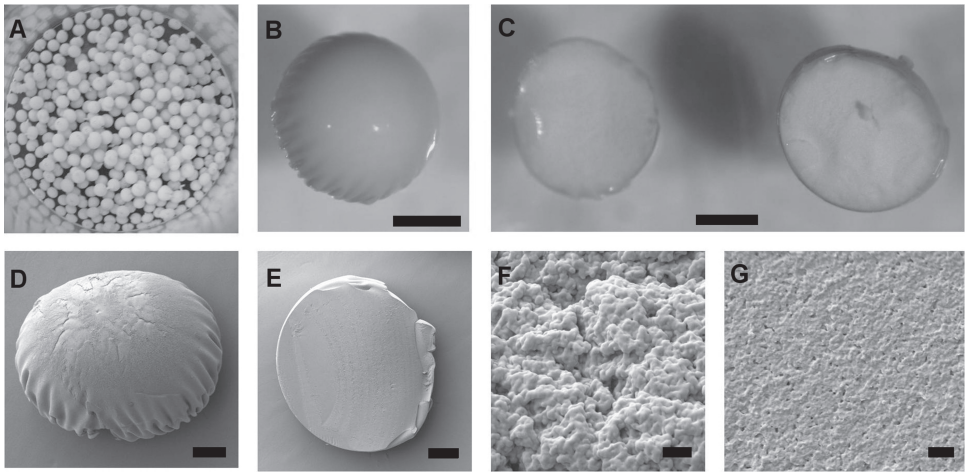

Figure 2. A) Photograph of microcapsules after minutes of formation. B,C) Optical microcopy images of an intact and cross-sectioned microcapsules (scale bars = 500 μm). D,E) SEM images of intact and cross sectioned microcapsule (scale bars = 100 μm). F,G) Magnification of the surface and internal structure respectively (scale bars = 200 nm).

which were then filtered and subjected to air drying at room temperature (≈20 °C). The average diameter of the microcapsules ranged from 800 to 1200 μm. **Figure 2** shows the photographs and images (from optical microscopy and scanning electron microscopy (SEM)) of the microcapsules generated using GL:SL, 1:6 wt/wt. As seen from the cross-sectioned images, the microcapsule had a homogeneously dense internal structure which was smooth in appearance. Though, under magnification, the surface appeared to be rough probably due to the shrinkage on air drying. The dried microcapsules were also tested for mechanical strength using a modification of 'squeezing capsule' technique previously described for microcapsules with a core-shell structure.[16] Since, in this case, the microcapsules were solid, the fracture force (where the microcapsules ruptured) can be easily obtained from the compression curves of the normal force (mN) plotted against the dimensionless displacement (distance relative to the first point of contact between probe and sample) (**Figure 3**). The fracture force which can be correlated to the hardness of the microcapsules

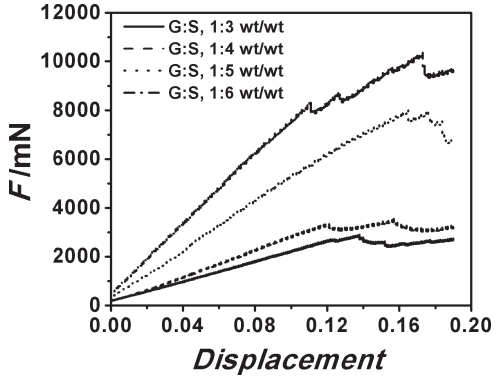

Figure 3. Force-displacement curves for microcapsules prepared at varying gelatin-shellac proportions. The reported values represent mean of five measurements, the standard deviation was less than 5% in all cases. (gelatin:shellac = G:S).

showed a strong dependence on the shellac concentrations as expected. The average values of fracture force (in mN) for various GL:SL ratios were as follows: 2707.6 ± 231.6 for 1:3 wt/wt; 3339.2 ± 304.5 for 1:4 wt/wt; 7982.6 ± 756.2 for 1:5 wt/wt and 8301.8 ± 823.7 for 1:6 wt/wt. The hardness values indicate that the microcapsules were quite rigid and hence can be considered as robust to withstand all the mechanical insults encountered during processing yet soft enough to be easily ruptured during mastication when used for oral applications.

A temperature scan of gelatin typically shows two basic transitions (i.e., glass transition, T_g and melting of crystallines, T_m), which are extremely close to each other and due to the hygroscopic nature of gelatin, water content strongly affects both T_g and T_m (e.g., the T_g of gelatin can vary from 217 °C for dry to 50 °C based on the water content).[33] The DSC thermogram of gelatin (**Figure 4**A) showed endothermic peaks at 71.87 and 82.67 °C corresponding to the T_g and T_m. The thermogram acquired on the second heating cycle of the same sample (graph not shown) revealed only one peak namely the glass transition of the gelatin matrix (T_g), indicating that the gelatin oligomers were completely amorphous after the DSC cooling cycle which is in agreement with recently published report.[34] The T_g of shellac in its acid form ranges from 37 to 49 °C whereas the ammonical salt of shellac shows slightly higher T_g values.[35,36] Figure 4A shows the thermogram of shellac with a prominent endothermic peak at 57.27 °C corresponsing to its T_g. The microcapsules on the other hand showed an endothermic peak at 60.25 °C thus indicating a shift of characteristic T_g values for both gelatin and shellac. The shift in the T_g value and complete absence of the melting of crystallines (T_m) related to gelatin suggests the interaction of shellac with gelatin and the amorphous nature of GL:SL composite in the formed microcapsules. The interactions were further characterized using FT-IR (Figure 4B). FT-IR has been frequently used to investigate the interpolymer complexes of proteins with other biopolymers such as chitosan,[37,38] alginate and pectins.[39] The characteristic absorption bands of gelatin in the IR spectra are situated in the amide band region; (3283 cm^{-1}) amide A representing vibration

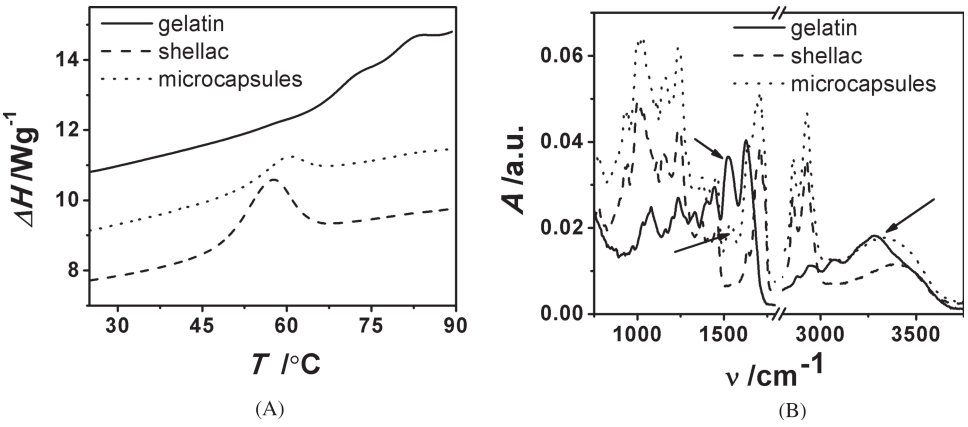

Figure 4. A) DSC heat flow graphs and B) FT-IR spectra for gelatin, shellac and microcapsules prepared using GL:SL ratio of 1:6 wt/wt.

of N-H groups, (1629 cm^{-1}) amide-I related to the C=O stretching/hydrogen bonding coupled with COO, (1527 cm^{-1}) amide-II representing the bending vibration of N-H groups and stretching vibrations of C-N groups and (1236 cm^{-1}) amide-III which is related to the vibrations in plane of C-N and N-H groups of bound amide.[40,41] In case of shellac, two prominent peaks appear at 1710 and 2925 cm^{-1} corresponding to the carbonyl bands and C-H strecthings and a weak peak related to the vinyl stretches and bends is seen at 1635 cm^{-1}.[36,42] On comparing the spectra of the microcapsules with the spectra of gelatin and shellac, it was found that the amide A and amide II band of gelatin were significantly affected indicating the involvement of the amide group in the interaction with shellac. The presence of a new peak at 1553 cm^{-1} in the spectra of the microcapsule further indicates an interaction of carboxylate group of shellac with a protonated amino group of gelatin. The interpretation is in line with earlier report on shellac-Eudragit E interactions.[36] The interpretation also supports the results obtained from ITC study as described previously.

2.2. Bio-Related Applications

2.2.1. Encapsulation and Release of Water Soluble and Water Insoluble Bioactives

Microencapsulation is a rapidly expanding technology especially for the stabilization and delivery of problematic drugs and bioactives for food and nutraceutical applications.[43,44] Accordingly, a lot of effort has been made to identify natural and edible polymers (such as vegetable proteins) which could serve as the shell materials for oral applications.[45]

To evaluate the encapsulation applications of novel GL:SL microcapsules, water soluble (epigallocatechin gallate, EGCG) and water insoluble (silibinin) polyphenols were loaded in the microcapsules followed by studying their release using in vitro release models simulating the gastric and intestinal digestion process. Epigallocatechin and silibinin were selected as the model bioactives for encapsulation because both of them have stability issues when delivered orally, resulting in a low bioavailability due to their degradation in the gastrointestinal tract and encapsulation has been proposed as an effective stratergy to overcome their bioavailability issues.[5,18,46–49] The bioactives were loaded at 10% solid weight of microcapsules prepared at lowest and highest shellac concentration (GL:SL, 1:3 and 1:6 wt/wt respectively). Due to the acid-resistant nature of shellac, it has been utilized as a popular choice for development of enteric release formulations[50] for delivery of bioactives which are degraded in the acidic conditions of the stomach and hence needs to be protected before they are released in the intestine.[51] The release patterns of EGCG and silibinin from microcapsules in gastric and instestinal phases are shown in **Figure 5**A,B respectively. As seen from the graphs, the release of encapsulated polyphenols (EGCG and silibinin) was higher in the intestinal phase as compared to the gastric phase probably due to the pH dependent solubility of shellac. In the case of silibinin, the sudden jump in the release pattern observed on changing the phases can also be explained from the fact that silibinin is more soluble at alkaline pH as compared to an acidic pH.[52,53] As seen from Figure 5, the release characteristics of encapsulated polyphenols were dependent on the concentration of shellac used in the preparation of microcapsules and thus, the release rates of enapsulated bioactives can be modulated by altering the concentrations of shellac.

2.2.2. Loading of Colorants and Flavors

Microencapsulation has found numerous applications in the food industry for coating colorants, flavors and other sensitive functional food ingredients, in an effort to increase their storage stability and also for the development of food products with enhanced aesthetic appeal.[54,55] The loading of food-grade colorants and especially the natural colorants in an aqueous based process is very challenging because of their inherent water insolubility. In the current work, a range of food grade colorants (natural and nature-identical) were encapsulated in the microcapsules resulting in the formation of visually appealing microcapsules with bright yellow, orange, blue and green colors. (**Figure 6**) The insoluble colorants (curcumin, and purpurin) were loaded by first dissolving them in a minute quantity of 0.1 N NaOH followed by the addition to the gelatin-shellac mixture and subsequent extrusion thereafter. Due to their low solubility at acidic pH, encapsulation was very effi-

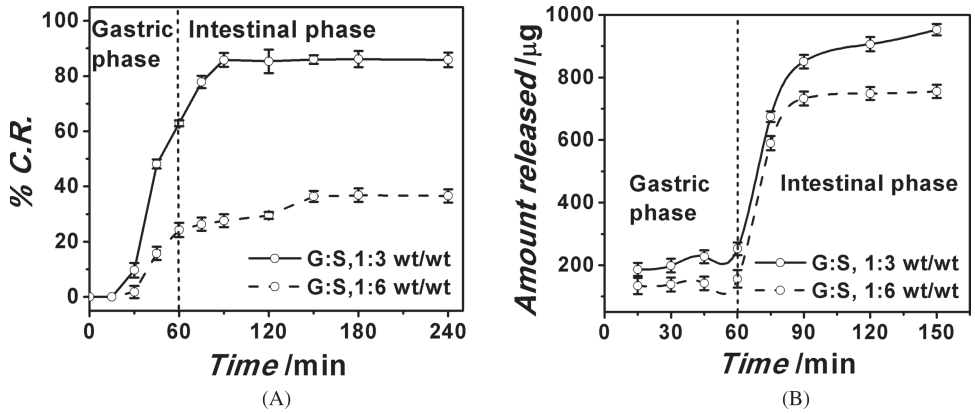

Figure 5. A) Percent cumulative release of EGCG and B) amount of silibinin released during gastric and intestinal phases of in vitro digestion. (G:S = gelatin:shellac).

cient with no leakage of colors during the formation of the microcapsules. On drying, the microcapsules retained their attractive hues and thus, these novel all-natural microcapsules can find important applications in the fields of foods and nutraceuticals to improve the visual appeal of the products.

In the last decade or so, the encapsulation of colorant/dyes (the ones which are sensitive to pH changes) in microcapsules have been utilized as an approach to develop microencapsulation based sensors.[56–58] In an attempt to show sensor application of the novel microcapsules described in this work, we encapsulated a pH sensitive dye-alizarin (1,2-dihydroxyanthraquinone). Alizarin, like other dyes from the group of hydroxyanthraquinones, displays a pH dependent color change and is accordingly used as an acid-base indicator.[59,60] The pH change property of alizarin was maintained after encapsulation, the color of the microcapsules could be changed reversibly between yellow and blue by simply altering the pH (**Figure 7**). This color changing property of our microcapsules could potentially be used for novel sensor applications such as visual indicators of shelf lives (via pH changes) in products which are susceptible to microbial degradation.

Flavors or aromatic oils represent a very important and valuable category of ingredients used in a range of foods, pharmaceuticals and home and personal care products. Due to the volatile and sensitive nature of flavors, microencapsulation has long been used as a preferred approach to protect and preserve them on one hand and to enable easier handling and controlled delivery on the other.[61,62] A flavor encapsulation application of

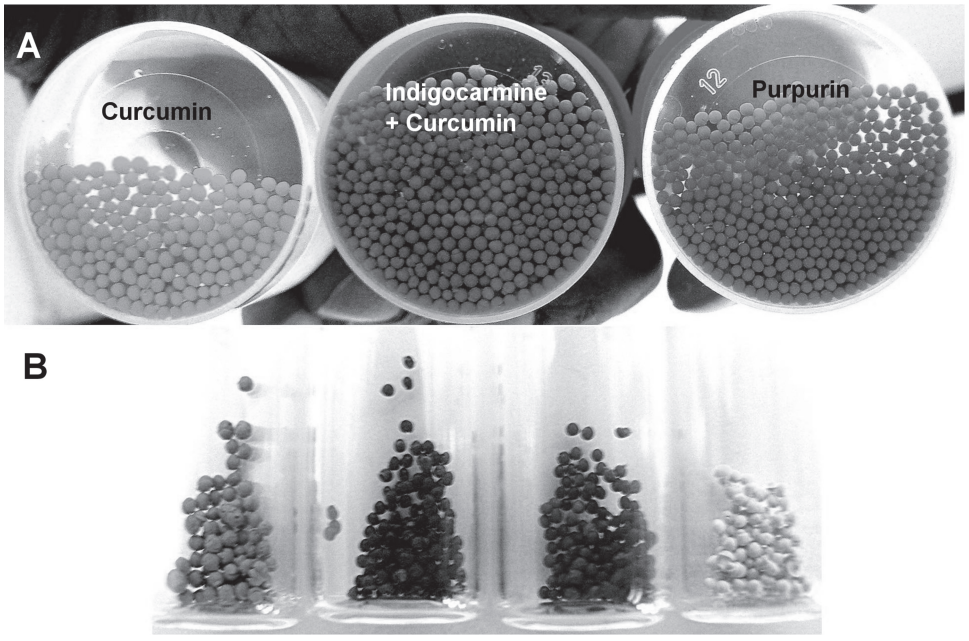

Figure 6. A) Photograph of brightly colored microcapsules (containing curcumin, mixture of indigocarmine + curcumin and purpurin) minutes after they are prepared by extrusion. B) Photograph of dried microcapsules, starting from left: with indigocarmine, indigocarmine+curcumin, purpurin and with no colorants. Color images are provided as Figure S1 (Supporting Information).

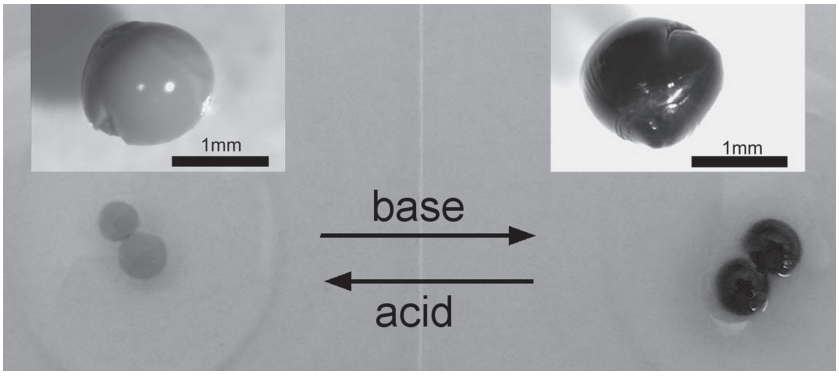

Figure 7. The photograph showing the acid-base color changes of alizarin loaded microcapsules between yellow and blue. Insets: Colored images of microcapsule from Leica Macroscope. Color images are provided as Figure S2 (Supporting Information).

our novel microcapsules was evaluated by loading d-Limonene and studying the release using hot-stage microscopy as described in our earlier work.[5] **Figure 8** shows the microscopy images of a single microcapsule (loaded with d-Limonene) captured at a range of increasing temperatures. As seen from the figure, the encapsulated flavor starts to ooze out at 60 °C with the rupture of the microcapsule structure at 80 °C. This temperature dependent release of flavor can find important applications in the development of food products (such as flavored teas and frozen foods for cooking) and non-food products such as scented detergents and medicated vaporisers.

2.2.3. Enzyme Immobilization

Enzyme immobilization has been continuously attracting a lot of interest from fields such as chemistry, biomedical, biotechnology and diagnostics.[63] The ease of separation from the reaction mixture and the possibility of re-use, gives immobilized enzymes distinct technical and economical advantages over soluble enzymes. Easy separation of the enzyme from the product after the reaction is complete, simplifies enzyme applications and the re-use of enzymes provides further cost advantages.[63] Among the numerous methods that can be used to carry out enzyme immobilization, microencapsulation through physical entrapment is considered advantageous especially because there is no chemical alteration of the enzyme during the immobilization resulting in a relatively lower deterioration of enzyme activity.[64]

An enzyme immobilization application of our GL:SL microcapsules was evaluated by loading α-amylase and studying the operational stability of the encapsulated enzyme. The loading of the enzyme was rather difficult due to its limited solubility in gelatin-shellac mixture which resulted in the formation of irregularly shaped microcapsules. To tackle this issue, the enzyme was first dispersed in glycerol before adding it to a GL:SL mixture followed by extrusion in the acid to generate α-amylase loaded spherical microcapsules. After separation of the formed microcapsules, the aqueous acidic solution and washing water did not show any enzyme activity indicating complete encapsulation and a theoretical load of 365 U enzyme per 50 mg of microcapsules. Microencapsulation generally leads to a loss of enzyme activity probably due to the confinement of some enzymes in polymer matrix of microcapsules making them in-assessable for the substrate interactions [5;63;64]. The loaded enzyme showed reasonable enzyme activity (≈50% of the load) but the operational stability was excellent (**Figure 9**). The

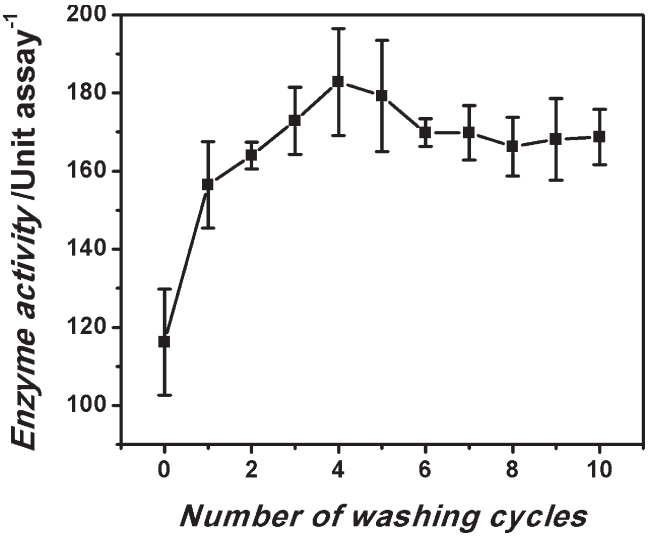

Figure 9. Enzyme activity reported as unit per assay over 10 cycles of washing for α-amylase immobilized in the microcapsules (prepared at GL:SL, 1:6 wt/wt).

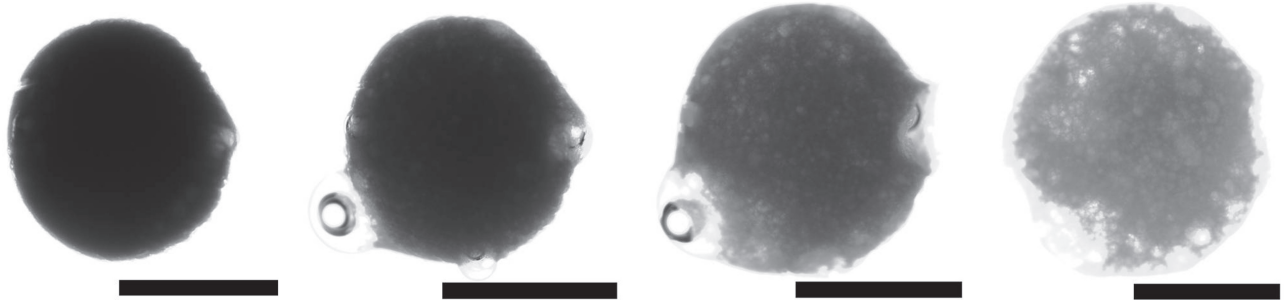

Figure 8. Microscopic images showing temperature triggered release of encapsulated aromatic oil with respect to the temperature (scale bars = 400 µm). Microcapsules were prepared at GL:SL, 1:6 wt/wt with 10 wt% d-Limonene. Temperature from left to right: 20, 60, 70 and 80 °C.

enzyme activity showed a gradual increase over first 4 cycles of washing with highest activity reaching up to 182.8 ± 13.7 U per assay followed by a constant enzyme activity over the last 5 cycles. The operational stability over 10 cycles of washing indicates that the encapsulated enzyme can be re-used with excellent retention of enzyme activity and thus, novel gelatin-shellac microcapsules qualify as a good candidate for enzyme immobilization.

3. Conclusion

In conclusion, we have successfully demonstrated the generation of novel microcapsules from two natural biopolymers-gelatin and shellac, both of which are FDA approved and edible. The all-natural microcapsules were generated using a simple extrusion method wherein the gelatin-shellac was dropped in acidic medium resulting in an instantaneous generation of spherical microcapsules that retained their shape on drying. The formation of the microcapsules was basically due to the strong interactions between two oppositely charged polymers (as confirmed from isothermal titration calorimetry and infrared spectroscopy) and the instant precipitation of acid-resistant shellac. The novel microcapsules were extensively characterized and several bio-related applications for pharmaceuticals (encapsulation and release of bioactives), foods (loading of colorants and flavors), sensors (encapsulation of pH sensitive dye) and biotechnology (enzyme immobilization) fields were successfully demonstrated.

Due to the nature of the components used, the reported study will be of major interest to researchers and scientists from varied field working in the field of green colloidal structuring with non-toxic and non-hazardous chemical compounds.

4. Experimental Section

Materials: Gelatin (from porcine skin, Type A) (bloom strength: 90-110, avgerage molecular weight 40 000 g/mol), α-amylase (43.9 U/mg), pepsin from porcine gastric mucosa (654 U/mg), pancreatin from porcine pancreas (activity equivalent to 8× USP specifications), 4-dimethylaminocinnamaldehyde (DMACA), alizarin, purpurin, indigocarmine, d-limonene and nile blue, sodium phosphate dibasic and sodium phosphate monobasic monohydrate were purchased from Sigma Aldrich, Switzerland. TEAVIGO (green tea extract containing minimum 90% epigallocatechin gallate as per supplier's claim). Shellac (AQUAGOLD) was generous gift sample from SSB Stroever GmbH & Co. KG (Bremen, Germany). Curcumin was purchased from Sanjivini Phytochemicals, Mumbai, India. Silibinin was re-crystallized in-house from ethanol. Glycerol, 1N HCL and 1N NaOH were bought from Merck KGaA, Germany. Water purified by the MilliQ system was used for all the experiments.

Preparation of Microcapsules: Stock solutions of gelatin (4 wt%) and shellac (24 wt%) were prepared beforehand. The pH values of gelatin and shellac stock solutions were 5.0 and 7.5 respectively. To prepare microcapsules, GL:SL mixtures were first prepared by rapid mixing of stock solutions under continuous stirring (1000 rpm) using magnetic stirrer (Model EM3300T, Labotech Inc, Germany) at different gelatin to shellac ratios. The gel-like mixtures of gelatin-shellac were then extruded drop wise in water acidified with 0.1 N HCl (pH ≈ 1.0) using a syringe and a needle (with internal diameter 0.3 mm). Spherical microcapsules were immediately formed due to the precipitation of associated GL:SL mixtures. The microcapsules were then separated and washed repeatedly using MilliQ water followed by air drying at room temperature (≈20 °C).

Loading of solid materials including bioactives and colorants was carried out by co-dissolving them with GL:SL mixtures followed by extrusion and precipitation. For components which did not dissolve fully in water, minute amount of 0.1 M NaOH was used to facilitate solubilization. Epigallocatechin gallate (EGCG) and enzyme loaded microcapsules were prepared in presence of glycerol, which was used to increase the viscosity of gelatin-shellac mixture.

For encapsulation of aromatic oil, it was first emulsified with gelatin using T 25 basic ULTRA-TURRAX (IKA-Werke GmbH & Co. KG, Germany) at a speed of 6500 rpm for 60 s followed by addition of shellac and extrusion in acidic medium.

Alizarin (a pH sensitive dye) was incorporated in the microcapsules by dissolving a small quantity of alizarin in GL:SL mixture using 0.1 M NaOH. On extruding the blue colored basic mixture of GL:SL-alizarin in the acidic medium, yellow colored beads were immediately formed. The dried yellow microcapsules were later used for demonstrating the reversible colour changing properties of microcapsules in response to the cycles of pH changes from acidic to alkaline and back using 0.1 M HCl and NaOH.

Isothermal Titration Calorimetry: The thermodynamics of the binding of gelatin with shellac was assessed using an isothermal titration calorimeter (VP-ITC Microcal, Northampton, MA). The solutions were degassed by using a Microcal Thermo Vac degassing unit. The reference cell was filled with degassed MilliQ water. The sample cell of volume (1.4422 mL) was filled with (2.7 μM) of gelatin and thermostated at 20 °C. The syringe of volume (250 μL) was filled with (0.1 mM) shellac and the rotating speed of the syringe was set at 307 rpm. The titrant solutions were added in (10 μL) aliquots (24 injections with 10 s duration each; first injection of 2 μL is rejected from analysis) at 180 s intervals. The heat released or absorbed upon each injection was measured as function of time. The heat of dilution from the blank titration of titrants into pure milliQ water was subtracted from the raw data. Data acquisition and analysis were performed with Microcal Origin software (version 7 SR4), and the single set of binding sites was applied to fit binding isotherms. Thermodynamic parameters, including the binding constant (K_a), observed binding enthalpy (ΔH), binding stoichiometry (n) and binding entropy (ΔS) were calculated by iterative curve fitting of the binding isotherms using Equation 1.

$$Q = V\Delta H \frac{(1+ [M]_t n K_a + K_a[L]_t) - \sqrt{\left[(1+[M]_t n K_a + K_a[L]_t)^2 - 4[M]_t n K_a^2 [L]_t\right]}}{2K_a} \quad (1)$$

where Q is the cumulative heat, $[M]_t$ is the total concentration of reactant in the sample cell, $[L]_t$ is the total concentration of titrant added, and V is the volume of the sample cell. The Gibbs free energy (ΔG) was determined from the binding constant using equation 2.

$$\Delta G = -RT \ln K_a \quad (2)$$

where R is the gas constant (8.314 J mol^{-1} K^{-1}) and T is the absolute temperature (in Kelvin). The entropy ΔS is calculated using equation 3.

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

Optical and Scanning Electron Microscopy: For optical microscopy, automated microscope system (Morphologi 3, Malvern Instruments, UK) quipped with a thermo electric stage PE 94 (Linkam Scientific Instruments Ltd, UK) was used. To study the temperature dependent release of flavour, a single microcapsule was heated gradually from room temperature (20 °C to 80 °C) and images were captured at regular intervals. Coloured samples were imaged using Leica M420 macroscope (Leica Microsystems Ltd, Switzerland) attached with GXCAM-3 (GT vision Ltd. UK). For SEM, samples were seeded on a holder with a sticky conductive surface and sputter-coated with Pt and examined in a Zeiss Auriga Field Emission SEM (Zeiss, Germany) operated at 3 kV.

ATR FT-IR Spectroscopy: IR analysis was carried out using Perkin Elmer Spectrum 100 (V6.1) in combination with a Universal Attenuated Total Reflectance (UATR) accessory equipped with a 1 reflection diamond/ZnSe top plate with a pressure arm. For both sample and background,

16 interferograms with a resolution of 4 cm^{-1} were co-added and Fourier transformed. The spectral information was obtained over a scanning range from 4000–400 cm^{-1}.

Differential Scanning Calorimetry: DSC analyses were carried out with a Perkin Elmer power compensated DSC8000 calorimeter, equipped with a controlled cooling accessory the intracooler 3. The samples (5–10 mg) were weighted into the stainless steel sample pans and subjected to a heat-cool-heat treatment from 5 to 90 °C at a scanning rate equals 10 °C/min.

Mechanical Testing: To investigate the mechanical strength of gelatin-shellac microcapsules, we squeezed single microcapsules using TA.XT plus Texture Analyser (Stable Microsystems Ltd., UK). The protocol for experiment was based on a modification of method reported earlier by Leick et. al. where they used Advanced Rheometric expansion System from TA instruments.[16] The sample was squeezed under compression test mode using a probe of diameter 4 mm at a test speed of 0.01 mm/s and the resulting force was measured as a function of distance. Each measurement was performed five times and the fracture force was obtained by averaging the values of normal force. Compression curves were further obtained by plotting normal force (mN) against dimensionless displacement.

Loading and Release of Epigallocatechin Gallate and Silibinin: The amount of EGCG and silibinin in the loaded microcapsules was determined as follows: Accurately weighed amounts of dried microcapsules were pulverised using mortar and pestle. The powdered samples were then dispersed in ethanol and subjected to sonication for 10 min in order to extract the bioactives. The suspensions were then centrifuged at 13000 rpm for 10 min using Centrifuge 5415 (Eppendorf, Hamburg, Germany) and the quantification of extracted bioactives in the supernatant was carried out using spectroscopy. For determination of EGCG, spectroscopy measurement of the green colored complex formed with DMACA was quantified by the integration of the absorbance peak at 637 nm using UV-1601 UV-vis spectrophotometer (SHIMADZU, Japan). For silibinin quantification, absorbance of supernatant was measured at 290 nm using SPECTRAmax 190 Microplate spectrophotometer (Molecular Devices Corp., USA).

A two-stage in-vitro digestive model mimicking gastric and intestinal phases was used to study the release of bioactives from the microcapsules. For the gastric phase, accurately weighed microcapsules were dispersed in (30 mL of 5 mM) phosphate buffer (pH 5.0) followed by the addition of (2 mL) of pepsin solution (12.5 mg/mL pepsin in 0.2 M HCl) to reach a pH 2.0. Subsequently, samples were incubated in a shaking water bath (Julabo SW22, Julabo Labotechnik GmbH, Seelbach, Germany) at 37 °C with mixing at 70 rpm for 60 min. Aliquots (0.5 mL) were taken every 15 min followed by centrifuged and quantification of bioactives in the supernatant. For intestinal phase, the samples from gastric phases were neutralized using (4.3 mL of 0.12 M) NaOH followed by the addition of (3 mL) pancreatin solution (16.7 mg/mL pancreatin dispersed in 0.2 M phosphate buffer pH 7.0 and further incubation, regular samplings and quantifications as described in the gastric phase.

Amylase Activity: Enzyme activity of α-amylase was measured using protocol provided by supplier of Amylazyme assay kit. The principle involved in this test includes breaking down the water dispersible (but insoluble) substrate, azurine-cross linked amylose (AZCL-Amylose) by enzyme into soluble fragment of dye resulting in an increase in absorbance at 590 nm. The procedure used was as follows: Accurately weighed enzyme sample (for control) and enzyme loaded microcapsules were first dispersed in acetate buffer pH 5.1 in a test tube followed by addition of single amylazyme tablet. The tube was then incubated at 40 °C for exactly 10 min followed by filtration. The absorbance of the filtrate was then read at 590 nm against reaction blank. Enzyme activity was reported as Unit per assay.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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ADVANCED FUNCTIONAL MATERIALS

Supporting Information

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Novel All-Natural Microcapsules from Gelatin and Shellac for Biorelated Applications

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Supplementary Information

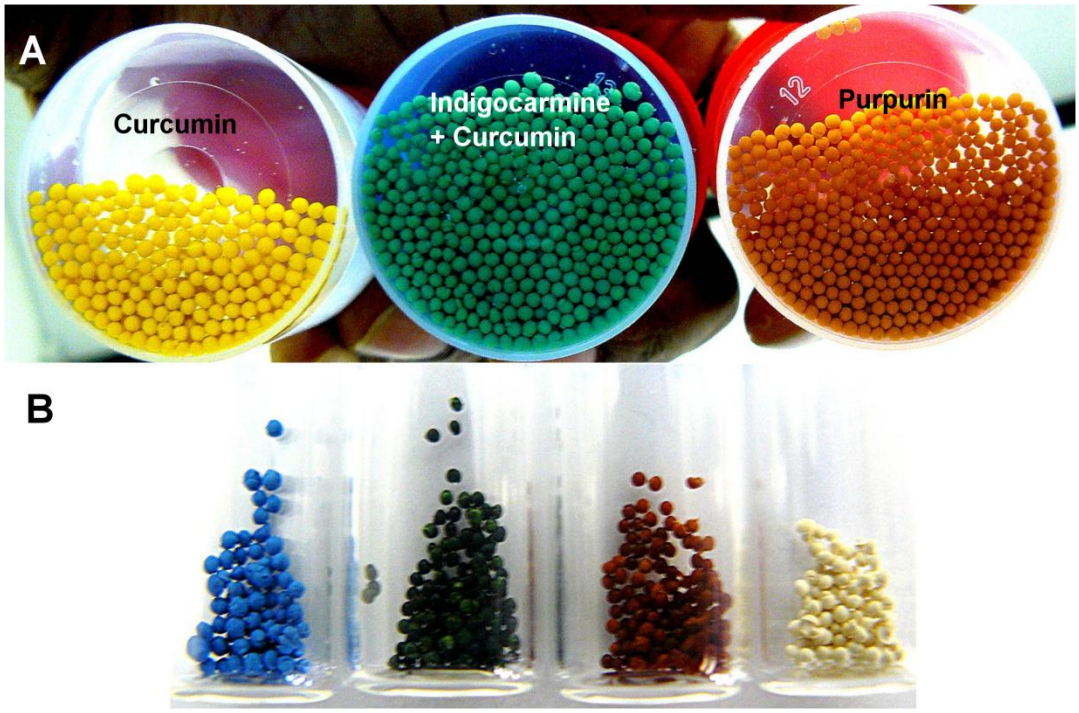

Figure S1

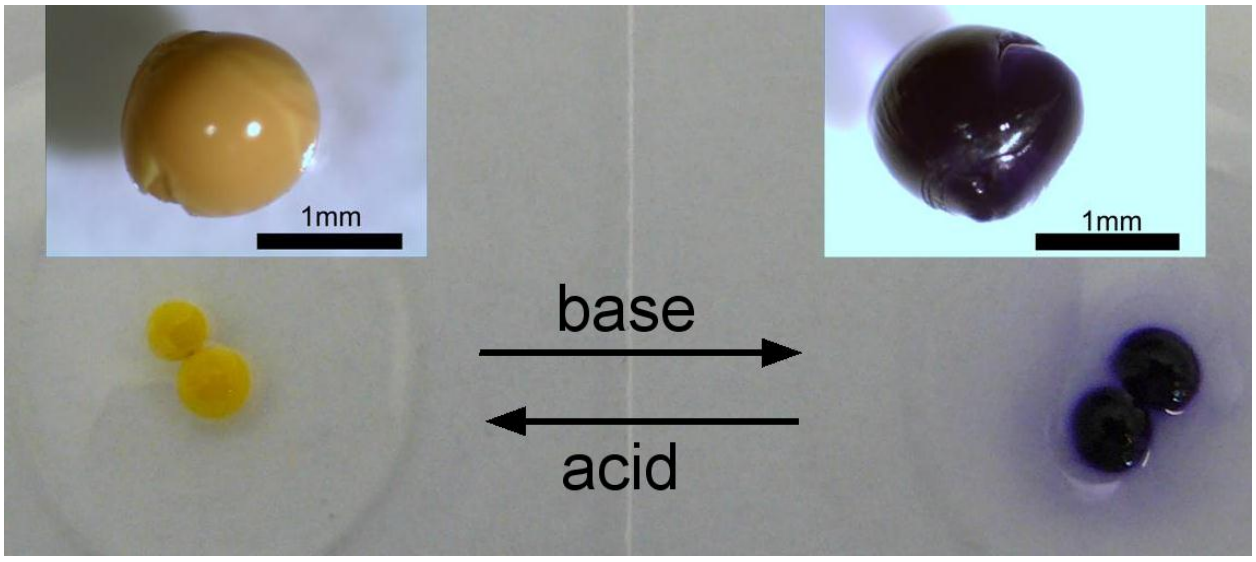

Figure S2