Investigation of Biopolymer-based Hydrogels as Green and Heterogeneous Catalysts in C-C Bond Formation

Dissertation

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Meiner Familie

„*Die Wissenschaft fängt eigentlich erst da an interessant zu werden, wo sie aufhört.*“

Justus von Liebig

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A Summary

The present dissertation evaluates the efficacy of different polysaccharides (e.g. chitosan, alginate and κ-carrageenan) and proteins (e.g. gelatin, collagen, silk fibroin) as possible catalysts for a variety of C-C bond formation reactions (Figure 1). These biopolymers[i] can be obtained in different forms (e.g. hydrogels, mesoporous materials). Among different forms hydrogels are one of the most interesting since they could act as biphasic and heterogeneous systems in chemical transformations and facilitate therefore the application as green and reusable catalysts. Overall, such malleability allows us to investigate the effect of the morphology and/or physical state of the potential biocatalyst on the reactivity.

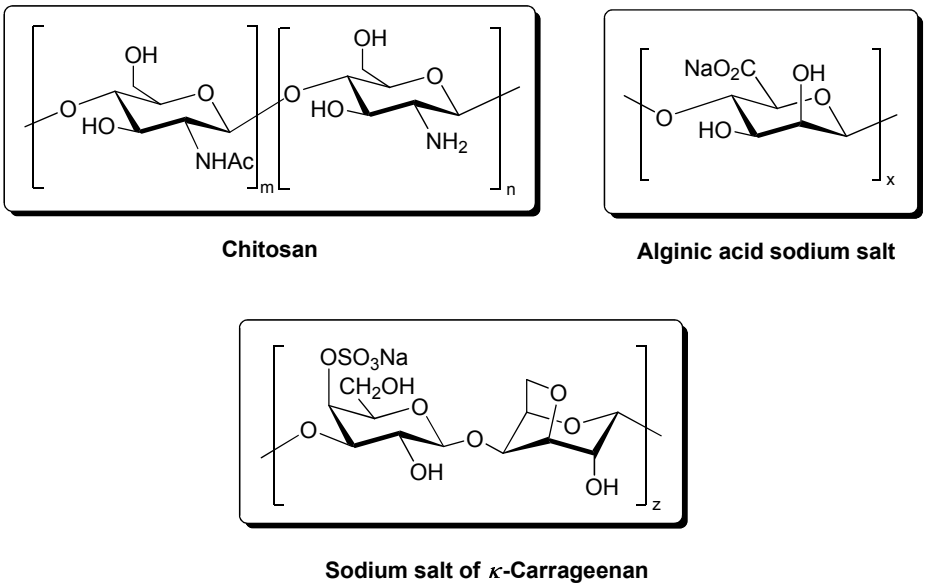


Figure 1. Repeating units of used polysaccharides in this work.

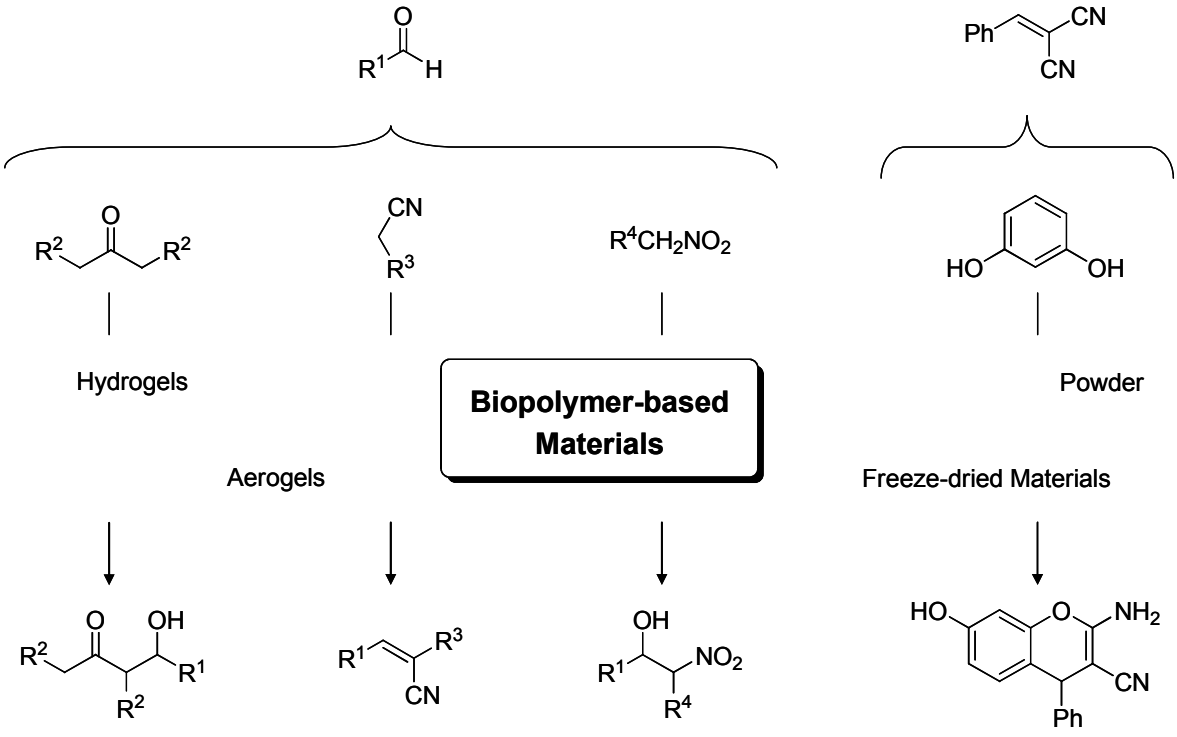
This work consists of mainly three parts: Introduction, Main Part (Chapters 1-5) and Supporting Information enclosed on a CD.

In the firs part of the introduction the importance and the main advantages of gels when used as reaction vessels and catalysts in organic transformations in the case of both LMW compound- and (bio)polymer-based hydro-/organogels are described. The second part of the introduction describes the most important efforts

[i] The term biopolymer includes both categories of polysaccharides and proteins.

during the last decade of polysaccharide-based gels as organocatalysts and support for catalytic active enzymes or metals.

Chapter 1 describes the results of the evaluation of chitosan-based materials as organocatalysts in different C-C bond formation reactions, i.e. aldol, Knoevenagel, nitroaldol (Henry) and Michael addition reaction (Scheme 1). The chitosan hydrogel and the commercial available powder showed almost similar activity, but from a practical point of view the recovery of the heterogeneous system in the case of the hydrogel beads was superior to the powder. The best results were obtained in the case of the Knoevenagel condensation, followed by the Henry, Michael addition and aldol reaction. Most importantly, a strong correlation between the pH of the hydrogel and the reaction outcome could be observed. Therefore, a meticulous washing of the prepared hydrogel was essential before the material was submitted as organocatalyst to the above mentioned reactions.



Scheme 1. Overview of tested C-C bond formation reactions.

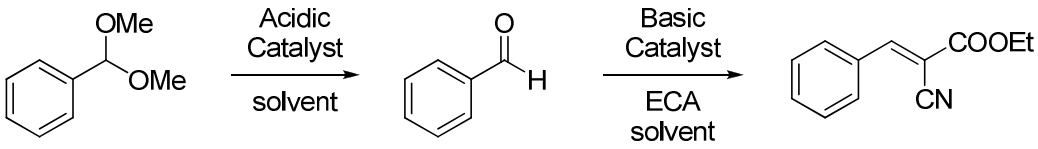
Chapter 2 summarizes the results obtained by gelatin- and collagen-based materials-mediated nitroaldol (Henry) and direct aldol reaction. Under physiological temperature in DMSO the powdered form of both proteins showed

the highest activity in both cases, whereas the use of the hydrogel was limited due to its low gel-*to*-sol transition. Moreover, the possible impact on food-containing aldehydes is considered and a comprehensive study between different biopolymers is discussed.

Chapter 3 describes a comparative evaluation of different metal (i.e. M = Ca, Cu, Co, Ni, Zn, Fe)-based alginate hydrogel beads as heterogeneous and recyclable catalysts in the nitroaldol (Henry) reaction. Thereby, corresponding control experiments showed that the possible metal leaching had no influence on the reaction outcome, emphasizing the importance of the basic carboxylate groups of alginate. Moreover, Ca^{2+}-AHG was superior to all other used materials.

In chapter 4 different materials based on the silk fibroin protein (i.e. FDSF, ASFS, MPSF and SFHG) obtained from the cocoons of the silkworm *Bombyx mori* were investigated as possible heterogeneous organocatalysts in the aldol, Knoevenagel condensation and nitroaldol (Henry) reaction. Among different physical forms of the protein tested, the freeze-dried form of silk fibroin (FDSF) showed the most promising results in DMSO in the case of the Henry reaction followed by the Knoevenagel condensation reaction, whereas in the aldol reaction only poor activities could be monitored.

Chapter 5 deals with the attempts to develop new bifunctional materials considering the polysaccharides chitosan, alginate and carrageenan to form polyelectrolyte complexes (PECs) offering acidic and basic sites. Aerogels (i.e. P-κ-CGAG, AA-κ-CGAG, HCA-κ-CGAG, CSAG and CS-κ-CGAG), hydrogels (CS-κ-CGPECHG and CS-AGPECHG), sulfonated chitosan powder (SFNCS) and the commercial available polysaccharide powders were tested in the one-pot deacetylation Knoevenagel condensation reaction (Scheme 2).



Scheme 2. One-pot deacetylation Knoevenagel condensation reaction.

B Zusammenfassung

Die vorliegende Dissertation bewertet die Auswirkung verschiedener Biopolymere als mögliche Katalysatoren auf eine Auswahl von C-C Bindungsknüpfungsreaktionen. Dabei wurden hauptsächlich Polysaccharide (Abbildung 1) wie Chitosan, Alginat und Carrageen berücksichtigt. Darüber hinaus, wurden in dieser Arbeit auch Proteine wie Gelatine, Kollagen und das Seidenprotein Fibroin mit aufgenommen. Diese Materialien zeichnen sich dadurch aus stabile Hydrogele zu bilden, welche als heterogene Systeme in chemischen Reaktionen eingesetzt werden können und dadurch eine Anwendung als grüne und wiederverwendbare katalytische Systeme ermöglichen.

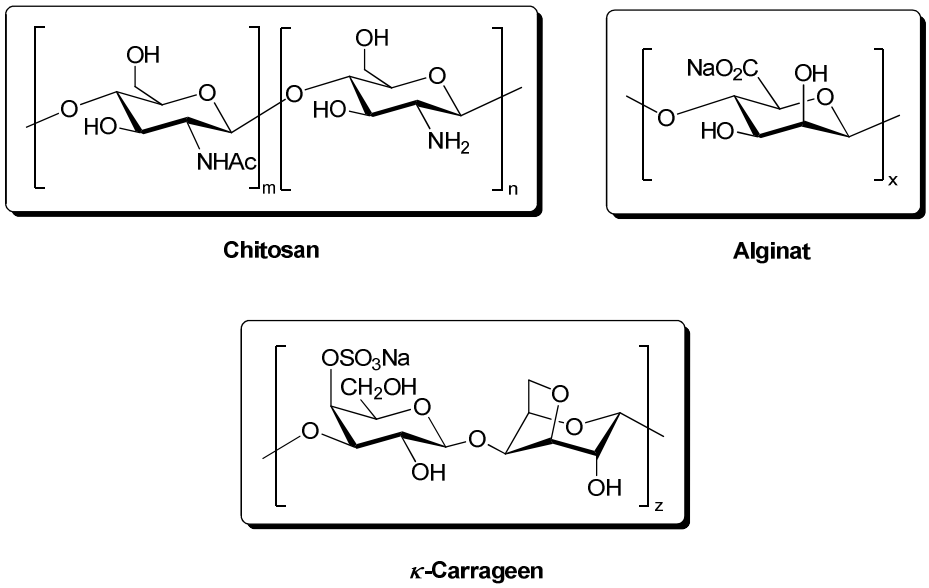
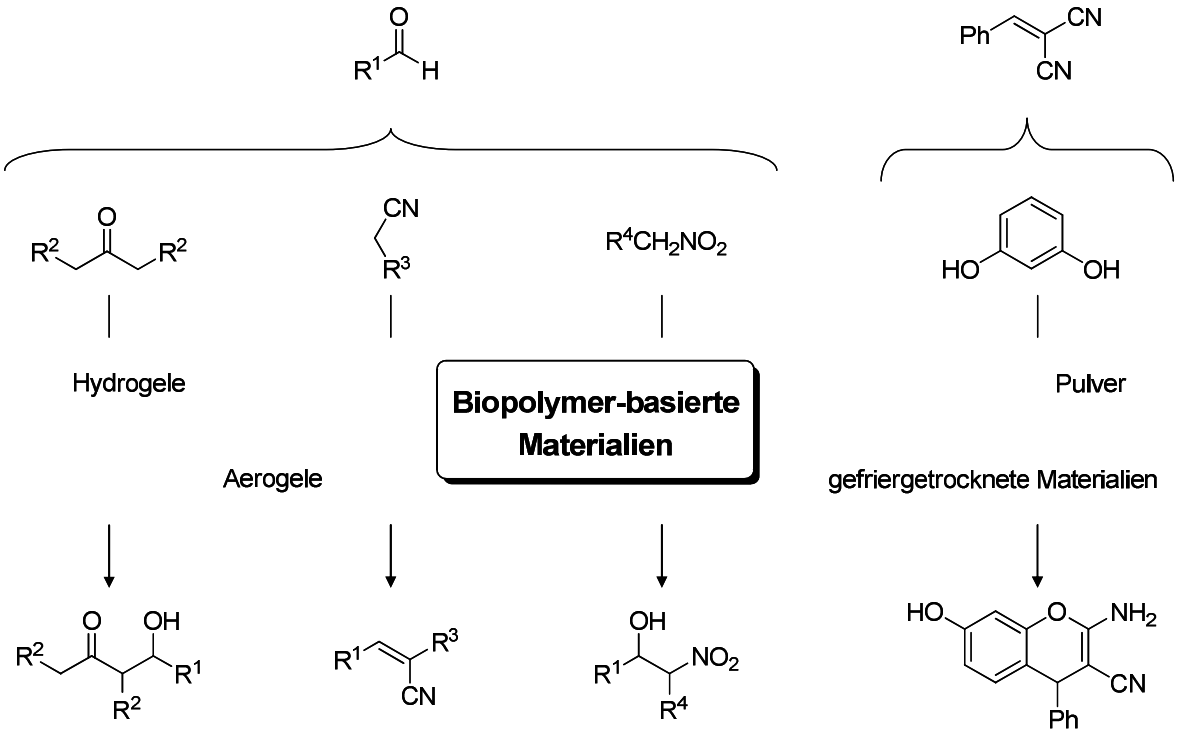


Abbildung 1. Wiederholungseinheiten, der in dieser Arbeit verwendeten Polysaccharide.

In der Einleitung werden die Wichtigkeit und die Vorteile von Gelen als Reaktionsmedium und als Katalysatoren in organischen Reaktionen bzw. Umwandlungen unter Berücksichtigung von „LMW“ Verbindungen- und (Bio)polymer-basierten Hydro-/Organogelen aufgeführt. Der zweite Teil fasst die wichtigsten Fortschritte der letzten zehn Jahre von Polysaccharid-basierten Gelen, die als Organokatalysatoren und Support für katalytisch aktive Enzyme und Metalle verwendet wurden, zusammen.

Das erste Kapitel beschreibt die kritische Bewertung von Chitosan-basierten Materialien, die als Organokatalysatoren in verschiedenen C-C

Bindungsknüpfungsreaktionen getestet wurden. Dabei wurden die Aldol, Knoevenagel Kondensations-, Nitroaldol (Henry) und Michael Additions-Reaktionen berücksichtigt (Schema 1). Das Chitosan Hydrogel und das kommerziell erhältiche Pulver zeigten ähnliches Verhalten, wobei aber bei der Aufarbeitung nach der Reaktion das Hydrogel erhebliche Vorteile gegenüber dem Pulver aufwies. Die höchsten Aktivitäten wurden im Fall der Knoevenagel Kondensations-Reaktion erhalten, gefolgt von der Henry, Michael Additions- und Aldol Reaktion. Die starke Korrelation zwischen pH Wert des Hydrogels und des Reaktionsumsatzes war eine der wichtigsten Erkenntnisse während dieser Studie. Aufgrund dessen, war das intensive Waschen des hergestellten Hydrogels essentiell, bevor dieses Material als Organokatalysator in den oben genannten Reaktionen eingesetzt wurde.



Schema 1. Überblick über die getesteten C-C Bindungsknüpfungsreaktionen.

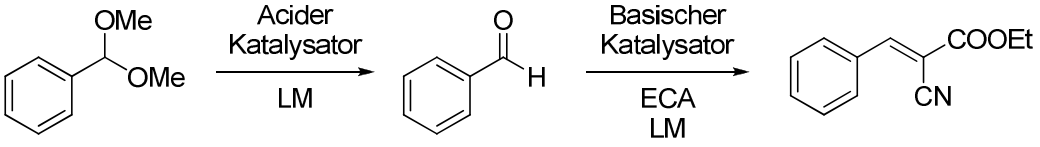
Im zweiten Kapitel wurden Gelatine- und Kollagen-basierte Materialen in der Nitroaldol (Henry) und Aldol Reaktion eingesetzt. In DMSO unter physiologischer Temperatur zeigten beide Proteine die höchsten Aktivitäten in beiden Reaktionen, wobei die Verwendung des Hydrogels auf Grund des niedrigen Schmelzpunktes unter diesen Bedingungen eingeschränkt war. Darüber

hinaus wurden die möglichen Einflüsse auf in Nahrung enthaltene Aldehyde diskutiert und eine vergleichende Studie in der Henry Reaktion unter Berücksichtigung unterschiedlicher Biopolymere durchgeführt.

Das dritte Kapitel beschreibt die Anwendung und Beurteilung der Auswirkung von verschiedenen Metall (M = Ca, Cu, Co, Ni, Zn, Fe)-basierten Alginat Hydrogelen als heterogene und wiederverwendbare Katalysatoren auf die Henry Reaktion. Dabei wurde durch entsprechende Kontrollexperimente gezeigt, dass mögliches „Metall-Leaching“ keinen Einfluss auf die Reaktion ausübt und somit das Zusammenspiel von Metall und den Carboxylatgruppen des Alginats für die Aktivität essentiell ist. Darüber hinaus konnte festgestellt werden, dass Ca^{2+}-AHG den anderen Materialien überlegen war.

Im vierten Kapitel wurden unterschiedliche Materialien basierend auf dem Seidenprotein Fibroin hergestellt und als heterogene Katalysatoren in der Aldol, Knoevenagel Kondensations- und Henry Reaktion getestet bzw. verglichen. Das gefriergetrocknete Fibroin erzielte dabei die höchsten Umsätze in DMSO im Fall der Henry Reaktion gefolgt von der Knoevenagel Kondensations-Reaktion, wobei in der Aldol Reaktion nur geringe Ausbeuten festgestellt werden konnten.

Kapitel 5 handelt von der Entwicklung neuer bifunktioneller Materialien unter Berücksichtigung von Polysacchariden wie Chitosan, Alginat und Carrageen um Polyelektrolytkomplexe (PECs) herzustellen. Dabei sollten diese zwei Funktionalitäten, saure und basische Stellen, aufweisen. Aerogele (d. h. P-κ-CGAG, AA-κ-CGAG, HCA-κ-CGAG, CSAG and CS-κ-CGAG), Hydrogele (d. h. CS-κ-CGPECHG and CS-AGPECHG), sulfoniertes Chitosan Pulver (SFNCS) und die dazugehörigen kommerziell erhältlichen Polysaccharid-Pulver wurden in der „one-pot“ Deacetylierungs-Knoevenagel Kondensations-Reaktion getestet und bewertet (Schema 2).



Schema 2. “One-pot” Deacetylierungs-Knoevenagel Kondensations-Reaktion.

C Introduction[i]

1. General

The interest in gels consisting of either water (hydrogels) or organic solvents (organogels) was considerably growing over the last one hundred years.[1-3] In general, these materials can be described as hierarchical, self-assembled and viscoelastic networks showing hard or soft rheological properties. Beside the most common classification considering the nature of the solvent, gels can be further sub-devided. Thereby, the driving forces for molecular aggregation are observed. Usually, terms like chemical gels[4-5] and physical gels[1],[6-7] can be found in literature. The former are based on covalent bonds, i. e. cross-linked polymers, whereas the latter on non-covalent bonds, i. e. hydrogen-bonds, van der Waals, charge transfer, dipole-dipole, π-π stacking and coordination interactions. Physical gels are formed by either low-molecular-weight (LMW) compounds or polymers and show reversible gel-*to*-sol phase transitions. In addition, combinations of both types are also possible.[8-9] In Figure 1, a few selected gelators both LMW compounds and polymers used especially for catalytic applications are highlighted.

From a macroscopical point of view, gels have a solid-like appearance, which arises from the entrapment of a liquid (major component) in the cavities of a solid 3D-matrix (minor component) by surface tension and capillary forces.[10] The minor solid part is thereby formed by the entanglement of 1D-polymeric (or suprapolymeric) strands of either macromolecules or LMW molecules through covalent or non-covalent interactions. In general, the strands own lengths of micrometer scale and diameters of nanometer scale. Consequently, one gelator molecule may be able to immobilize up to 10^5 solvent molecules and enhance the viscosity of the gel by a factor of 10^{10}. This phenomenon can be described through a metastable dissolution-crystallization equilibrium of the gelator and opens the possibility to respond to a variety of external stimuli.[11, 12]

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[i] In this introduction parts from the reference “D. D. Díaz, D. Kühbeck, R. J. Koopmans, *Chem. Soc. Rev.* **2011**, *40*, 427-448: Stimuli-responsive gels as reaction vessels and reusable catalysts” were amended and included.

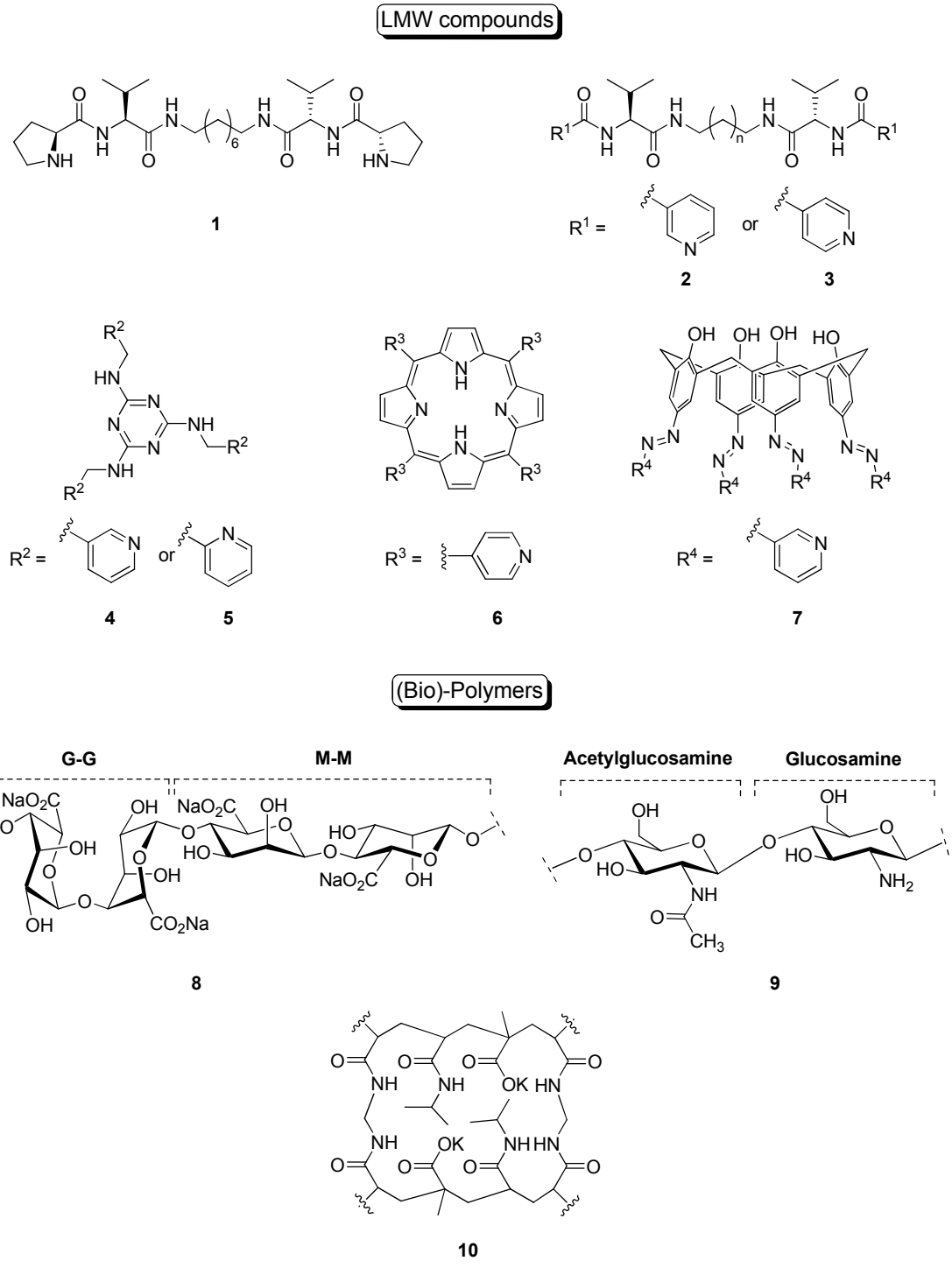


Figure 1. Overview of selected gelators used for catalysis: **1**[14]: *L*-proline modified LMW gelator used for the catalyzed Henry reaction; **2**-**3**[15], **4**-**7**[16]: Different molecules bearing pyridine moieties for Pd(II) complexation and the use in the oxidation of benzyl alcohol; **8**: General structure of sodium alginate which can be used directly as basic organocatalyst or as support for enzymes or metals; **9**: General structure of chitosan which can be applied as support for basic organocatalyst or as support for enzymes or metals; **10**[17]: Synthetic polymer obtained by polymerization of MBA, NIPA and PMA as support for Pd NPs and catalytic system in the Suzuki-Miyaura cross-coupling reaction.

Gels are used in different classical approaches like regenerative medicine, drug-delivery, sensors, actuators, cosmetic, foods, environmental remediation, and nanoelectronic (‘bottom–up’ approaches).[13] Due to their highly ordered 3D-network, confined environment, high surface areas based on porous interstices and ability to incorporate different catalytic moieties, these materials also find application in catalysis. Beside macromolecular nanoreactors, i. e. organic polymers, micelles or zeolites,[18] gels may open a new competitive area for chiral implification in chemical transformations.[19]

When different types of gels are compared, biopolymer-based hydrogels may own predominant properties. Biopolymers (e.g. alginate, chitosan, carrageenan and several proteins) are in general nontoxic, biocompatible and biodegradable. Therefore, they can be used especially in green applications. Due to their wide abundance in nature and generation for example from (sea)food waste, wood, etc. these polymers are also cheap compared to the synthetic ones. From a practical point of view commercial available biopolymers can be already used for gel formation without further modifications, whereas synthetic polymers and LMW gelators first have to be synthesized in several steps and generally have to be heated before gelation can take place. Biopolymer-based gels can be obtained in many cases in beneficial form of beads in a variable diameter range from micrometer to millimeter scale, which is attributed to the ionotropic gelation mechanism. Moreover, these hydrogels show either no or high gel-*to*-sol transitions and can be therefore also used at high temperatures. Due to the heterogeneous nature and spherical appearance the gels can be easily recovered from the reaction mixture by simple filtration and used in the next cycle, whereas the recovery of LMW gelators is more time extensive. In the latter case the gel has to be transferred to the solution state by applying an external stimulus (e. g. heat, pH gradient, UV irradiation, etc.) to get access to the reaction products. After successful extraction and separation, the LMW molecules have to be purified and dried before they can be used again.

Based on these superior benefits the following overview highlights the most important results obtained in the last decade for biopolymer-based gels in organo-, bio- and metal catalysis. The materials described in this section are in general classical gels consisting of a gelator (i. e. biopolymer) and solvent (in most cases

water). In addition, advances in the case of aerogels, gels in which the liquid phase is exchanged by a gas phase *via* CO_2 supercritical drying, are also considered.

2. Catalysis

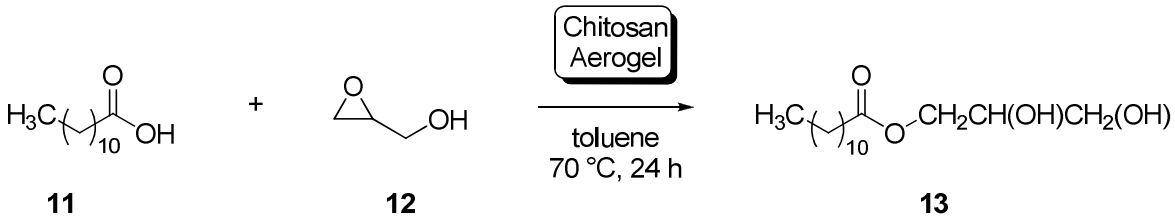
2.1 Biopolymer-based Gels

2.1.1 Organo- and Biocatalysis

One of the very first investigations dealing with a catalytic active and unmodified chitosan-based hydrogel was reported by Reddy and co-workers in 2006.[20] The environmental benign hydrogel was successfully introduced to the non-stereoselective aldol reaction and the (*E*)-selective Knoevenagel condensation. Thereby the corresponding products were obtained in good conversions and chemo-selectivity (aldol *vs.* dehydration) under heterogeneous conditions. The application of the hydrogel in form of uniform beads facilitated the work-up remarkable compared to the powdered counterpart and supported the recyclability of the organocatalyst. But most importantly, the hydrogel-based catalyst showed the highest activity of all applied materials, among others commercial chitosan powder and air-dried chitosan beads. This observation may be explained by the higher accessibility of the functional amine group in the former (55-65%, 2.5% and 1.65%, respectively, based on ca. 80% deacetylated commercial chitosan).

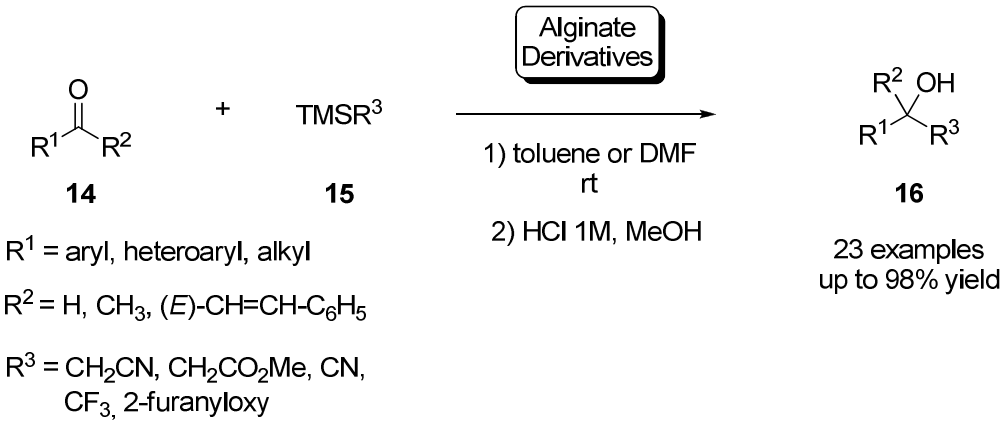
As the slow diffusion through hydrogels in biphasic reaction systems may hinder the accessibility of intrinsic functional groups and therefore the catalytic efficiency in other reaction types, the development of a porous analogon might be of interest. In this sense, Quignard and co-workers[21] have made investigations about the exchange of the solvent in the gel by a gas phase to obtain a mesoporous material with a high surface area (ca. 110 $m^2\ g^{-1}$ *vs.* 1 $m^2\ g^{-1}$ for the freeze-dried gel). Thereby, the hydrogel first were dehydrated by insertion in a series of successive ethanol-water baths with increasing alcohol concentration before the material could be dried under supercritical CO_2 conditions. The so obtained chitosan aerogel beads showed good yields as well as selectivies

(addition product vs. glycidol polymerization) in the formation of α-monoglyceride (**13**) by addition of a fatty acid (**11**) to glycidol (**12**), whereas the freeze-dried material was not active at all (Scheme 1).



Scheme 1. Chitosan aerogel-catalyzed formation of α-monoglyceride (**13**).

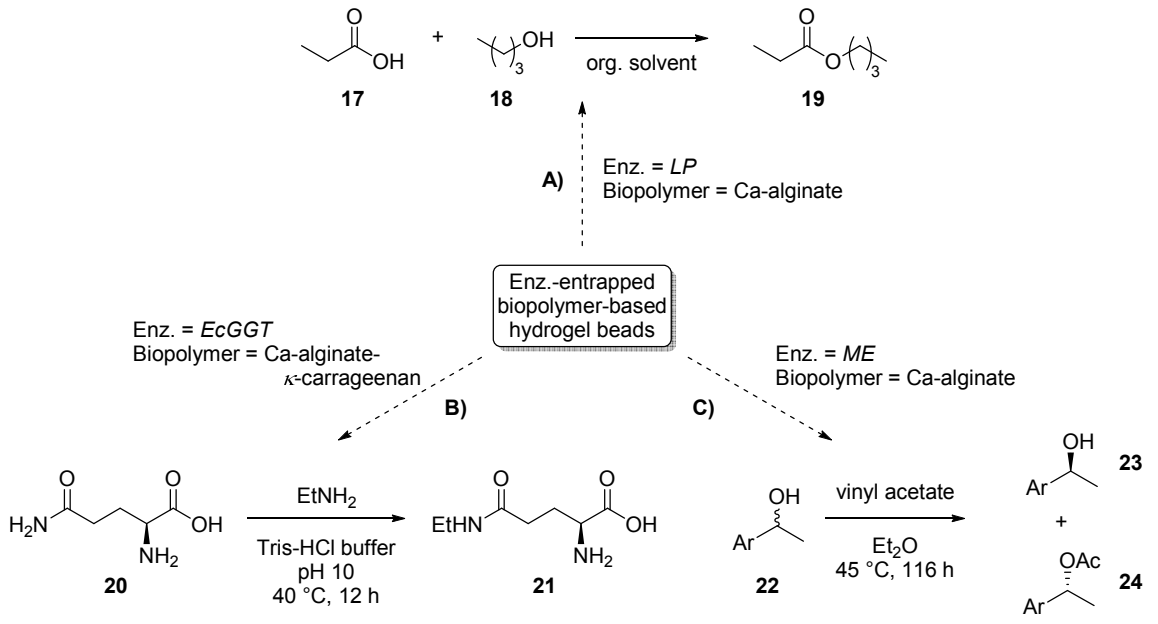
The efficacy of chitosan aerogel beads were further tested by the same group in the asymmetric direct aldol reaction in water.[22] The aldol products were obtained in high yields and with good stereoselectivities (up to 93% ee) and the catalyst could be reused at least for four consecutive cycles without loss of activity. The use of additives like DNP and stearic acid enhanced both yield and stereoselectivity. It is worth to mention that this material was not superior to the commercial chitosan powder and the corresponding hydrogel, showing that enhanced surface area and access to the intrinsic functional amine groups was in this case not the key feature.



Scheme 2. 1,2-Addition of different nucleophiles (**15**) to carbonyl compounds (**14**) catalyzed by alginate derivatives.

More recently, Levacher's group[23] reported the preparation and the use of a sodium alginate acetogel and a freeze-dried calcium (Ca)-alginate hydrogel in the cyanomethylation (Scheme 2) of carbonyl compounds (**14**) with (trimethylsilyl)derivatives (**15**). In both cases the products (**16**) could be obtained only in moderate yields, whereas the ammonium alginate salt (*n*-Bu_4N^+alginate-COO^-) showed consiberably enhanced activity. Interestingly, the reaction with Ca-alginate hydrogel was totally inhibited.

Beside the direct application of biopolymer-based gels as basic organocatalysts in organic synthesis, a few reports were also made about the immobilization of enzymes on biopolymer hydrogels, mainly on Ca-crosslinked alginate beads. The aim of this support is based on the protection of the enzyme in an aqueous environment when it should accelerate chemical transformations in organic media. Further useful benefits of such systems may be enhanced activity and good reusability when compared to the free form of the enzyme. Scheme 3 summarizes the most important investigations in this direction.



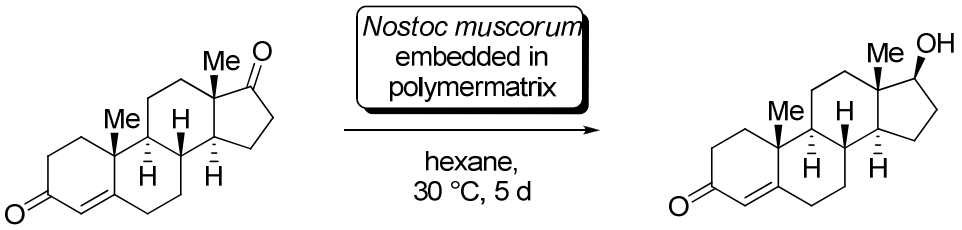
Scheme 3. Overview of the most important contributions to enzyme encapsulation on alginate-based hydrogels and their application in different reactions. (Scheme 3 is adapted from reference [24])

In this context, Ansorge-Schumacher and co-workers[25] have developed a biocatalytic system based on the entrapment of lipase (*LP*) from *Candida rugosa*

in Ca-alginate hydrogel beads with mean diameters between 200 and 400 μm. This enzyme loaded gel was successfully applied to the esterification reaction between propionic acid (**17**) and *n*-butanol (**18**) in organic solvents. The highest ester productivity of all tested solvents was obtained in a highly-dense mixture of bromohexane:cyclohexane (1:1) containing the surfactant CTAC (0.1 vol%) as additive to avoid beads agglomeration. Moreover, Lin's group[26] observed a biocatalytic activity of γ-glutamyltraspeptidase from *Escherichia coli* (*EcGGT*) immobilized on Ca-alginate-κ-carrageenan hydrogel composites in the synthesis of *L*-theanine (**21**). Thereby, an alginate concentration of 2% (w/v), a bead size of 1.9 mm and an enzyme loading of 1.5 mg/g alginate was found to be the ideal condition for maximum enzyme activity. The applied biocatalyst showed also good reusability without a significant loss of enzyme activity in five consecutive runs. More recently, Gotor and co-workers[27] reported the entrapment of *Manihot esculenta* (*ME*) in Ca-alginate hydrogel beads and their use in enzymatic kinetic resolution of racemates *via* stereoselective acetylation of a wide range of alcohols (**22**), whereas Chen and co-workers[28] were testing the efficacy of *Candida antarctica* lipase B immobilized on the same type of beads in the chiral resolution of α-phenyl ethanol as a model in organic phase. In the former case excellent enantioselectivities were obtained with substituted phenylethan-1-ols bearing electron-withdrawing groups. Moreover, the catalyst could be reused at least for four cycles with only a small loss in the activity and selectivity. The latter biocatalyst showed very poor enantiomeric excess for the free and hydrogel entrapped enzyme, whereas high enantioselectivities with the freeze-dried hydrogel could be obtained. In addition, this biocatalyst showed still high activity after the fifth cycle. The development of immobilized proteases of *Bacillus subtilis* on Ca-alginate hydrogels was reported by Azhar and co-workers.[29] This material was tested for its activity using casein as subsrate considering the impact of different parameters (i.e. temperature, pH, calcium chloride, salts, substrate concentrations and incubation time). It is important to mention that the encapsulated proteases were more stable and showed reusability with consistent deactivation over three cycles when compared to the free enzyme. Beside the application of these biocatalysts in a variety of different reactions, enzymes like glucoamylase and pullulanase were encapsulated in Ca-alginate beads with a

sodium alginate concentration of 2.5% and used in the hydrolysis of starch.[30] During this investigation the entrapped enzymes showed enhanced thermostability at 55 °C and ca. four times higher sugar productivity.

In the previous listed references, nearly in all cases Ca-alginate hydrogels were used as enzyme support. For further comparative studies the change of the morphology surrounding the enzyme by the simple exchange of the biopolymer source could be an efficient methodology to tune or enhance enzyme activities, recyclabilities and/or stabilities in biocatalytic transformations. For this approach polysaccharides like chitosan and carrageenan could be of interest, as they are also able to form stable hydrogels. Considering these thoughts, Faramarzi's group[31] investigated a comprehensive study of biopolymers as well as synthetic polymers (i. e. agar, agarose, κ-carrageenan, polyacrylamide, polyvinyl alchohol, and sodium alginate) encapsulated microalgal cells (*Nostoc muscorum*) in the bioconversion of androst-4-en-3,17-dione (**25**) to testosterone (**26**). Nevertheless, the cells immobilized on the sodium alginate matrix with a concentration of 2% (w/v) showed the highest activities (e. g. 72% *vs.* 31% conversion by κ-carrageenan) of all and were three times more efficient than the free form. Interestingly, the reusability of the beads could be enhanced up to five consecutive runs by increasing the sodium alginate concentration to 3% (w/v).



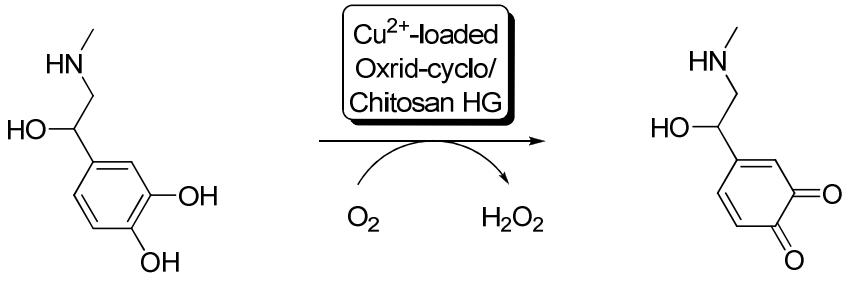
25 **26**

Scheme 4. Bioconversion of androst-4-en-3,17-dione (**25**) to testosterone (**26**) catalyzed by microalgal cells in different polymer matrices.

2.1.2 Metal Catalysis

Biopolymers are not only known for their entrapment of enzymes, but also offering the possibility to bind a variety of metal cations. In this context, the development of

a Cu(II)-doped chitosan-based hydrogel using oxidized β-cyclodextrin (oxrid-cyclo) as cross-linker was reported by Paradossi and co-workers.[32] This hybrid material was successfully introduced as a heterogeneous and recyclable catalyst to the oxidation of adrenaline (**27**) to adrenochrome (**28**) by molecular oxygen in 0.1 M HEPES buffer (Scheme 5). Reddy's group[33] developed, however, the immobilization of Cu(II) cations onto a different support, i. e. sodium alginate, *via* ionotropic gelation based on metal-carboxylate interactions. This kind of hydrogel was successfully applied as a heterogeneous catalyst in the 1,3-dipolar cycloaddition of alkynes with azides (AAC), as well as for the oxidative coupling of 2-naphthols and phenols in water to obtain the corresponding 1,4-disubstituted 1,2,3-triazoles and biaryl compounds in good to excellent yields with an exclusive 1,4 regioselectivity in the former.



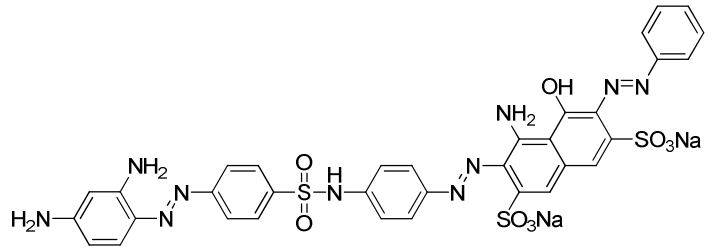
27 **28**

Scheme 5. Oxidation of adrenaline (**27**) to adrenochrome (**28**) by Cu(II) bound to an oxidized form of β-cyclodextrin/chitosan composite hydrogel at pH 6.78, 25 °C.

Moreover, the beads could be recovered very easily by simple filtration and introduced after washing to the next cycles showing a reusability at least for three consecutive runs without loss of activity. Beside Cu(II) cations, also other divalent/polyvalent metals (e.g. Ca(II), Co(II), Fe(III), Ni(II) and Zn(II), etc) are able to form stable hydrogel beads in combination with aqueous sodium alginate solutions. In this sense, Ding and co-workers[34] used Fe(III)-alginate hydrogel beads as efficient and heterogeneous catalyst for the oxidative degradation of azo dyes (i.e. Acid Black 234 (**29**) and Reactive Blue 222 (**30**)) in the presence of H_2O_2 under visible light irradiation (Figure 2)).

As previously mentioned, in general the use of aerogels may improve the intrinsic accessibility of catalytic centers considerably. Therefore Quignard's

group[35] prepared homogeneously and highly dispersed Pd NPs in a Ca-alginate aerogel matrix. This material showed porous properties with surface areas between 500 and 700 m^2 g^{-1} and was highly active in the Suzuki C-C coupling reaction. The drop of the recycling efficiency of the catalyst from 95 to 70% conversion could be explained by poisoning of the pores by the substrates/product and was neither attributed to the aggregation of NPs nor to the leaching of the metal.



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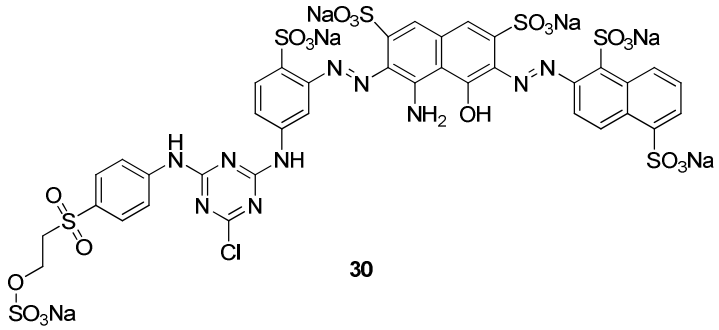
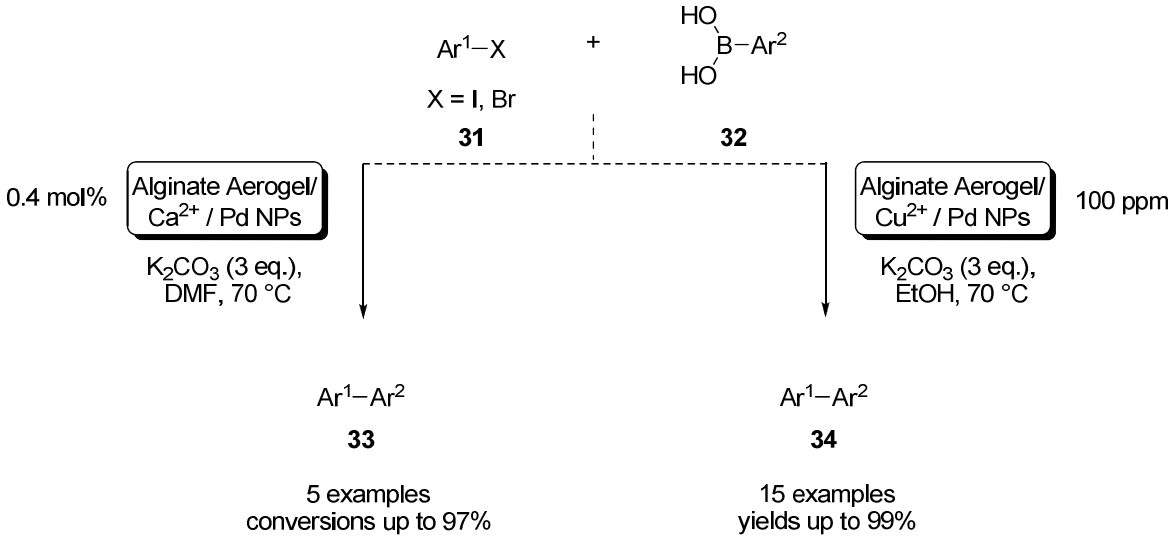


30

Figure 2. Azo-dyes: Acid Black 234 (**29**) and Reactive Blue 222 (**30**) degradated by the use of visible light irradiation, Fe(III)-alginate hydrogel beads and H_2O_2.

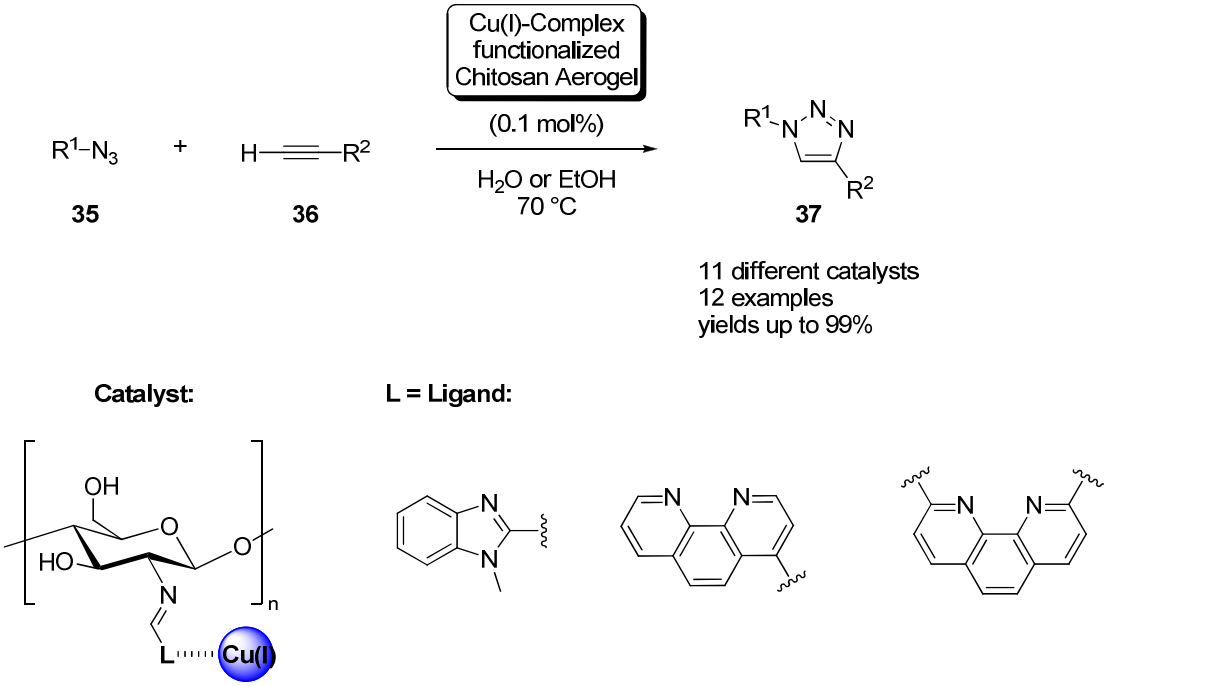
Further studies[36] showed that the gelling metal has a high influence on the catalyst activity. In this case Pd NPs were loaded onto different metal (i.e. Ca, Ba, Mn, Zn, Ni, Ce, Cu and Co) alginate aerogels, which were tested in the Suzuki-Miyaura reaction (Scheme 7) between aryl halides (**31**) and aryl boronic acids (**32**). Thereby the Cu-based aerogel showed the highest activity of all tested materials and could be reused at least for six cycles without loss of activity. Beside Pd NPs, it was also possible to encapsulate Au NPs in such biopolymer-based matrices.[37] For this approach chitosan as support was used instead of alginate. The porous catalyst was highly active in both Sonogashira cross-coupling and phenylboronic acid homocoupling and showed at least a constant lifetime over

three consecutive runs in both cases. This could be attributed to homogeneously dispered NPs in co-existance with Au(I) and Au(III) ions and to the high surface area (248 m^2 g^{-1}) obtained within these aerogels. The same group[38] extended the research in this area considering the immobilization of metal cations instead of NPs onto modified chitosan aerogels. Thereby, chitosan aerogels were reacted with different *N*-based ligands *via* Schiff base formation and loaded with Cu(I) ions in a range between 0.5 and 2.1 mmol Cu g^{-1} catalyst.



Scheme 7. Comparison of the influence of Pd NPs embedded in Ca^{2+}- and Cu^{2+}-alginate aerogels on the Suzuki-Miyaura reaction.

The modified gel was applied in the (3+2) Huisgen cycloaddition (Scheme 8) between different azides (**35**) and alkynes (**36**) in EtOH or water. Thereby the corresponding products could be obtained with high yields and an exclusive 1,4-regioselectivity.



Scheme 8. (3+2) Huisgen cycloaddition between different azides and alkynes catalyzed by Cu(I) immobilized on ligand-modified chitosan aerogels. The three most active ligands are summarized.

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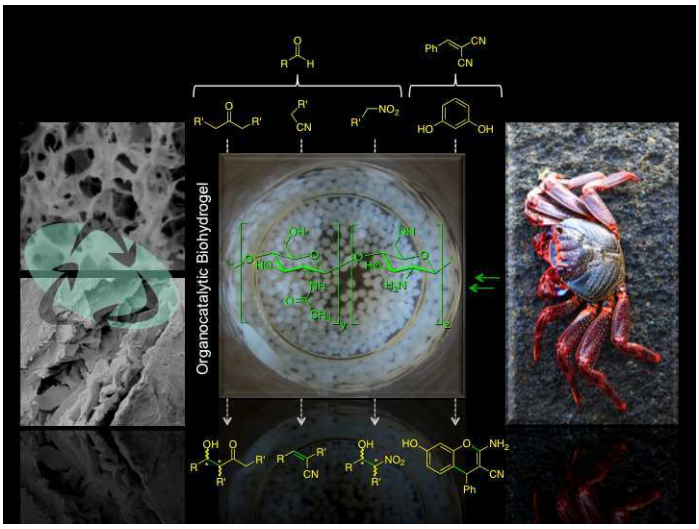
D Main Part

1. Critical Assessment of the Efficiency of Chitosan Biohydrogel Beads as Recyclable and Heterogeneous Organocatalyst for C-C Bond Formation[i]



The effectiveness of neutral pH chitosan hydrogel beads (CSHB) as a green organocatalyst for a variety of C-C bond forming reactions (i.e. aldol reaction, Knoevenagel condensation, nitroaldol (Henry) reaction, Michael addition) has been comprehensively evaluated. Reaction rates, conversions and selectivities were studied as a function of a series of input variables including size, pH and reactive surface area of the beads, catalyst loading, temperature, molecular weight of the biopolymer, concentration, solvent system and molar ratio of reactants. Moreover, the catalytic biohydrogel beads were characterized by a variety of techniques including, among others, SEM, FT-IR, TGA and DSC.[ii]

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[i] Reproduced with permission from: D. Kühbeck, G. Saidulu, K. R. Reddy, D. D. Díaz, *Green Chem.* **2012**, *14*, 378-392. Copyright 2012 The Royal Society of Chemistry.

[ii] Entries 3-6 and 9-11 in Table 1, entries 1-2 in Table 3 and entries 1-2 in Table 4 and AAS measurements were performed by G. Saidulu. All other experiments were carried out by D. Kühbeck.

1.1 Introduction

With growing concern for our environment and stringent environmental regulations by governments, the emphasis of science and technology is shifting more and more from petrochemical-based feedstocks towards the optimal use of environmentally friendly and sustainable resources and processes.[1] In this regard, direct utilization of products derived from naturally occurring materials has become a prevalent means for a number of high-tech applications.

Within this context, and during the past few decades, biopolymers have attracted increasing attention in both academic and industrial worlds owing to their unique properties, such as biodegradability, biocompatibility and antibacterial activity.[2] Among these biopolymers, cellulose and chitin are the most important biomass resources and the most abundant organic compounds on Earth.[3] Chitin, poly(β-(1 → 4)-*N*-acetyl-D-glucosamine), is the main component of the cell walls of fungi, the exoskeletons of arthropods such as crustaceans and insects, the radulas of molluscs and the beaks of cephalopods.[4] Depending on its source, chitin occurs as two allomorphs, namely the α and β forms, and it is usually extracted by acid treatment to dissolve calcium carbonate followed by alkaline treatment to solubilise proteins.[5] Chitosan, the most important derivative of chitin, can be obtained by extensive deacetylation under alkaline conditions (Figure 1).[6] However, chitosan is rarely 100% deacetylated resulting actually in a hydrophilic random copolymer of β-(1–4)-linked glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit). Their relative ratio defines the degree of deacetylation (DDA) that controls important properties of the polymer such as its basicity, viscosity and solubility, which are also influenced by the polymer's molecular weight.[7] Indeed, the intrinsic pK_a of chitosan depends on the DDA, the ionic strength and the charge neutralization of amine groups. In practice, it usually lies within 6.3-6.7 for completely neutralized amine functions when the deacetylation does not exceed 50%, which leads to protonation in aqueous acidic solution with a charge density of the resulting polyelectrolyte dependent on the exact pH and DDA values.[8],[9] Perhaps one of the biggest advantages of chitosan as a raw material is that its dilute acidic solutions can be readily cast into films and fibers, or coagulated into well-defined spherical particles by spraying into alkaline solution.

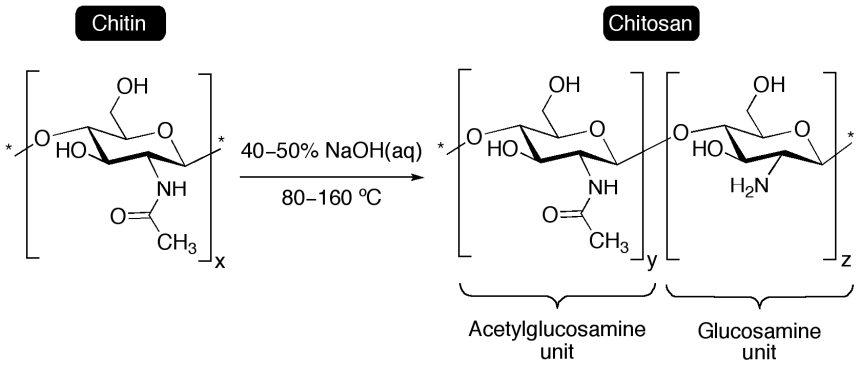


Figure 1. General preparation of chitosan by deacetylation of chitin under alkaline conditions, which are chosen depending on the biopolymer source and the desired DDA.[10]

While chitosan has been widely used in agriculture, food, and biomedical applications,[4],[11] such physical-chemical versatility and good processability has driven its use also in the field of heterogeneous catalysis, especially during the last decade.[12] The presence of both hydroxyl and amino groups in the chitosan make it useful as a chelating agent. Most studies in this area have focused on exploiting its complexation properties with metal ions and as a polymeric matrix for the synthesis of nanoparticles.[12],[13],[14] Although chitosan-supported organocatalysts have been recently reported,[15] the direct use of this amine-containing biopolymer, as a green chemistry approach,[16] in base catalysis has so far been scarcely investigated. In the other hand, it is known that the chitosan normally has a very low surface area (ca. 1.58 $m^2\ g^{-1}$), their aerogels can display a surface area up to 350 $m^2\ g^{-1}$ with high content of accessible basic sites (up to 5.8 mmol g^{-1} of $-NH_2$ groups).[17] This was exploited by Quignard and co-workers to prepare chitosan aerogel microspheres, obtained under supercritical CO_2 conditions, and used as a catalyst for the synthesis of monoglyceride by fatty acid addition to glycidol in toluene at 70 °C.[18] They have also reported very recently the use of chitosan aerogel as a recyclable, heterogeneous organocatalyst for the asymmetric direct aldol reaction in water.[19] Shukla and co-workers have described the use of powered chitosan, prepared through the hydrogel synthesis route, as a high-temperature catalyst for the synthesis of jasminaldehyde by the Claisen-Schmidt condensation of 1-heptanal and benzaldehyde under solvent-free conditions.[20] In 2006, some of us showed a preliminary study on the potential of chitosan hydrogel beads (CSHB) as a recyclable organocatalyst for both aldol and Knoevenagel reactions in DMSO.[21] In the field of low-molecular-weight (LMW) gels (i.e. gels

made of proline-containing LMW gelators) as self-supported heterogeneous selective catalysts, the more recent seminal work from Miravet, Escuder and co-workers[22] should also be featured.

The preliminary studies carried out in our group dealing with the use of CSHB in organocatalysis provided some ambiguous results, which motivated us to investigate this material in more detail. Thus, we report here the results of a comprehensive study aimed to gain a better understanding of the exact role of chitosan hydrogel used directly as a green organocatalyst for C-C bond forming reactions (i.e. atom-economical reactions) - which are in the broad sense a prerequisite for all life on earth - and the variables that can impact its performance.

1.2 Results and Discussion

Preparation and characterization of the catalyst

Catalyst preparation

In order to evaluate the scope of CSHB as organocatalyst, uniform-size spherical hydrogel beads were prepared by adaptation and optimization of reported procedures based on the alkaline coagulation of an acidic viscous chitosan solution added using a dropping funnel (Figure 2A). Thus, almost spherical shaped beads with narrow size distribution (average diameter = 4.0 ± 0.1 mm) were reproducibly obtained by adjusting the distance between the tip of the dropping funnel and the coagulating medium to 1.5 cm and a falling rate of drops controlled at approximately one drop per second (Figure 2B-C). CSHB with a mean diameter of 2.2 ± 0.2 mm were obtained using a syringe equipped with a 0.8 mm diameter needle. One of the most critical aspects during the evaluation of the CSHB catalyst is the meticulous washing protocol of the matured beads, thereby ensuring the removal of trapped hydroxyl ions (OH^-), which otherwise might influence the expected base catalysis by the free amino groups $-NH_2$ on the chitosan backbone, upon slow diffusion-controlled leaching of OH^- during the reaction. In order to demonstrate this hypothesis, different batches of beads at different pH were prepared by tuning the wash procedure. The exact pH of the filtrate in each case

was measured using a pre-calibrated pH meter. The use of previously reported pH indicators like phenolphthaleine,[21] proved to be unreliable for this study.[23] The general correlation of the pH of the filtrate with the internal pH of the beads was checked by extensive trituration-dissolution of the beads and measuring the pH of the resulting solution.

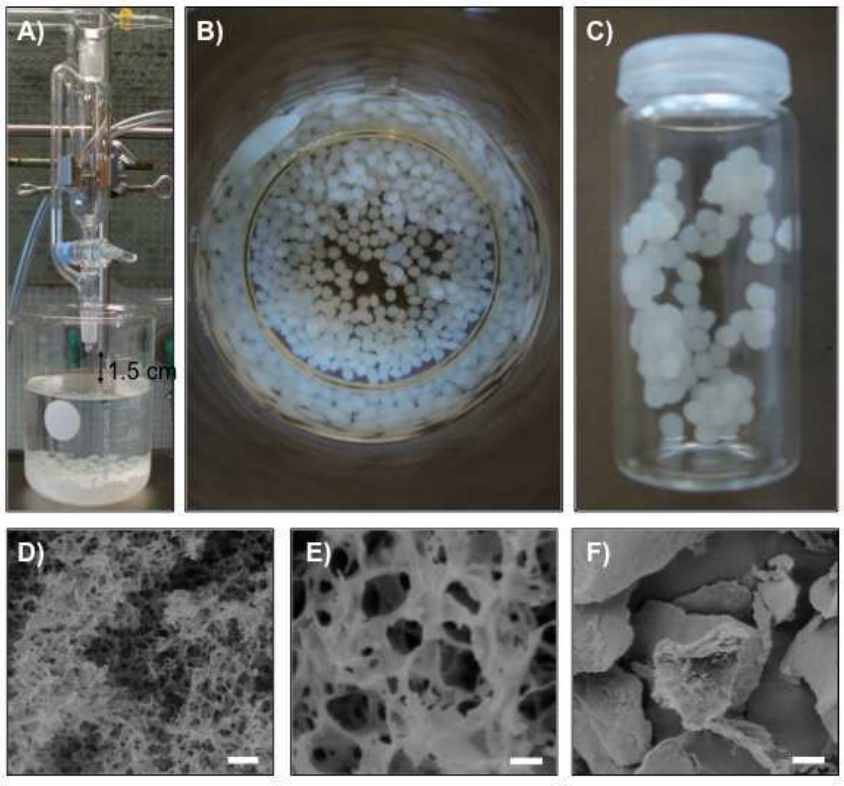


Figure 2. A) Experimental setup used for the preparation of spherical CSHB with an average diameter of 4.0 ± 0.1 mm from commercially LMW chitosan. B) Aspect of CSHB during the maturing process in NaOH aqueous solution. C) Macroscopic view of milk-white coloured CSHB after maturing. D) Representative SEM image of the freeze-dried cryogel beads made from the CSHB (scale bar 5 μm; magnification 2000×). E) Zoom in of picture D) (scale bar 1 μm; magnification 10000×). F) SEM image of commercially powdered chitosan (PCS) (scale bar 20 μm; magnification 500×).

Morphology

Scanning electron microscopy (SEM) investigations of the corresponding freeze-dried CSHB showed the heterogeneous porous nature and well-developed networks of the beads with internal pores up to 2 μm in diameter (Figure 2D-E), in contrast to the amorphous structure of commercially powdered chitosan (Figure

2F). Such heterogeneous and layered structure of the CSHB surface can strongly favour the adsorption of small molecules and ions presented in the medium via electrostatic interactions (non-specific or physical adsorption), hydrogen bonding and/or van der Waals forces.[24]

The average porosity of the beads regardless of the diameter was estimated in 74 ± 2%, with a calculated moisture content of 94 ± 1%. The aqueous swelling of the chitosan was translated in a much higher percentage of accessible -NH_2 groups (55–65%) in comparison to both powdered commercially chitosan and dried chitosan beads (2.5 and 1.7%, respectively),[21] which should enhance the potential base catalytic activity of the former.

FTIR spectroscopy

The FT-IR spectrum of PCS showed the expected bands at 1645 cm^{-1} (amide I, C=O stretching), 1588 cm^{-1} (N-H angular deformation of amino groups), 1420 cm^{-1} (-CH_2 bending vibration), 1377 cm^{-1} (C-O stretching of primary alcoholic groups -polysaccharides conformation sensitive area-), 1321 cm^{-1} (amide III), as well as the bands corresponding to the symmetric stretching of C-O-C in the region 1010-1090 cm^{-1}. The broad band between 2990-3600 cm^{-1} corresponds to -OH and -NH stretching absorption, whereas the aliphatic C-H stretching can be observed between 2850-2950 cm^{-1} (Figure 3).

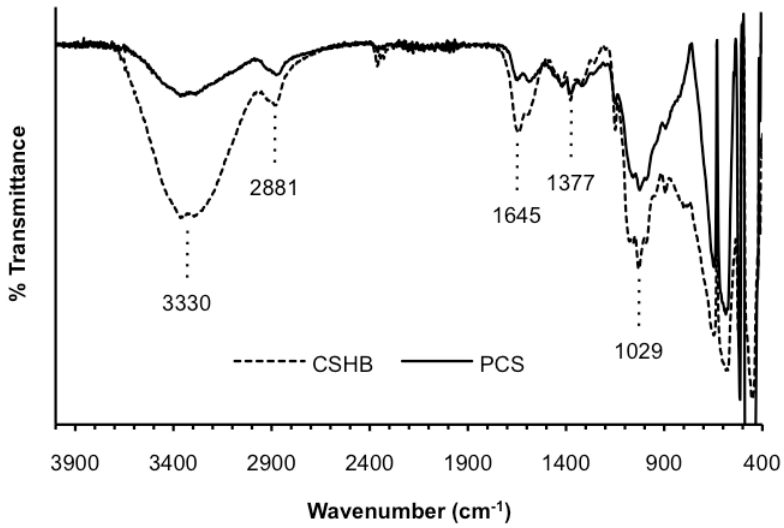


Figure 3. FT-IR spectra of PCS and CSHB (4 mm diameter).

In the other hand, 4 mm diameter CSHB showed also the broad but more intense peak between 2990-3650 cm^{-1} related to the stretching vibrations of the -OH and -NH groups also involved in hydrogen bonding. The band at 2881 cm^{-1} is again attributed to C-H stretching, whereas amide II band, N-H bending, C-O stretching of acetyl groups and free $-NH_2$ groups converge in the area between 1600-1645 cm^{-1}. As for PCS, the zeta potential of CSHB[25] is also defined by the protonation/deprotonation features of the amine groups since it is positive in acidic solutions and negative in basic solutions, with a point of zero found at about pH 6.6, which is close to the pK_a values for the $-NH_2$.[26]

Thermal characterization

(1) Thermogravimetric analysis (TGA): TGA curve of CSHB showed expected weight loss at two stages (see ESI). The first one (ca. 95% weight loss) was found in the region below 250 °C, which is attributed to the water content and in agreement with the estimated value (the weight loss in the same region for solid samples due to absorbed atmospheric water was ca. 1.8%). The second weight loss for CSHB (ca. 2.3%) was observed in the region between 250 and 450 °C, which is attributed to the decomposition of the polysaccharide chain by comparison with the TGA spectrum of the solid samples (ca. 50-60% weight loss in the same region). In general, the decomposition temperature of chitosan is molecular weight dependent (the lower the molecular weight, the lower the degradation temperature).[27] The lower decomposition pattern of the CSXG with respect to PCS is attributed to the higher packing density of the former. (2) Differential scanning calorimetry (DSC): The DSC thermograms (see ESI) of commercially PCS and CSHB were consistent with the above TGA and literature data.[28] PCS showed an expected exothermic peak centered at 294 °C, which corresponds to the degradation of the biopolymer backbone, whereas the CSHB showed also a broad endothermic peak centered at 115 °C, which is properly ascribed to the loss of water (the equivalent peak due to evaporation of the absorbed water in PCS was centered at 96 °C). The corresponding exothermic peak of the CSHB was centered at 289 °C.

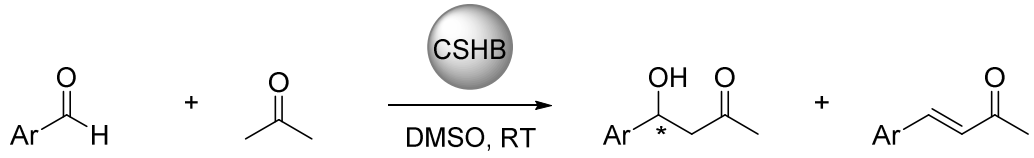
Catalyst performance in the aldol reaction

Despite the low pK_a value of the amine group in the chitosan, there are a few aspects that should be taken into consideration when testing the catalytic potential of chitosan-based materials: 1) For monoamines, there is only a single pK_a value, but for polyamines the actual number of pK_as is related to the total number of amine groups in the polymer. Thus, the pK_as are used to calculate the overall concentration of conjugate base present for a given amine,[29] which ultimately is influenced by the polymer polydispersity index (PDI), polymer chain length and the length of the spacer between amines. 2) In general, amines are more basic in polar aprotic solvents (e.g. DMSO) than in water. In the context of gel systems, it is also worth considering the potential enhancement of basicity of the system upon gel formation,[30] which could take place on the aminated surface of CSHB. 3) The possibility of thermodynamic control in amine-catalyzed Aldol-type reactions involves several reversible steps and a modest exothermicity in reaction with aldehydes, which contribute to the success of the reaction even when weak bases are used to produce only low concentrations of the corresponding nucleophilic intermediates.

Astoundingly, and apparently in contrast with previous observations,[21] neutral pH CSHB with average diameters of 4.0 ± 0.1 mm showed very low activity towards direct aldol reactions between 4-nitrobenzaldehyde (**1a**) as acceptor and acetone (**2**) (model reaction I) or cyclohexanone (**5**) (model reaction II) as donor in DMSO. The reaction was initially run at RT using 17 mol% of catalyst[23] in agreement with previous report.[21] A molar ratio aldehyde:ketone 1:13.6 was employed to minimize self-condensation of the acceptor and favour cross-condensantion.[18],[20],[21] No product formation was detected in control experiments without catalyst, using dried gel beads instead of CSHB or using commercial PCS (see ESI, Table S2).[31] Nevertheless, instead the expected quantitative conversion of the aldehyde,[21] only 4% conversion was achieved after 24 h.[32] No significant improvement was observed neither by increasing three-fold the catalyst loading nor at higher temperature.[23] The small differences were observed within the experimental error. In spite of the extremely low conversions, the ratio aldol product:dehydration product (99:1) was in agreement with that previously reported.[21],[33]

With this set of data in our hands, and during the preparation of several CSBH batches, we realized that “just” a problem in controlling the washing step in the catalyst preparation could perhaps explain, at least to some extent, the observed enormous discrepancy with the previous report where phenolphthalein-indicator was used to monitor the pH of the filtrates.[21] In order to demonstrate this hypothesis, several CSHB batches were prepared under different washing protocols to guarantee hydrogel beads with different basicity. Thus, pH dependent experiments could be performed as described in Table 1. Neutral CSHB afforded only 12.3% (Table 1, entry 6) conversion when 4-nitrobenzaldehyde was used as model substrate, which did not show conversion in the absence of the catalyst (Table 1, entry 13). In general, the use of slightly basic CSHB resulted in an expected conversion enhancement (Table 1, entries 7, 9, 10, 12), which could be further enhanced by longer reaction times (Table 1, entry 8 *vs.* 7). Such correlation is even reflected in those cases where further washings were done for the beads from the same batch, resulting in a drastic reduction in conversion (Table 1, entry 7 vs. 1, entry 9 *vs.* 6 or 2). Therefore, the low activity of the phenolphthalein-indicator at pH < 8.3 (colour change interval = 8.3-10, from colourless at pH < 8.3 to fuchsia at higher pH) should be taken into consideration in order to ensure the preparation of neutral CSHB, which should be cross-checked with the electrical measurement of the proton concentration inside the beads and/or conductivity measurements of the filtrates.[34] Hence, we anticipate that the earlier reported aldol conversions using CSHB could be determined under basic conditions rather than a neutral environment due to sufficient trapping of hydroxide ions,[21] which would indeed enhance the catalysis. This could also explain the drop of the conversion after a second run of the CSHB at pH 7.87 (Table 1, entry 10 *vs.* 11). In contrast, CSHB batches displaying pH values between 6.57 and 6.87 showed very little activity in the case of 4-nitrobenzaldehyde (Table 1, entries 1-2) and no activity whatsoever in less activated substrates like 4-chlorobenzaldehyde (Table 1, entry 4) or 2-napthaldehyde (Table 1, entry 5) under identical conditions to those previously reported,[21] indicating that simply the accessible free primary amino groups presented in these hydrogel beads of the native chitosan are not active enough to promote satisfactorily the formation of the required enamine intermediate[35] under the present hydrogel conditions.

Table 1. Correlation between pH of CSHB and conversion to the β-hydroxycarbonyl aldol product.[a]



1a-c **2** **3a-c** **4a-c**

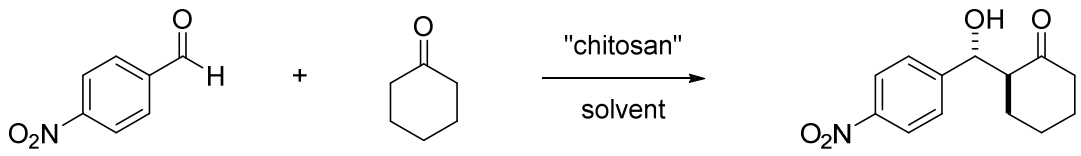
Entry	ArCHO	pH[b]	Time (h)	Conversion (%)[c]
1	4-Nitrobenzaldehyde (**1a**)	6.57	24	6
2	4-Nitrobenzaldehyde (**1a**)	6.61	24	8
3	4-Nitrobenzaldehyde (**1a**)	6.87	24	4
4	4-Chlorobenzaldeyhde (**1b**)	6.87	18	0
5	2-Naphthaldehyde (**1c**)	6.87	18	0
6	4-Nitrobenzaldehyde (**1a**)	7.00	24	12
7	4-Nitrobenzaldehyde (**1a**)	7.34	24	39
8	4-Nitrobenzaldehyde (**1a**)	7.34	30	59
9	4-Nitrobenzaldehyde (**1a**)	7.62	24	30
10	4-Nitrobenzaldehyde (**1a**)	7.87	24	45
11	4-Nitrobenzaldehyde (**1a**)	7.87	24	31[d]
12	4-Nitrobenzaldehyde (**1a**)	10.96	24	42
13	4-Nitrobenzaldehyde (**1a**)	-[e]	24	0

[a] Reaction conditions: **1a**-**c** (1.0 mmol), **2** (1 mL, 13.6 mmol), beads number = 20 (corresponding to 17 mol% of free amino groups with respect to the aldehyde), DMSO (3 mL), RT. CSHB used in the following entries correspond to the same batch preparation: Entries 1, 8 and 9 (batch 1); entries 2, 6 and 12 (batch 2); entries 11 and 12 (batch 3); [b] Herein, the reported relative pH values correspond to the filtrate after washing; [c] Determined by ^{1}H NMR of the crude product based on the aldehyde proton (see ESI). Estimated relative error = ± 2; [d] Result of the first recycling of entry 10; [e] Control experiment in which no catalyst was employed. Note: The selectivity **3**:**4** was estimated as 99:1 based on ^{1}H NMR analysis.

Despite the fact that CSHB would in principle fulfil the requirements to alter the selectivity of the reaction (in the case in which the beads act as a nanoreactor),[36] no induction of stereoselectivity due to the chiral backbone of the biopolymer was observed.

To ensure that partial volatilization of acetone (b.p. = 50.5 °C at 760 mm Hg) was not decreasing the reaction rate, we also tested the reaction between **1a** and the non-volatile cyclohexanone (**5**) (b.p. = 155.7 °C at 760 mm Hg), which has similar basicity in DMSO (pK_a = 26.4 for **2** and 26.5 for **5**) and represents a well-studied substrate for comparison in the same reaction.[15a],[19] Taking into consideration the latest results reported on the use of chitosan aerogels as catalyst for the asymmetric aldol reaction in water,[19] both DMSO and water were used to evaluate the performance of CSHB. No conversion was observed when the reaction was carried out in absence of any chitosan-based material (Table 2, entries 1 and 3). Similarly, no conversion was observed after 48 h when commercially LMW PCS was used in DMSO (Table 2, entry 2), and only 8% when the reaction was carried out in H_2O (Table 2, entry 4). In the latter case, a moderate anti:syn diastereoselectivity (68:32) was achieved. As expected from these results, the use of PCS in H_2O:DMSO 1:1 (v/v) afforded almost no conversion whatsoever (Table 2, entry 5). Disappointedly, when we tested our CSHB as catalyst, the reaction conversion was only slightly increased to 13% in H_2O (Table 2, entry 6) with almost no detriment in the diastereoselectivity (69:31), 6% in H_2O:DMSO 1:1 (v/v) (Table 2, entry 8), and 3% in DMSO (Table 2, entry 7). In the latter case, the anti:syn ratio dropped by ca. 60% in comparison to the reaction in pure water. Addition of toluene as a co-solvent to improve the solubility of the reactants in the aqueous medium provoked slightly deterioration of both conversion and selectivity (Table 2, entry 9 vs. 6). These findings would be in agreement with the key role of solvent, and especially the beneficial effect of water, in base-catalyzed aldol-type reactions.[21],[37] Unfortunately, our CSHB were found to be ca. 5 times less effective (in terms of yield but not in terms of selectivity) than the chitosan aerogel, PCS and CSHB formulations reported previously by Quignard and co-workers under analogous conditions (Table 2, entry 6 vs. 10-11).[19] At this point, and in agreement with previous observations,[19] we checked that the use of slightly more diluted conditions (0.2 M vs. 0.3 M), a higher excess of ketone (20 eq. vs. 13.6 eq.) and 5 mol% increased catalyst loading, provided a remarkable enhancement of the conversion in both PCS and CSHB-catalyzed processes with almost no variation in the diastereoselectivity (Table 2, entries 4 and 6).

Table 2. Aldol model reaction II between 4-nitrobenzaldehyde (**1a**) and cyclohexanone (**5**) under different conditions.[a]



1a **5** **6a**

Entry	Solvent[b]	Catalyst	Conversion (%)[c]	dr (anti/syn)[c]
1	DMSO	-	0	-
2	DMSO	PCS[d]	0	-
3	H_2O	-	0	-
4	H_2O	PCS[d]	8 (46[e])	68:32 (69:31[e])
5	H_2O:DMSO (1:1)	PCS[d]	2	61:39
6	H_2O	CSHB	3	56:44
7	DMSO	CSHB	3	56:44
8	H_2O:DMSO (1:1)	CSHB	6	70:30
9	H_2O:toluene (4:1)	CSHB	9	66:34
10[f]	H_2O	CSHB	75[h]	68:32
11[g]	H_2O	CSAB	86[h]	70:30

[a] Reaction conditions: **1a** (1.0 mmol), **5** (13.6 mmol), pH = 6.80, beads number = 20 (corresponding to 17 mol% of free amino groups with respect to the aldehyde), solvent (3 mL), 48 h, RT; [b] The amount of water held by the CSHB (20 beads) was estimated in ca. 0.5 mL, which is not included in the total volume of the solvent described in the reaction conditions; [c] Determined by ^{1}H NMR spectroscopy of the crude product. Batch-to-batch estimated relative error = ± 0.5%. Relative configurations were assigned by comparison with reported literature data. [d] Powdered chitosan: 28 mg (corresponding to 17 mol% free amino units with respect to the aldehyde); [e] Result obtained under the following conditions: 22 mol% free amino groups with respect to the aldehyde, 0.1 mmol **1a**, 2.0 mmol **5**, 0.5 mL H_2O, 48 h, RT; [f] Data reported in ref. [19] using different CSHB under the conditions specified in footnote [e]. No specific details regarding the preparation and characterization of those CSHB used were given, which would be necessary for a precise comparison with our beads; [g] Data reported in ref. 19 using chitosan aerogel beads (CSAB) as catalyst under the conditions described in footnote [e]; [h] Isolated yield of the β-hydroxycarbonyl compound **6a**.

Hence, we decided to evaluate which of the different parameters represent a significant contribution to the conversion (Figure 4). The results of the

experimental design clearly showed that only the reaction scale caused a major impact (e.g. 75% conversion for F vs. 7% for E).

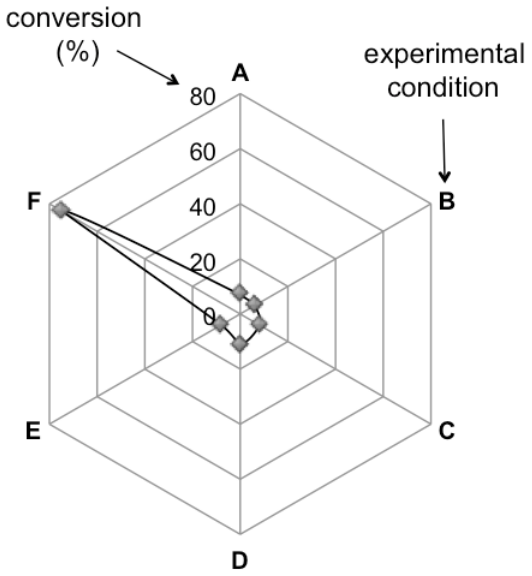


Figure 4. Radial diagram showing the effect of catalyst loading (**B** vs. **C**), molar ratio (**A** vs. **B**; **C** vs. **D**), concentration (**A** vs. **E**) and reaction scale (**E** vs. **F**) on the conversion of the aldol reaction between **1a** and **5** catalyzed by 4 mm diameter CSHB. Experimental conditions: **A** = 22 mol% catalyst, 1 mmol **1a**, 20 mmol **5**, 0.3 M in **1a**; **B** = 22 mol% catalyst, 1 mmol **1a**, 13.6 mmol **5**, 0.3 M in **1a**; **C** = 17 mol% catalyst, 1 mmol **1a**, 20 mmol **5**, 0.3 M in **1a**; **D** = 17 mol% catalyst, 1 mmol **1a**, 13.6 mmol **5**, 0.3 M in **1a**; **E** = 22 mol% catalyst, 1 mmol **1a**, 20 mmol **5**, 0.2 M in **1a**; **F** = 22 mol% catalyst, 0.1 mmol **1a**, 2 mmol **5**, 0.2 M in **1a**. Constant conditions: 48 h, RT. Estimated error = ± 0.5%.

In the other hand, the effect of the molecular weight of the chitosan was found to be statistically insignificant in this reaction in terms of conversion and selectivity. For example, the use of medium (MMW) or high molecular weight (HMW) PCS lead to 51% conversion (anti/syn = 71:29) or 78% conversion (anti/syn = 68:32), respectively. These values are in the same range than those obtained under the same conditions using LMW PCS (46% conversion, anti/syn = 69:31 (Table 2, entry 4). A similar behaviour was observed with the hydrogel beads, which lead to 13% conversion (anti/syn = 70: 30) in the case of MMW chitosan or 7% conversion (anti/syn = 72:28) in the case of HMW chitosan (for comparison, see Table 2, entry 6).[38] With these results in hand, we decided to explore also some other important variables that could greatly influence the catalyst performance in the case of the hydrogel beads. The foregoing findings and a meticulous study of the experimental

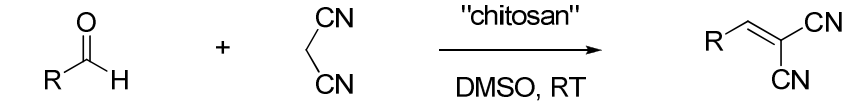
details provided for the preparation of similar CSHB,[39] motivated us to evaluate first the foreseeable effect of the surface area to volume ratio (SA:V) of the CSHB in the aldol reaction rate. For a given shape, high SA:V decreases linearly with increasing size and provides a strong driving force to speed up chemical reactions upon minimization of thermodynamic free energy. In this context, smaller spherical CSHB (2.2 ± 0.2 mm in diameter) were also tested in the aldol model reactions. However, no significant difference was observed compared to 4-mm CSHB in terms of conversion and selectivity. The result most likely suggests, at least within the studied size range, a basicity mismatch effect rather than a SA:V effect. Moreover, in contrast to the aerogels,[19] the addition of 20 mol% of 2,4-dinitrophenol as a catalyst for the formation of the enamine intermediate did not provide better results in the case of CSHB (17 mol%) for the model aldol reaction I (conditions: 18 h, RT).

Knoevenagel reaction

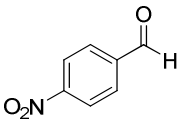
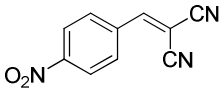
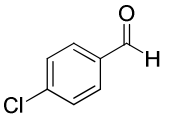
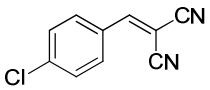
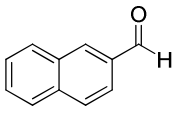
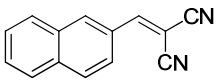
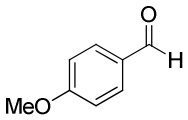
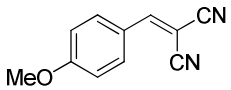
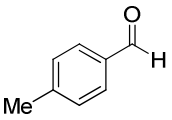
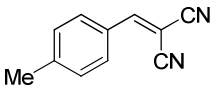
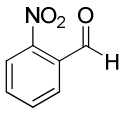
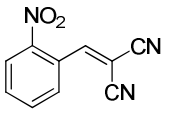
With the lessons learned from the case of the aldol reaction, we further re-evaluated the neutral CSHB as organocatalyst for the Knoevenagel condensation reaction at RT in DMSO. This modification of the aldol condensation was tested for an expanded variety of aromatic, heteroaromatic and aliphatic aldehydes in combination with activated methylene compounds as donors including malononitrile (pK_a(water) = 11.1; pK_a(DMSO) = 11.10), ethylcyanonacetate (pK_a(DMSO) = 13.10), barbituric acid (pK_a(water) = 4.01) and Meldrum's acid (pK_a(DMSO) = 7.33). Good to excellent conversions (i.e. 55-100%) to the desired condensation product were quickly achieved in the reaction between malononitrile and a variety of aromatic, heteroaromatic and aliphatic aldehydes (Table 3). Thereby, aryl compounds with electron withdrawing groups (e.g.Table 3, entries 1-2) afforded approximately 1.6-fold higher conversion[40] rate than those with electron donating groups (e.g.Table 3, entries 4-5). The desired condensation product was also obtained in good yield with more hydrophobic substrates such as 2-naphthaldehyde (Table 3, entry 3). The reaction rates in the case of aliphatic aldehydes (Table 3, entries 9-10) were comparable to those observed for activated aromatic aldehydes. Double condensation in the case of terephthalaldehyde was also achieved in very good yield without difficulties (Table 3, entry 8). In

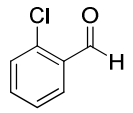
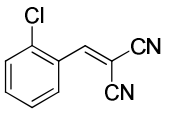
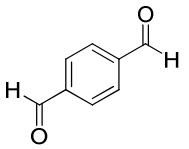
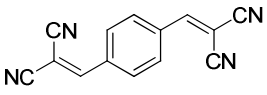
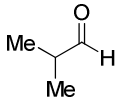
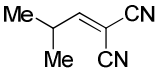
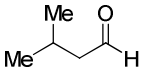
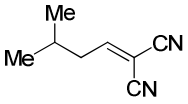
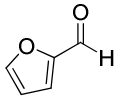
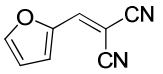
agreement with previous observations,[41] heteroaromatic substrates like 2-furaldehyde were found to react more slowly than other aromatic aldehydes (Table 3, entry 11). It was previously reported that the reaction rates in Knoevenagel condensations are slowed when bulky reagents were used.[42] However, the use of 2-substituted isomers (Table 3, entries 6-7) provided the same results as the 4-substitued isomers (Table 3, entries 1-2), which indicates a negligible effect of the steric effect of these 2-substitued isomers in our system, albeit other substituents with higher A-values were not evaluated (A-value(Cl) = 0.43 kcal mol^{-1}; A-value(NO_2) = 1.1 kcal mol^{-1}).

Table 3. Knoevenagel condensation reaction between different aldehydes **1a**-**k** and malononitrile (**7**) catalyzed by CSHB in DMSO at RT.[a]



R = aryl, alkyl **1a-k** | **7** | **8a-k**

Entry	Aldehyde 1a-k	Product 8a-k	Time (min)	Conversion (%)[b]
1	 **1a**	 **8a**	5	99
2	 **1b**	 **8b**	5	100 (92[c])
3	 **1c**	 **8c**	5	83
4	 **1d**	 **8d**	5	62
5	 **1e**	 **8e**	5	100 (93, 80)[d]
6	 **1f**	 **8f**	5	100

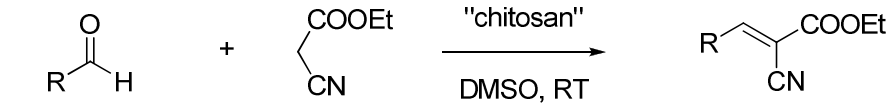
7	 **1g**	 **8g**	60[e]	100
8	 **1h**	 **8h**	5	93
9	 **1i**	 **8i**	5	55[f]
10	 **1j**	 **8j**	5	84[f]
11	 **1k**	 **8k**	30	100 (84[c])

[a] Reaction conditions: **1a**-**k** (1.0 mmol), **7** (1.1 mmol), DMSO (3 mL), mean pH = 6.9, beads number = 20 (corresponding to 17 mol% of free amine groups with respect to the aldehyde), RT; [b] Determined by ^{1}H NMR spectroscopy of the crude product based on the aldehyde proton. Batch-to-batch estimated error = ± 0.5%; [c] Yield previously reported in the literature for the uncatalyzed process in water reaction. Reaction time = 3 min for entry 2 and 30 min for entry 11; [d] Experiments using CSHB from different batches with slightly different pH values: 6.7 for 93% conversion and 6.5 for 80% conversion; [e] Reaction time was not optimized; [f] Conversion calculated in respect to malononitrile instead of the aldehyde due to the lower boiling point of the latter.

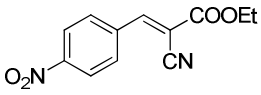
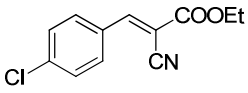
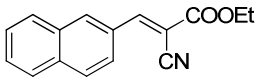
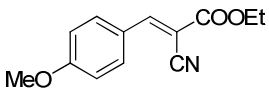
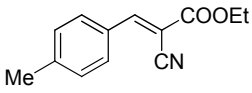
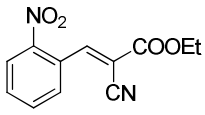
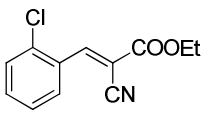
In contrast to some reports in the literature,[43],[23] at least in our hands and using the conditions depicted in Table 3, we could not observe conversion of aldehydes **1a**-**1b** within 5 min in pure water and in the absence of any catalyst. In any event, the use of efficient green base-catalysts in aprotic solvents like DMSO would overcome the limitations of working with unstable aldehydes (e.g. aldehydes containing hydrolyzable silanes, aldehydes containing water-sensitive functional groups, aliphatic or water-insoluble aldehydes like **1a**-**1b** with slow kinetic in/on pure water). As a proof-of-concept, highly reactive *tert*-butyl chloride was quantitatively hydrolyzed to *tert*-butanol in 0.3 M water after 1.5 h at RT, whereas it remained stable in DMSO and in the presence of 20 hydrogel beads (17 mol% of free -NH_2 groups; estimated amount of trapped water = 0.5 mL).

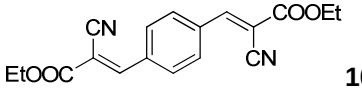
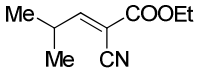
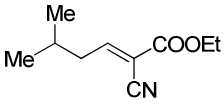
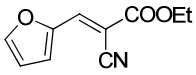
As expected, the reactions with ethylcyanoacetate were in general slower than with more acidic malonitrile (Table 4), albeit no difference in terms of catalyst stability was observed in any case. Moreover, only one geometric isomer (*E*-isomer) was obtained in all cases. Amongst the tested aryl aldehydes, only 4-nitrobenzaldehyde reacted quantitatively in 5 min (Table 4, entry 1). Less activated aldehydes needed 1 h to react completely (Table 4, entries 2, 8), which was not possible with more electron rich aryl substrates even after 5 h (Table 4, entries 4-5). Herein, low reactive substrates like 2-naphthaldehyde produced only modest conversion after 1 h (Table 4, entry 3).

Table 4. Knoevenagel condensation reaction between different aldehydes **1a**-**k** and ethylcyanoacetate (**9**) catalyzed by CSHB in DMSO at RT.[a]



R = aryl, alkyl **1a-k** | **9** | **10a-k**

Entry	Aldehyde 1a-k	Product 10a-k	Time (min)	Conversion (%)[b]	*E/Z* ratio[c]
1	**1a**	 **10a**	5	100	100/0
2	**1b**	 **10b**	5	31 (100[d], 3[e], 0[f])	100/0
3	**1c**	 **10c**	5	9 (35[d])	100/0
4	**1d**	 **10d**	5	28 (29[d], 75[g])	100/0
5	**1e**	 **10e**	5	29 (35[d], 83[g])	100/0
6	**1f**	 **10f**	20	97	100/0
7	**1g**	 **10g**	60	92	100/0

8	**1h**	 **10h**	40	100	100/0
9	**1i**	 **10i**	60	55[h]	100/0
10	**1j**	 **10j**	60	12[h]	100/0
11	**1k**	 **10k**	60	100 (46[i])	100/0

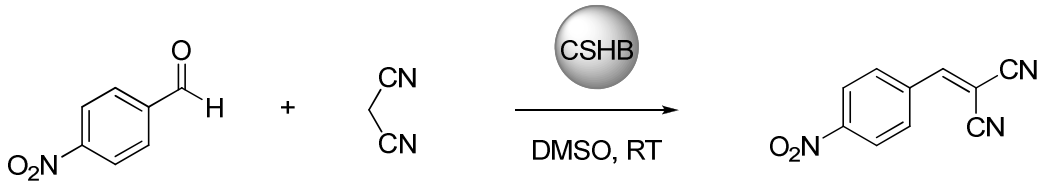
[a] Reaction conditions: **1a**-**k** (1.0 mmol), **9** (1.1 mmol), DMSO (3 mL), mean pH = 6.9, beads number = 20 (corresponding to 17 mol% of free amine groups with respect to the aldehyde), RT; [b] Determined by ^{1}H NMR spectroscopy of the crude product based on the aldehyde proton. Batch-to-batch estimated error = ± 0.5%; [c] Determined by ^{1}H NMR of the crude product; [d] Conversion after 60 min; [e] Control experiment using PCS (50 mg), reaction time = 5 min; [f] Control experiment without catalyst, reaction time = 5 min; [g] Conversion after 5 h; [h] Conversion calculated with respect to ethylcyanoacetate instead of the aldehyde due to the lower boiling point of the latter; [i] Conversion obtained under solvent-free conditions (60 min, RT).

Moreover, the steric effects showed relatively high influence in comparison to the reaction with malononitrile, which can be also overcome with longer reaction times (Table 4, entries 6-7). In general, kinetic rates for activated aromatic aldehydes were found to be higher than for their aliphatic or heteroaromatic partners (Table 4, entries 9-11). Control experiments carried out in the absence of CSHB or in the presence of PCS (Table 4, entry 2) confirmed the utility of the CSHB as heterogeneous catalyst for the Knoevenagel condensation reaction. It is also worth mentioning that in none of the cases, side reactions like self-condensation or Cannizaro products were observed. Although the studied beads also showed activity under solvent-free conditions, the use of solvent clearly facilitates the molecular collisions and the access to the CSHB -NH_2 groups (Table 4, entry 11).

Kinetic studies of a model Knoevenagel reaction between **1f** and **9** catalyzed by CSHB in DMSO (Table 4, entry 6) led to a first-order rate constant = $4.9 \pm 0.1 \times 10^{-2}$ min^{-1}, which is in the same range as that reported in the literature for comparable processes.[23] Moreover, a much lower reaction rate was observed in the case of other activated methylenes containing heterocyclic compounds such as barbituric acid or Meldrum's salt (see ESI, Table S3), most likely due to steric effects.

With the "ideal synthesis/catalysts" concept[44] in mind, we further evaluated the recovery and reusability of the catalyst in the model condensation between **1a** and **7** (Table 5). After the work-up of the reaction, no visible physical changes were observed on the CSHB catalyst surface. The catalyst was found to be easily recovered and retain full activity for at least 4 runs with high TON (> 5800) and TOF (> 1100 min^{-1}), which indicate both efficient catalyst recovery and good catalyst lifetime.[44]

Table 5. Recycling experiments in the Knoevenagel condensation reaction between **1a** and **7** catalyzed by CSHB in DMSO at RT.[a]



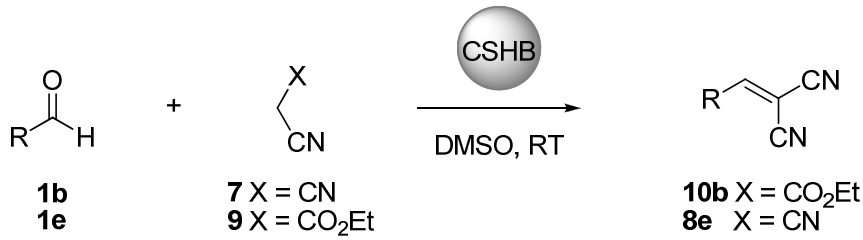
1a **7** **8a**

Entry	Catalytic cycle	Time (min)	Conversion (%)[b]	TON[c] (± 59)	TOF (min^{-1})[d] (± 12)
1	Fresh catalyst	5	99	5823	1164
2	First cycle	5	99	5823	1164
3	Second cycle	5	99	5823	1164
4	Third cycle	5	99	5823	1164

[a] Reaction conditions: **1a** (1.0 mmol), **7** (1.1 mmol), DMSO (3 mL), mean pH = 6.9, beads number = 20 (corresponding to 17 mol% free amine units with respect to the aldehyde), RT; [b] Determined by ^{1}H NMR spectroscopy of the crude product based on the aldehyde proton. Batch-to-batch estimated error = ± 0.5%; [c] Turnover number defined as the molar ratio of converted substrate to catalyst loading; [d] Turnover frequency defined as the molar ratio of converted substrate to catalyst loading per unit of time.

The general greater effectiveness of the Knoevenagel condensation in comparison to the previous aldol reaction disguises the expected pH dependence activity in the former. In spite of this, a similar trend of pH-triggered conversion could also be perceived for model Knoevenagel condensations between **1b** and **9** or **1e** and **7** (Table 6).

Table 6. Correlation between pH of CSHB and conversion towards the Knoevenagel product in DMSO at RT.[a]



1b **1e** | **7** X = CN **9** X = CO_2Et | **10b** X = CO_2Et **8e** X = CN

Entry	Aldehyde	Donor	Product	pH	Conversion (%)[b]	TON[c] (± 117)
1	**1b**	**9**	**10b**	6.49	15	882
2	**1b**	**9**	**10b**	6.99	31	1824
3	**1b**	**9**	**10b**	10.48	72	4235
4	**1e**	**7**	**8e**	6.49	80	4706
5	**1e**	**7**	**8e**	6.99	87	5118
6	**1e**	**7**	**8e**	8.44	100	5823

[a] Reaction conditions: **1b** or **1e** (1.0 mmol), **7** or **9** (1.1 mmol), DMSO (3 mL), mean pH = 6.9, beads number = 20 (corresponding to 17 mol% free amine units with respect to the aldehydeI, RT, reaction time = 5 min; [b] Determined by ^{1}H NMR spectroscopy of the crude product based on the aldehyde proton. Batch-to-batch estimated error = ± 0.5%; [c] Turnover number defined as the molar ratio of converted substrate to catalyst loading.

The concrete participation of the primary -NH_2 groups of the chitosan in the aldol-like reactions was further demonstrated by submitting the CSHB to an imine cross-link process with glutaraldehyde to form CS-N=CH$(CH_2)_3$CH=N-CS.[45] This heterogeneous cross-linking reductive amination slightly reduced the positive ζ-potential at pH < 6.7 and enhanced the mechanical stability of the beads. However, blocking the primary -NH_2 groups should cause an erosion of the CSHB for catalysis by decreasing the accessibility of substrate molecules to active basic sites. Indeed, when the model reaction between aldehyde **1f** and ethylcyanoacetate (**9**) (Table 4, entry 6; conversion = 97%) was carried out in the presence of the cross-linked CSHB, the conversion dropped drastically to ca. 36%. Some conversion is still observed most probably due to an incomplete imine cross-linking process caused by partial polymerization and/or irreversible entrapment of glutaraldehyde within the cross-linked beads.[45]

However, the foregoing results point out another significant factor that may influence the -NH_2 catalysis of CSHB. The ability of chitosan to readily form imines

in the presence of aldehydes under mild conditions could support also a potential change from amine to imine catalysis, at least to a certain extent, with a consequent modification of the surroundings of the active site. Hence, a possible combination of both amine and imine base catalysis (apparently favourable to the former) should be considered in the mechanism of CSHB-catalyzed reactions like Knoevenagel condensations, where imines are usually the key intermediates. In this sense, imine grafted silicas have been already described as mild and effective base catalysts for Knoevenagel and Michael reactions.[46]

At the core of our research perspective, the particular case of biohydrogel materials in catalysis is framed within a scientific challenge devoted towards altering the selectivity of chemical transformations by arranging the potential reactants in organized and confining media.[36] In combination with the inherent chirality of the chitosan, the high active surface area of CSHB and its 3D porous network might amplify the potential stereoselection in the aldol-like reactions. However, the fact that almost negligible enantiomeric excess (< 1%) was observed in the aldol reaction may denote a role of the biohydrogel as an immobilized base catalyst instead of a chiral nanoreactor, which is likely to be the case for aerogel microspheres. Indeed, no apparent change in light-scattering was observed for CSHB in water between 0 °C and 60 °C, which suggest a permanent close packed structure within that range of temperature. In order to further verify or falsify the above hypothesis we run some model reactions using hemispherical CSHB obtained by cutting in half the original spherical beads. For the same number of beads, the total reactive surface area (SA) will be higher for the hemispherical CSHB (Figure 5, top). Thus, the comparison between the ratio of the surface areas [$SA_{2(\text{hemispherical CSHB})}/SA_{1(\text{spherical CSHB})}$] with the ratio of the specific reaction rates [$K_{2(\text{hemispherical CSHB})}/K_{1(\text{spherical CSHB})}$] should provide insight about the reaction pathway. No major difference between these ratios would be expected if most of the substrate molecules were transformed on the CSHB surface (Figure 5, top, path b), whereas a much higher kinetic ratio would underline primary reactivity inside the beads (Figure 5, top, path a). In our case, a fairly good linear correlation between surface area of the CSHB and the ratio of the reaction rates was observed (Figure 5, bottom). Thus, the studied CSHB seems to behave most likely as a base-supported catalyst, which is also consistent with the fact that no trace of reaction product could be detected at any reaction time inside the beads. Hence,

the modest differences observed between conversions and isolated yields[40] should be attributed to partial adsorption/absorption of the starting materials onto/inside the beads and/or minor lost during product isolation, mainly due to volatility issues and/or deficient washing steps.

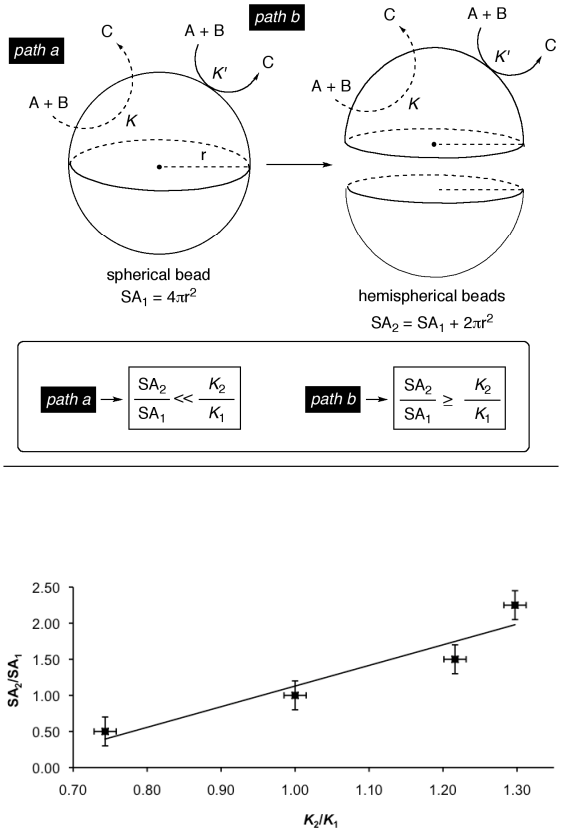


Figure 5. *Top*: Relationships between reactive surface areas (SA_1, SA_2) and reaction rate (K_1, K_2) for two potential modes of action of CSHB with diameter *r*: (1) as a nanoreactor (path a); and (2) as a supported base. Theoretical considerations were made on the basis of perfect spherical beads. *Bottom*: Surface area to reaction rate plot for the Knoevenagel condensation reaction catalyzed by CSHB.

In the other hand, the possibility of hydrogen bonding at the surface of the beads between the aldehyde and the water molecules of the hydrogel network could assist the condensation reaction at the water/organic solvent interface. In this regard, UV-vis experiments showed for example a significant adsorption of 2-nitrobenzaldehyde onto CSHB (ca. 20 mol% after 1 h at RT).[23] Diffusion studies of other small molecules in CSHB have confirmed that the solute transport through the gel beads occurs faster at higher temperatures and primarily by a Fickian

diffusion mechanism with general activation energies in the range of 20-30 kJ mol^{-1}.[47] At neutral pH, the negative zeta potential of the CSHB would indeed favour the adsorption of electron-deficient aldehydes. Experiments with metal-doped CSHB have also showed that the catalytic reaction is more likely located on the external layers of catalyst particles. In this case, the strong decrease of kinetic rates with increasing size of catalytic beads confirms a higher contribution of the resistance to intraparticle diffusion in the control of reaction kinetics.[48]

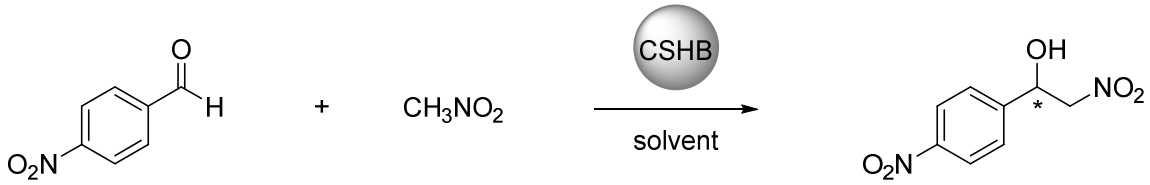
Nitroaldol (Henry) reaction

Encouraged by the latter results we decided to evaluate also the CSHB as a heterogeneous catalyst for the classical and valuable nitroaldol (Henry) reaction, which involves the reaction of nitroalkanes with carbonyl compounds (i.e. aldehydes, ketones) in the presence of an ionic or non-ionic base-catalyst to form β-nitroalcohols under a wide range of experimental conditions.[49] One of the main drawbacks of this powerful atom-economical reaction is the formation of several by-products that complicate the isolation of the desired compounds. These by-products include mainly polymerizable nitroalkenes (formed upon dehydration of the β-nitroalcohols, especially in the case of aryl aldehydes), self-condensed products in the case of sterically hindered substrates (i.e. Cannizzaro reaction epimerized β-nitroalcohols and products derived from the Nef reaction.[49a] In order to optimize the formation of β-nitroalcohols, a careful control of the basicity of the reaction medium and long reaction times are usually required. In the case of aromatic aldehydes, the selectivity of the Henry reaction is strongly dictated by the electronic nature of the substituents and their ability to favour either the imine or ion-pair mechanism.[49]

In order to evaluate the performance of the CSHB as an organocatalyst for the Henry reaction, we first investigated the model reaction between 4-nitrobenzaldehyde (**1a**) and excess nitromethane (**11**) in the presence of nearly neutral CSHB in both protic and aprotic polar solvents that are frequently used in this transformation (Table 7). As expected, the solvent used did not show a very large influence on the outcome of the reaction.[50] The reaction was driven to full conversion in 12 h at RT when either water or DMSO were used as solvent (Table

7, entries 5-6),[51] whereas in the case of MeOH or EtOH 24 h and 30 °C were needed to achieve similar results (Table 7, entries 2, 4).

Table 7. CSHB-catalyzed model nitroaldol (Henry) reaction in different solvents.[a]



1a **11** **12a**

Entry	Solvent	Catalyst	T (°C)	Time (h)	Conversion (%)[b]
1	MeOH	CSHB	20	24	33
2	MeOH	CSHB	30	24	99 (98[c], 80[d])
3	MeOH	CSHB	30	24	97[e], 92[f], 78[g], 27[h]
4	EtOH	CSHB	30	24	99
5	H_2O	CSHB	25	12	98 (15[i], 5[j])
6	DMSO	CSHB	25	12	100 (74[i], 33[j])
7	H_2O	-	25	16	2
8	DMSO	-	25	24	< 1
9	MeOH	-	30	24	0
10	MeOH	ADCSHB	30	24	0[k]
11	MeOH	PCS	30	24	37[l]
12	MeOH	PCS	30	24	99[m]

[a] Reaction conditions: **1a** (1.0 mmol), **11** (10.0 mmol), solvent (3 mL), mean pH = 6.9, catalyst beads number = 20 (corresponding to 17 mol% of free amine groups with respect to the aldehyde); [b] Determined by ^{1}H NMR spectroscopy of the crude product based on the aldehyde proton. Batch-to-batch estimated error = ± 0.5%. [c] Conversion using 15 CSHB units; [d] Conversion using 10 CSHB units; [e] Conversion using 7 equivalents of **11** with respect to **1a**; [f] Conversion using 5 equivalents of **11** with respect to **1a**; [g] Conversion using 3 equivalents of **11** with respect to **1a**; [h] Conversion using 1 equivalent of **11** with respect to **1a**; [i] Recycling experiment: Conversion after second cycle. [j] Recycling experiment: Conversion after third cycle; [k] ADCSHB = Air dried chitosan hydrogel beads ($m_{(20\ units)}$ = 28 mg); [l] Control experiment performed using 28 mg of PCS as catalyst; [m] 28 mg of PCS was used as catalyst and 0.54 mL of H_2O was added.

A temperature increment of only 10 °C was enough to increase the conversion to the desired nitroaldol product **12a** from 33% to 99% (Table 7, entry 1 vs. 2).

Regarding the aldehyde:nitromethane molar ratio, 1:10 was found to be optimum for a catalyst loading of 17 mol% (Table 7, entries 2-3).

In contrast, equimolar amounts of reactants in MeOH afforded only 27% conversion after 24 h at 30 °C (Table 7, entry 3). Nearly no conversion was observed when the reaction was run either in H_2O, DMSO or MeOH in the absence of CSHB (Table 7, entries 7-9). Interestingly, the use of air dried CSHB was also unsuccessful (Table 7, entry 10), whereas the commercialy PCS afforded a modest 37% conversion (Table 7, entry 11). The latter could be driven to 99% conversion by adding to the reaction mixture approximately the amount of H_2O estimated in 20 hydrogel beads (ca. 30 eq. of H_2O with respect to the aldehyde) (Table 7, entry 12). As pointed out by Quignard and co-workers, these results could be explained by the dramatic effect that the method used for drying these biomaterials could have on the accessibility of the surface catalytic groups.[19] In agreement with previous observations for the aldol reaction, almost no enantiomeric excess (< 1%) was detected in MeOH, H_2O or DMSO either for the nitroaldol version. In contrast to the Knoevenagel condensation reaction, the recycling model experiments carried out showed a remarkable catalyst deactivation right after the first virtually quantitative cycle (Table 7, entries 5-6), being even more severe in water than in DMSO. Although the exact deactivation mechanism in the case of hydrogel beads remains unclear, blocking of the basic catalytic sites by chemical poisoning of the surface of the beads seems to play a major role. In this sense, factors like the chemical evolution of intermediate imines, large excess of nitromethane, slow reaction kinetics and the presence of a protic solvent could contribute to the formation of an inactive coat blocking the active surface of the CSHB. In order to support this hypothesis the model nitroaldol reaction between **1a** and **11** (Table 7, entry 6) was carried out using beads previously matured under three different conditions: (A) beads matured in a solution of **1a** (1 mmol) in DMSO (3 mL) for 12 h at RT; (B) beads matured in a solution of **11** (10 mmol) in DMSO (3 mL) for 12 h at RT; (C) beads matured in a solution of **1a** (1 mmol) in DMSO (3 mL) for 5 min at RT. The conversion values obtained in each case were 23% (A), 95% (B) and 95% (C). The results point out that the time in which the reactants are in contact with the beads can be crucial for performance of the catalyst. In agreement to these results, the recycled beads in the case of the Knoevenagel condensation (reaction time = 5 min) did not show a

major detriment of the catalytic activity. In contrast, the longer reaction time observed in nitroaldol facilitates the blocking of catalytic amine groups. The results of the experiments (A) and (B) point out that the chemical evolution of intermediate imines on the bead surface, by reduction Cannizzro-like processes or formation of cross-linked aminals, play a main role in the catalyst deactivation (Figure 6, a-b).

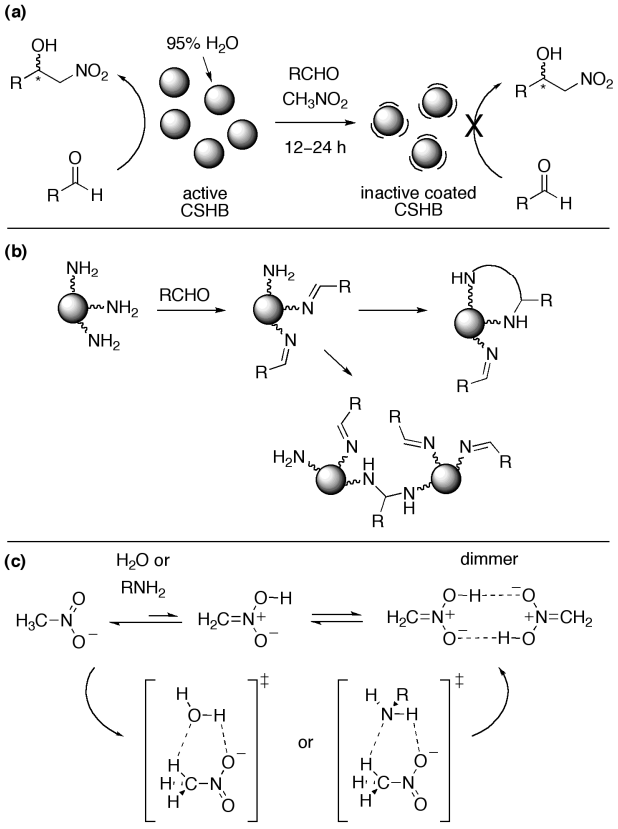


Figure 6. a) Plausible contributions to the catalyst deactivation in the nitroaldol reaction; b) formation of lineal or cyclic aminals from intermediate imines; c) formation and H-bonding stabilization of *aci*-nitromethane catalyzed by hydroxyl or amine groups.

To a lesser extent, nitroalkanes could be also partially associated with this process since they can interact with the free amine groups by formation of molecular (or charge-transfer) complexes, or with the hydroxyl groups of polysaccharides forming nitroalkyl ethers,[52] which would be in tautomeric equilibrium with the corresponding nitronic acid species (*aci*-form, $R^1R^2C{=}NOOH$). Moreover, the formation of the *aci*-form is also known to be catalyzed by water[53] or amines[54] through two stabilizing hydrogen bonds, which could also support the faster catalyst deactivation observed in pure water. Moreover, remarkable stable dimers of the *aci*-form could be established like in the case of carboxylic acids.[55] In any

event, the overall effect would be a hindered access to the free amino groups on the surface of the hydrogel beads causing a detriment in the catalytic activity (Figure 6, c). Moreover, SEM images of the catalytic beads after the nitroaldol reaction suggested the presence of both layered structures and new agglomerated moieties on the surface (Figure 7). Such clustered structure could be also observed in the case of less active glutaraldehyde cross-linked beads.

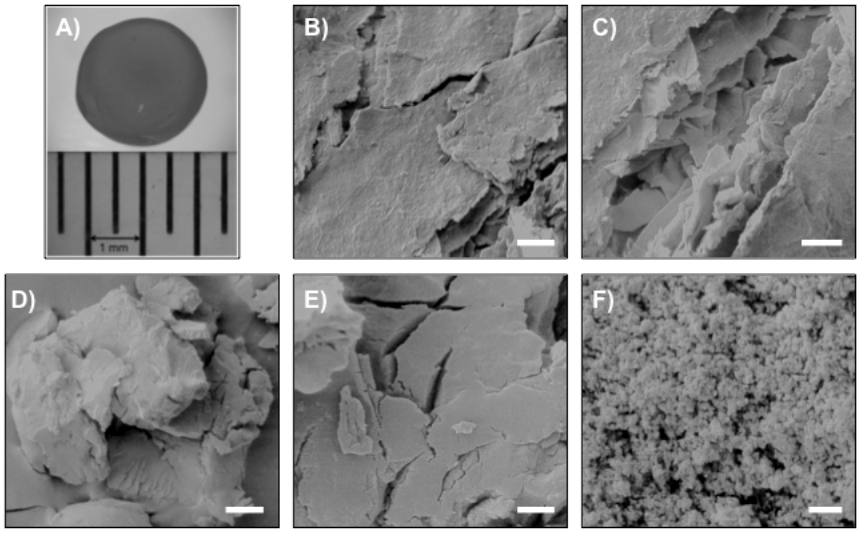
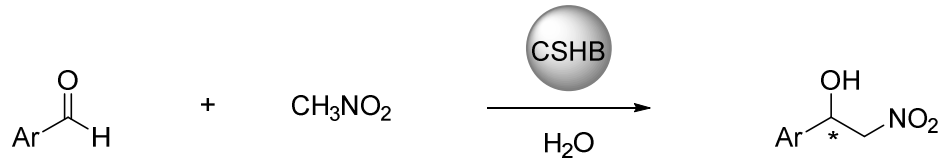


Figure 7. A) Digital photograph of a CSHB under an optical microscopy (magnification 10×). B-C) Representative SEM images showing the layered surface structure of the CSHB (B: scale bar 20 μm; magnification 500×; C: scale bar 10 μm; magnification 1000×). E)-F) Representative SEM images showing the clustered surface structure of the CSHB after the 3rd cycle in the nitroaldol reaction in water (E: scale bar 50 μm; magnification 200×; F: scale bar 10 μm; magnification 1000×). F) SEM image of the freeze-dried cryogel beads made from the glutaraldehyde cross-linked CSHB (scale bar 2 μm; magnification 5000×).

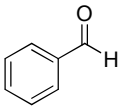
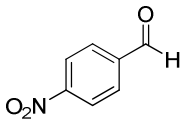
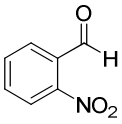
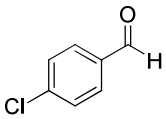
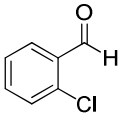
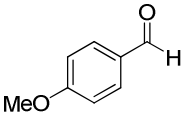
Due to the high toxicity of methanol and the best results obtained in pure water, the latter was used as the green solvent[56] to study the performance of the biohydrogel catalyst in the Henry reaction with different aldehydes (Table 8). As expected, aldehydes bearing strong electron-withdrawing groups (i.e.**1a**, **1f**) were converted to the desired nitroaldol product **12** much faster and high TON than less electrophilic aldehydes (i.e.**1l**, **1b**, **1g**) (Table 8, entries 2-3 vs. 1, 4-5). For 2-substituted isomers **1f** and **1g** no major steric effect was observed during the

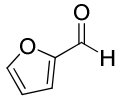
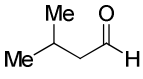
reaction (Table 8, entries 3, 5 vs. 2, 4, respectively). In the case of the less reactive chloro-substituted aldehydes like **1b**, the conversion could be enhanced to ca. 70% after 4 days at RT or up to 85% after 24 h at 40 °C. As is shown, *β*-hydroxy nitroalkanes were always obtained as the major product, except for 4-methoxybenzaldehyde and furfuraldehyde, which afforded a mixture of nitroalkane and nitroalkene (dehydrated product) in a ratio 2:1 (Table 8, entries 6-7). With aliphatic aldehydes such as isovaleraldehyde (Table 8, entry 8), almost only dehydrated product was detected.

Table 8. CSHB-catalyzed nitroaldol (Henry) reaction between different aldehydes **1** and nitromethane (**11**) in water at RT.[a]



1 **11** **12**

Entry	ArCHO	Product	Conversion (%)[b]	TON[c] (± 117)	TOF (h^{-1})[d] (± 5)
1	 **1l**	**12l**	91 (99[e])	5353	223
2	 **1a**	**12a**[f]	99[g] (99[h])	5824	243
3	 **1f**	**12f**	96[g] (99[h])	5647	235
4	 **1b**	**12b**	13 (12[e])	765	32
5	 **1g**	**12g**	10 (10[e])	588	24
6	 **1d**	**12d**	23[i]	1353	25

7	 **1k**	**12k**	16[i]	941	39
8	 **1j**	**12j**	76[k]	4471	186

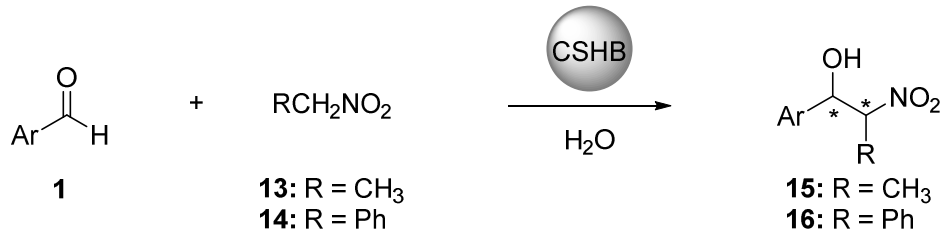
[a] Reaction conditions: Aldehyde (1.0 mmol), **11** (10.0 mmol), mean pH = 6.9, catalyst beads number = 20 (corresponding to 17 mol% of free amine groups with respect to the aldehyde), solvent (3 mL), 24 h, RT; [b] Conversion was determined by ^{1}H NMR spectroscopy based on the aldehyde proton. Batch-to-batch estimated relative error = ± 0.5%; [c] Tunrover number defined as the molar ratio of converted substrate to catalyst loading; [d] Turnover frequency defined as the molar ratio of converted substrate to catalyst loading per unit of time; [e] Conversion obtained using 28 mg of PCS as catalyst after 24 h; [f] The enantiomeric excess was determined by chiral-phase HPLC for this model reaction run: a) DMSO (ee = 0.62%), b) MeOH (ee = 0.34%), c) H_2O (ee = 0.05%). Conditions: eluent = n-Heptane/i-propanol 70/30, flow rate = 0.5 mL min^{-1}, wavelength = 215 nm, run time = 30 min; retention time (RT): RT_1 = 14.34 min, RT_2 =16.86 min; [g] Conversion obtained after 12 h using CSHB as catalyst; [h] Conversion obtained using 28 mg of PCS as catalyst after 12 h; [i] Molar ratio nitroalkane:nitroalkene product = 2:1, reaction time = 54 h; [j] Molar ratio nitroalkane:nitroalkene product = 2:1; [k] Only dehydrated product with traces of nitroalkane was observed.

We should indicate that our observations made with commercial PCS in the Henry reaction are in reasonable agreement with the data recently provided by Cui and co-workers.[15b] Nevertheless, the absence of data regarding the properties of the used chitosan makes a reliable comparison very difficult. For instance, aldehyde **1f** was nearly fully converted into the corresponding nitroalcohol product in 24 h (Table 8, entry 3), which was previously obtained in 47% (isolated yield) in 16 h.[15b] Under the assumption that the same solid chitosan material was used in both cases (e.g. same molecular weight and DDA), the observed deviations might be justified considering the difference in reaction times (e.g. 24 h vs. 16 h) and solvent concentrations (e.g. 0.3 M vs. 0.1 M in aldehyde), as well as possible loss of product during isolation/purification, to which the use of higher amounts of nitroalkane could also contribute (e.g. the ratio aldehyde:nitromethane 1:10 vs. 1:23).[15b]

We finally confirmed that the size of the nucleophilic carbanion might play a more critical role than the pK_a of the donor in the mechanism of the CSHB-catalyzed nitroaldol reaction, in a similar way that has been demonstrated for some enzyme-catalyzed examples.[57] We found that the reaction rates and

conversions in the presence of CSHB dropped significantly in the case of nitroethane (**13**) in comparison to nitromethane (**11**) (pK_a values:[57] **11** = 10.2; **13** = 8.6) (Table 9). In the other hand, several products were detected by TLC when phenylnitromethane was used as donor, but the NMR analysis of the reaction crude did not allow the unequivocal identification of the signals for the expected product. In general, CSHB performed comparatively better than PCS (Table 9, entry 2). Moreover, the experiments with nitroethane afforded anti/syn ratios 41:59, 45:55 and 45:55 for aldehydes **1l**, **1a** and **1b**, respectively, with negligible enantiomeric excess.[58] Again, a dramatic inactivation of the catalyst was observed during the recycling experiments (Table 9, entry 3).

Table 9. CSHB-catalyzed nitroaldol (Henry) reaction between aldehydes **1** and nitrolkanes **13**-**14** in water at RT.[a]



1

13: R = CH_3
14: R = Ph

15: R = CH_3
16: R = Ph

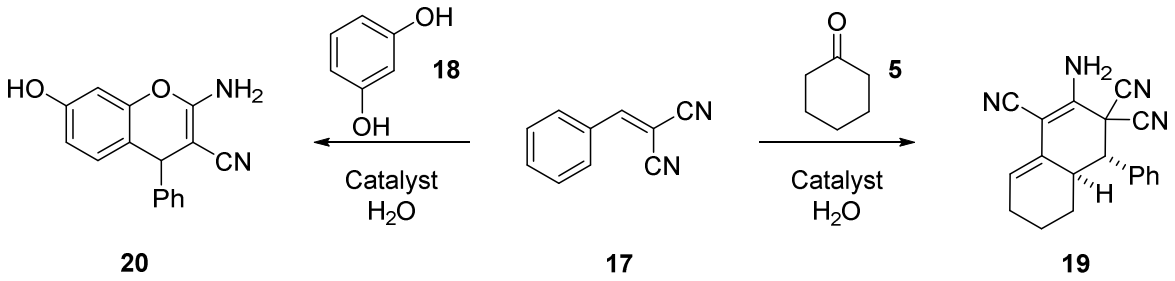
Entry	ArCHO	Product	Time (h)	Conversion (%)[b]	dr[c] (anti/syn)	TON[d] (± 117)	TOF[e] (h^{-1})(± 5)
1	**1l**	**15l**	26	43	41/59	2529	97
2	**1a**	**15a**	24	85 (62[f], 2[g])	41/59 (45/55[f])	4470	186
3	**1a**	**15a**	24	36[h]	45/55	2118	88
				12[i]	47/53	706	29
4	**1b**	**15b**	26	26	45/55	823	32

[a] Reaction conditions: Aldehyde (1.0 mmol), **13** or **14** (10.0 mmol), mean pH = 6.9, catalyst beads number = 20 (corresponding to 17 mol% of free amine groups with respect to the aldehyde), H_2O (3 mL), RT; [b] Conversion was determined by ^{1}H NMR spectroscopy based on the aldehyde proton; [c] Determined by ^{1}H NMR spectroscopy of the crude product. Batch-to-batch estimated relative error = 0.5%. Relative configurations were assigned by comparison with reported literature data; [d] Turnover number defined as the molar ratio of converted substrate to catalyst loading; [e] Turnover frequency defined as the molar ratio of converted substrate to catalyst loading per unit of time; [f] Result of a control experiment performed with 28 mg of PCS as catalyst after 24 h; [g] Result of a control experiment performed without catalyst after 24 h; [h] Recycling experiment: Conversion after first cycle. i) Recycling experiment: Conversion after second cycle.

Michael addition

Finally, the performance of neutral CSHB was also preliminarily evaluated towards two model Michael-like additions involving the reaction between benzylidinemalonitrile (**17**) and either (a) cyclohexanone (**5**) or (b) resorcinol (**18**) in water. The choice of these reactions was made on the basis of the catalytic effect of PCS previously observed in these transformations.[59]

Table 10. Performance of CSHB as organocatalyst in Michael additions in water in comparison with the uncatalyzed and PCS-catalyzed processes.[a]



20 **17** **19**

Entry	Catal.	Yield 19 (%)[b]	Yield 20 (%)[b]	TON[c] 19	TON[c] 20	TOF[d] 19 (h^{-1})	TOF[d] 20 (h^{-1})
1	None	12 (20[e])	8 (15[e])	-	-	-	-
2	PCS	26 (36[e])	19 (24[e])	1182 ± 91	864 ± 91	394 ± 30	288 ± 30
3	CSHB	27	23	1227 ± 91	1045 ± 91	409 ± 30	348 ± 30

[a] Reaction conditions: **17** (1.0 mmol), **5** or **18** (1.0 mmol), mean pH = 6.9, catalyst = Catal.: hydrogel beads number = 26, amount of PCS = 36 mg (corresponding to ca. 22 mol% of free amine groups with respect to 17), H_2O (5 mL), reflux, 3 h; [b] Isolated yield. Batch-to-batch estimated relative error = ± 0.5%; [c] Turnover number defined as the molar ratio of converted substrate to catalyst loading. [d] Turnover frequency defined as the molar ratio of converted substrate to catalyst loading per unit of time; [e] Values as reported in ref. [59]. The obtained lower yields in comparison with the reported values could be attributed to product loss during filtration/recrystallization (entry 1) or to possible critical differences between the properties of the specific chitosan used for the experiments - no specific technical data regarding the DDA of the biopolymer were previously reported - (entry 2).

As shown in Table 10 (entry 1), the addition of either **5** or **18** to **17** in refluxing water and in the absence of catalyst yielded the corresponding racemic products aminopyrancarbonitrile **19** or chromene **20** albeit in much lower yields. However,

the use of both PCS and CSHB under the same conditions afforded the desired products in higher yields (Table 10, entries 2-3). In terms of TON and TOF, no significant differences were observed between CSHB and PCS, suggesting a similar reactive surface of both catalysts for the Michael reaction under prescribed conditions.

Evaluation/optimization of Michael-like addition reactions catalyzed by CSHB, as well as further explorations of the scope and limitations of the direct use of this and related biohydrogels in organocatalysis for biomedical purposes are in progress in our laboratory.

1.3 Conclusion

In conclusion, we have demonstrated that neutral LMW chitosan hydrogel beads with diameters ranging from 2 to 4 mm, is very limited in catalyzing the aldol reaction in both DMSO and aqueous conditions at a catalyst loading of 17 mol% (mol% of free amine groups with respect to the aldehyde). A meticulous washing protocol of the matured hydrogel beads was found to be critical to ensure the removal of trapped OH^- ions, which otherwise would become the actual catalytic species upon their slow diffusion-controlled leaching during the reaction. The detailed study on the impact of the washing protocol on the catalytic activity of the hydrogel beads aims to provide a better standardization of production process to be able to prepare reproducible CSHB batches.

In contrast, neutral pH chitosan hydrogel beads displayed high catalytic activity and selectivity in both the Knoevenagel and nitroaldol (Henry) reactions with a variety of acceptors and donors. TONs up to ca. 5823 were achieved in both cases, whereas the highest TOFs ranged between ca. 1164 min^{-1} for the Knoevenagel and ca. 243 h^{-1} for the nitroaldol (Henry) reaction. In general, higher TONs and TOFs were routinely found for CSHB in comparison to PCS. Experiments with different nitroalkanes revealed that the carbanions with bigger size provided the lower reaction rates and conversions despite the higher acidity of the donor. A similar effect has been also observed in some enzymatic catalysis of this reaction. Nevertheless, the observed negligible enantioselectivites and the reactive surface-reactivity relationship studies confirmed that the biohydrogel

beads act as an immobilized base catalyst rather than as a bionanoreactor. In general, no significant correlation between reaction time and selectivity was observed, and neither the SA:V ratio nor the molecular weight of the biopolymer were found to have a major influence on the catalytic performance of the hydrogel beads. Interestingly, CSHB were also found to be catalytically active towards model Michael additions, albeit with modest TON/TOF comparable to PCS. Optimization of the experimental variables leads to the conclusion that the reaction scale plays a major role in the performance of the biohydrogel catalyst.

Catalytic CSHB are readily available, inexpensive, nontoxic nonhazardous, and requires no initiation step. In terms of recyclability, the CSHB catalyst could be efficiently recovered after the Knoevenagel condensation reaction by a simple filtration/washing protocol and reused several times without noticeable loss in activity. However, poisoning of the catalyst in the presence of aldehydes and nitroalkanes during extended periods of time was observed, thus seriously hindering its reusability in the nitroaldol (Henry) reaction. Moreover, the direct use of CSHB as organocatalyst could complement the use of other forms of the catalyst (e.g. PCS, CSAB) by (1) being compatible with biodegradable and low toxic organic solvents like DMSO and, therefore, with hydrolysable aldehydes or low reactive aldehydes in aqueous medium, (2) avoiding undesired alterations on the functionalized surface of the biopolymer due to imprecise drying protocols, and (3) enhancing the mechanical stability of the catalyst due to its elastic properties, without risking the accessibility of the functional groups.

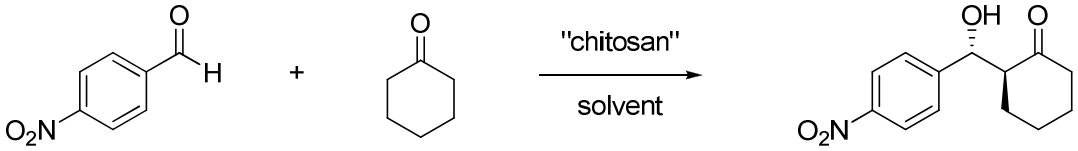
1.4 Addendum

Regarding the enantioselectivity it was found that in the case of the nitroaldol reaction no enantiomeric excess could be obtained with our CSHG, CSAG and PCS catalysts although chitosan should have the potential to induce selectivity due to its chiral backbone. In the case of the model aldol reaction II Quignard and co-workers could show that CSAB, CSHB and chitosan from a commercial source can promote the aldol reaction under stereoselective control and with high yields in water. The fact only very poor conversions towards the aldol product **6a** under our conditions were obtained, hindered the investigation for HPLC analysis of the

isolated pure product. In addition the chiral-phase separation of the enantiomers of **6a** was dissatisfying. Thus no exact statement about the enantiomeric excess could be made for our catalysts in the past. But for a proof of concept further HPLC measurements were applied for the cross aldol product **6a**. Under recently optimized conditions an effectual separation of these enantiomers could be achieved. A comparison of the results of the enantiomeric excess for our CSHB in either H_2O or DMSO as well as the outcome of PCS and Quignard's catalysts are summarized in Table 11.

As previously reported our catalysts were not as powerful as those reported from Quignard's group under consideration of the conversion. In the case of our CSHB and PCS only 3% (entry 1) and 8% conversion (entry 3) could be reached, respectively. In contrast to our values Quignard's CSHB and PCS could perform the reaction in good to high yields, i.e. 75% (entry 4) and 86% isolated yield (entry 6), respectively. One important reason for the big difference in the outcome in terms of conversion/yield could be the fact that in our conditions 17 mol% of the catalyst and ca. 14 equivalents of cyclohexanone (**5**) were used, whereas in Quignard's case 22 mol% and 20 eq. were used. To check this statement our PCS was applied under Quignard's conditions. Interstingly, the conversion could be increased from 8% to 46% (entry 3), but the reaction conversion is still much lower than reported for Quignard's catalyst. Accordingly, the difference in the outcome of the reaction could be also derived from a batch variation phenomenon of the commercial available chitosan. In terms of stereoselectivity in the aldol reaction the CSHB catalyst could reach compareable ee values for the major *anti* isomer, i.e. 78% ee (entry 1) for our CSHB and 80% ee (entry 4) reported for Quignard's CSHB. In the case of PCS the reported ee value of 83% (entry 6) for the major *anti* isomer could not be reached with our PCS catalyst, which was yielding only 64% ee (entry 3). When our CSHB catalyst was applied in a different solvent from water, i.e. in DMSO, a significant decrease of ee of the *anti* isomer to 52% could be monitored. In conclusion, our chitosan-based catalysts were not as powerful in terms of conversion as those reported by Quignard in the aldol reaction between **1a** and **5**, but they were able to reach comparable ee values, which was a proof that chitosan is able to induce stereoselctivity due to its chiral backbone.

Table 11. Determination of the enantiomeric excess induced by "chitosan" in the case of the aldol model reaction II.[a]



1a **5** **6a**

Entry	Solvent[b]	Catalyst	Conversion (%)[c]	ee (%)[d]	dr (anti/syn)[c]
1	H_2O	CSHB	3	78 (33)	56:44
2	DMSO	CSHB	3	52 (29)	56:44
3	H_2O	PCS[e]	8 (46[f])	64 (36)	69:31 (69:31[f])
4[g]	H_2O	CSHB	75[i]	80 (53)	68:32
5[h]	H_2O	CSAB	85[i]	84 (60)	70:30
6[j]	H_2O	PCS	86[i]	83 (62)	70:30

[a] Reaction conditions: **1a** (1.0 mmol), **5** (13.6 mmol), pH = 6.80, beads number = 20 (corresponding to 17 mol% of free amino groups with respect to the aldehyde), solvent (3 mL), 48 h, RT; [b] The amount of water held by the CSHB (20 beads) was estimated in ca. 0.5 mL, which is not included in the total volume of the solvent described in the reaction conditions; [c] Determined by ^{1}H NMR spectroscopy of the crude product. Relative configurations were assigned by comparison with reported literature data; [d] The enantiomeric excess was determined by chiral-phase HPLC using these conditions: eluent = n-Heptane/i-propanol 95/5, flow rate = 1.0 mL min^{-1}, wavelength = 254 nm, run time = 120 min; retention time (RT): *syn* isomer: $RT_{1\ (min)}$ = 62.46 min, $RT_{2\ (maj)}$ = 101.08 min; *anti* isomer: $RT_{1(maj)}$ = 74.23 min, $RT_{2\ (min)}$ = 89.55 min. (RT values are the average of 3 independent samples); [e] Powdered chitosan: 28 mg (corresponding to 17 mol% free amino units with respect to the aldehyde); [f] Result obtained under the following conditions: 22 mol% free amino groups with respect to the aldehyde, 0.1 mmol **1a**, 2.0 mmol **5**, 0.5 mL H_2O, 48 h, RT; [g] Data reported in ref. 19 using different CSHB under the conditions specified in footnote [f]. No specific details regarding the preparation and characterization of those CSHB used were given, which would be necessary for a precise comparison with our beads; [h] Data reported in ref. [19] using chitosan aerogel beads (CSAB) as catalyst under the conditions described in footnote [e]; [i] Isolated yield of the β-hydroxycarbonyl compound **6a**; [j] Data reported in ref. [19] using powdered chitosan from a commercial source.

1.5 Experimental Section

Materials and Methods

^{1}H and ^{13}C NMR spectra were recorded at 25 °C on Bruker Avance 300 or 400 MHz spectrometers. Chemical shifts are denoted in δ (ppm) relative to tetramethylsilane (TMS $\delta = 0$) as internal standard or relative to residual solvent peaks. Coupling constants J are given in Hertz. The following standard abbreviations are used for characterization of ^{1}H NMR signals: s = singlet, d = doublet, t = triplett, m = multiplet. Error of reported values: chemical shift 0.01 ppm (^{1}H NMR), 0.1 ppm (^{13}C NMR), coupling constant 0.1 Hz. The enantiomeric excess (ee) values were determined by chiral-phase HPLC using a Varian 920-LC Liquid Chromatograph and a) Chiralcel AS-H, 4.6 × 250 mm, 10 μm (in the case of the aldol model product) and b) a column Phenomenex Lux Cellulose-1, 4.6 × 250 mm, 5 μm (in the case of the nitroaldol (Henry) model product). Melting points (m. p.) are uncorrected and were measured in a Büchi 504392-S or Opti Melt MPA 100 equipped with a digital camera. IR spectra were recorded using a Diamond ATR (attenuated total reflection) accessory (Golden Gate) or in a VARIAN 1000 FT-IR (Scimitar™ Series). TGA measurements were carried out under nitrogen with the following program heating rate: (1) equilibration step for 30 min @ 30 °C; (2) heating profile from 30 °C to 600 °C @ 10 °C/min; (3) 15 min @ 600 °C. UV-vis measurements were performed on a Varian Cary 50 Instrument. DSC measurements were performed in a SETERAM TMA 92 16.18. The DSC thermograms were obtained under dynamic argon atmosphere (1 l h^{-1}, low-rate gas flow measured with a Brook flowmeter equipped with a Tube R-2-15-AA P-073) at a heating rate of 5 °C min^{-1}. Samples were placed an open Al_2O_3 crucible. In, Zn, Ag and Al metals were used to calibrate the DSC modulus in relation to temperature and enthalpy. An empty sample holder was used as reference and the runs were performed by heating the samples from 25 up to 400 °C. Height of the peaks were measured by the difference between the heat flow at the peak and the DSC curve baseline. The average diameter of the hydrogel beads was calculated by taking pictures of 20 beads under an optical microscope (Wild Makroskop M420 1.25×) equipped with a digital camera (Canon Power shot

A640). pH values were determined using a HANNA instuments Microprocessor pH 211 Meter. In general, 30 mL of the corresponding filtrate were filtrated via a Rotilabo®-Spritzenfilter purchased from Carl Roth GmbH (diameter 33 mm, Nylon-Membran, 0.45 μm) in a Falcon tube and stirred slowly at RT until a constant pH value was adjusted. SEM images were obtained with a Zeiss DSM 950 scanning electron microscope operated at 10 kV. The samples were sputtered with Au prior to imaging by a SCD 040 Balzers Union. TLC was facilitated by the use of the following stains in addition to UV light (254 nm) with fluorescent-indicating plates (aluminium sheets precoated with silica gel 60 F_{254}, Merck): phosphomolybdic acid, vanillin, iodine. Atomic Absorption Spectroscopy was performed using Perkin-Elmer model A300 (USA) Atomic Absorption Spectrophotometry (AAS). Standard used for Na^+ determination was purchased from Qualigens Fine Chemicals (Fischer Scientific – Ireland). Na^+ AAS standard solution contains 1000 mg/L Na^+ in 0.5 M HNO_3. Subsequent dilutions of the standard solution provided AAS values of 5.26 and 10.83 for Na^+ 5 ppm and 10 ppm standard solutions repectively. Chitosan bead solutions for AAS studies were prepared as following: 10 beads of chitosan were taken from different batches prepared at different pH and dissolved in 1 mL of concentrated acetic acid and the clear solution was diluted to 50 mL with distilled water in a 50 mL standard flask, which was used for AAS measurements. Statistical validation of representative results from random experiments was performed by simple one-way analysis of variance yielding overall significance ($p < 0.05$). The values in the text, tables and figures are expressed as mean ± standard deviation.

All reactions were carried out in a 5 mL round-bottom-flask under stirring at 500 rpm. Analytical grade solvents and commercially available reagents were purchased from TCI Europe or Aldrich and were used as received. Low molecular weight chitosan (Cat. No. 448869; Batch No. MKBB9037; CAS 9012-76-4; viscosity 20-200 cP, 1% in 1% acetic acide; actual DDA = 91.7%); medium molecular weight (MMW) chitosan (Cat. No. 448877; Batch No. MKBC3804; CAS 9012-76-4; viscosity 200-800 cP, 1% in 1% acetic acid; DDA = 75-85%) and high molecular weight (HMW) chitosan from crab shells (Cat. No. 48165; Batch No. BCBC2236V; CAS 9012-76-4; viscosity > 400 mPa.s, 1% in acetic acid; DDA = 75-85%) were purchased from Aldrich and used without further purification.

General preparation of chitosan hydrogel beads (CSHB)

0.64 g of LMW chitosan were placed in a beaker and dissolved in 0.1 M HCl (40 mL) during 1 h at RT. The as-prepared viscous clear solution was added drop wise into 0.1 M NaOH aqueous solution (600 mL) at RT using a dropping funnel (50 mL, diameter of the tip = 4mm), resulting in immediate coagulation of droplets into beads. The distance between the dropping funnel tip and solution surface was adapted between 1.0 and 1.5 cm to ensure almost uniform beads with a spherical like geometry (4.0 ± 0.1 mm). The obtained beads were matured in this solution for 1 h without stirring. After this time, the hydrogel beads were collected on a Buchner funnel (without filter paper) and washed with 250 mL portions of Mill-Q water until the pH value of the filtrate was found to be in the desired range. The hydrogel beads were placed on a filter paper to remove the excess of water before using them in the catalytic experiments. The beads were found to be stable, in terms of aspect and reactivity, for at least one month when stored at RT. For the preparation of hydrogel beads of 2.2 ± 0.2 mm in diameter, a 20 mL syringe with a needle of 0.8 mm in diameter was used instead of the dropping funnel. A similar protocol was used to prepare the beads from MMW and HMW chitosan. Different number of washings during the isolation of the beads usually provided batches with different basicity.

Determination of number of accessible amine groups

The number of accessible amine groups of the CSHB catalyst was calculated by analyzing its reaction with salicylaldehyde (SA). 20 CSHB units were mixed with a solution of SA (2.5 mL from a 0.16 M stock solution in EtOH) and nitrobenzene (NB) (0.4 mmol) as GC internal standard. After 1 h, the formation of the Schiff base complex between SA and accessible -NH_2 groups of the CSHB was quantified by analyzing the GC peak area of the remaining unreacted salicylaldehyde. A master calibration curve (i.e. NB/SA areas plotted against NB/SA concentrations) was used to determine the actual concentrations in solution. The number of accessible -NH_2 groups was further determined taking into consideration the actual DDA of the chitosan used for the preparation of the hydrogel beads: DDA (degree of deacetylation) = 91.7%, M_d (molar mass of

deacetylated unit) 161.16 g/mol, M_a (molar mass of acetylated unit) = 203.19 g/mol, m (weight of 20 dried beads) = 28 mg, loading = 6.093 mmol/g. Accessible -NH_2 groups = [(1/161,16 g/mol x 0.917) + (1/203.19 g/mol x 0.083)] x 0.028 g = 0.00017 mol. (Note: This method does not consider some degree of data artefact caused by potential adsorption/absorption of unreacted SA and/or NB on/in the beads occurred during the formation of the salicylaldimine Schiff base complex).

Procedure for the preparation of cross-linked CSHB

60 units of chitosan hydrogel beads (mean pH 6.9, 10.2 mmol of -NH_2 groups) were placed into a 20 mL glass vial and mixed with 10 mL of aqueous glutaraldehyde solution (25 wt.%, c = 25 mM, molar ratio glutaraldehyde/NH_2 = 2.45). This mixture was shaken for 48 h at room temperature. After this time, the beads were washed/shaken 10 times with 10 mL of water during 5 min every time to remove the unreacted glutaraldehyde as indicated by TLC analysis.

General procedure for aldol model reaction I

To a solution of 4-nitrobenzaldehyde (151.1 mg, 1.0 mmol) and acetone (1 mL, 13.6 mmol) in DMSO (3 mL), 20 hydrogel bead units (corresponding to 17 mol% of free amine groups with respect to the aldehyde) were added in one portion. The resulting reaction mixture was gently stirred (500 rpm) under the specified conditions of time and temperature. TLC was used to monitor the reaction. After the indicated reaction time, the work-up was initiated by decanting the supernatant. The hydrogel beads were resuspended in DMSO (2 mL), stirred for 5 min and decanted. This process was repeated 3 times and the organic phases combined. Water (5 mL) was added to the solution and extracted with EtOAc (3 x 10 mL). The combined organic layers were further washed with water (3 x 15 mL), dried over Na_2SO_4 (20 min) and evaporated under reduced pressure. Conversion was determined by ^{1}H NMR spectroscopy of the crude product. The use of different stirring rate during the reaction might cause small deviations on the kinetic and therefore on the conversion.

General procedure for aldol model reaction II

To a mixture of 20 hydrogel bead units (corresponding to 17 mol% of free amine groups with respect to the aldehyde) in the corresponding solvent system (3 mL), a solution of 4-nitrobenzaldehyde (151.1 mg, 1.0 mmol) in cyclohexanone (1.4 mL, 13.6 mmol) was added. The resulting reaction mixture was gently stirred (500 rpm) for 48 h at RT. TLC was used to monitor the reaction. After the indicated reaction time, the supernatant was diluted with EtOAc (2 mL) and decanted. The hydrogel beads were resuspended in DMSO (2 mL), stirred for 5 min and decanted. This process was repeated 3 times and the organic phases combined. Water (5 mL) was added to the solution and extracted with EtOAc (3 x 10 mL). The combined organic layers were further washed with water (3 x 15 mL), dried over Na_2SO_4 (20 min), filtrated and evaporated under reduced pressure. Conversion and diasteromeric ratio was determined by ^{1}H NMR spectroscopy of the crude product.

General procedure for the Knoevenagel condensation reaction

To a solution of the appropriate aldehyde (1.0 mmol) and malononitrile or ethyl-2-cyanoacetate (1.1 mmol, 72.7 mg or 118 μL respectively) in DMSO (3 mL), 20 hydrogel bead units (corresponding to 17 mol% of free amine groups with respect to the aldehyde) were added in one portion. The resulting reaction mixture was gently stirred (500 rpm) at RT until no more aldehyde was observed (TLC). After this time, the supernatant was decanted and the remaining hydrogel beads were resuspended in DMSO (2 mL), stirred for 5 min and decanted. This process was repeated 3 times. Water (5 mL) was added to the solution and extracted with EtOAc (3 x 10 mL), dried over Na_2SO_4 and evaporated under reduced pressure. Conversion was determined by ^{1}H NMR spectroscopy of the crude product. When malononitrile was used as donor, the water employed during the work-up was replaced by brine.

General procedure for nitro-aldol (Henry) reaction

A mixture of the appropriate aldeyhde (1.0 mmol) and nitromethane (0.54 mL, 10.0 mmol) or nitroethane (0.72 mL, 10.0 mmol) was dissolved in the corresponding

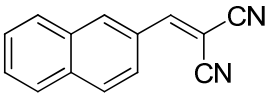
solvent (emulgated in the case of water) (3 mL) under stirring. To this mixture, 20 units of chitosan hydrogel beads (corresponding to 17 mol% of free amine groups with respect to the aldehyde) were added in one portion. The resulting reaction mixture was gently stirred (500 rpm) under the specified conditions of time and temperature. For the work-up, the supernatant was decanted and the remaining hydrogel beads were washed with the solvent used in the reaction (2 mL) (i.e EtOH, MeOH) and the filtrate evaporated. In the case of water, EtOAc (3 mL) was used to wash the beads by stirring during 5 min and further decantation. This process was repeated 3 times. The combined aqueous layers were extracted with EtOAc (3 x 10 mL), dried over Na_2SO_4 and evaporated under reduced pressure. In the case of DMSO, water (5 mL) was added to the organic layers followed by extraction with EtOAc (3 x 10 mL). The combined organic layers were further washed with water (3 x 15 mL), dried over Na_2SO_4, filtrated and evaporated under reduced pressure. Conversion was determined by ^{1}H NMR spectroscopy of the crude product.

General procedure for Michael addition

A mixture of benzylidenemalononitrile (**17**) (154 mg, 1.0 mmo), cyclohexanone (**5**) (0.11 mL, 1.0 mmol) or resorcinol (**18**) (110 mg, 1.0 mmol) and chitosan catalyst (LMW PCS = 36 mg or CSHB = 26 beads, 22 mol% of free amine groups with respect to 17) in water (5 mL) was refluxed during 3 h. After this time, the yellow precipitate was filtered off and washed with water (2 x 5 mL). The crude residue was resuspended in absolute EtOH and heated until boiling. The hot mixture was filtered and the procedure repeated three times with the isolated chitosan catalyst. In spite of the probable diffusion of EtOH into the CSHB during this procedure, the spherical beads could be recovered and reused in a second run without significan loss of catalytic activity. Aminopyrancarbonitrlie **19** and chromene **20** were finally isolated by filtration upon recrystallization from the hot ethanolic solution.

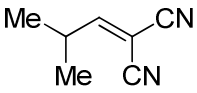
Reaction products **6a**,[60] **8f**,[61] **10e**,[62] **10l**,[63] **10m**,[64] **12d**,[65] **12j**,[65],[66] **12k**,[65] **19**[59] and **20**[59] are known compounds and showed spectroscopic data identical with the literature.

2-(Naphthalen-2-ylmethylene)malononitrile (8c)



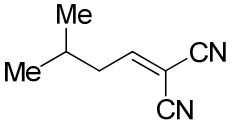
Yellow solid, **m.p.**: 131-132 °C; **R_f** (30% EtOAc/n-hexane): 0.50; **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm) = 8.27 (d, J = 1.5 Hz, 1H), 8.07 (dd, J = 8.8, 1.9 Hz, 1H), 8.00–7.84 (m, 4H), 7.65 (m, 2H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm) = 159.8, 135.9, 134.5, 132.6, 130.0, 129.7 (2C), 128.6, 128.1, 127.8, 124.2, 114.0, 112.9, 82.2; **FT-IR ν_{max}** (cm^{-1}): 2922, 2225, 1733, 1583, 1464, 1583, 1464, 1375, 1270, 1185, 965, 868, 810, 745; **Elemental analysis** (%): Calculated for $C_{14}H_8N_2 \cdot 1/5\ H_2O$: C, 80.91; H, 4.07; N: 13.48. Found: C, 81.02; H, 4.21; N: 12.73.

2-(2-Methylpropylidene)malononitrile (8i)



Yellow liquid; **R_f** (20% EtOAc/n-hexane): 0.47; **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm) = 7.14 (d, J = 10.6 Hz, 1H), 3.02 (ddt, J = 13.3, 10.6, 6.6 Hz, 1H), 1.18 (d, J = 6.6 Hz, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm) = 175.0, 112.1, 110.4, 87.9, 32.8, 21.0; **FT-IR ν_{max}** (cm^{-1}): 2973, 2239, 1734, 1607, 1469, 1367, 1094, 955, 620; **HRMS**: Calculated for $(M+H)^+$ 121.0766, found 121.0752 (+APCI GC-MS).

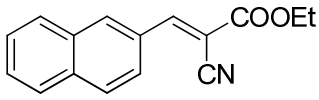
2-(3-Methylbutylidene)malononitrile (8j)



Yellowish liquid; **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm) = 7.35 (t, J = 8.1 Hz, 1H), 2.50 (dd, J = 8.0, 6.8 Hz, 2H), 1.94 (tt, J = 13.4, 6.7 Hz, 1H), 1.01 (d, J = 6.7 Hz, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ □(ppm) = 168.9, 112.1, 110.6, 90.4, 41.5, 28.2, 22.2; **FT-IR ν_{max}** (cm^{-1}): 2962, 2936, 2877, 2240, 1715, 1606, 1467, 1389, 1372,

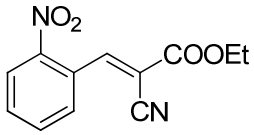
1168,1048, 632; **HRMS**: Calculated for $C_8H_{10}N_2$ $(M+H)^+$ 135.0922, found 135.0914 (+APCI GC-MS).

(E)-Ethyl 2-cyano-3-(naphthalen-2-yl)acrylate (10c)



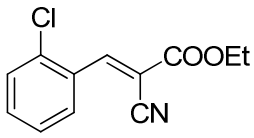
Bright yellow solid, **m.p**.: 111-112 °C; **R_f** (30 EtOAc/hexanes): 0.50; **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm) = 8.37 (d, J = 6.7 Hz, 2H), 8.18 (dd, J = 8.7, 1.8 Hz, 1H), 7.98–7.82 (m, 3H), 7.68–7.51 (m, 2H), 4.41 (q, J = 7.1 Hz, 2H), 1.42 (t, J = 7.1 Hz, 3H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm) = 162.7, 155.0, 135.4, 134.2, 132.8, 129.4, 129.2, 129.1, 129.1, 127.9, 127.2, 125.3, 115.8, 102.7, 62.8, 14.2; **FT-IR ν_{max}** (cm^{-1}): 2989, 2220, 1723, 1598, 1364, 1245, 1161, 1093, 1021, 815, 752; **Elemental analysis** (%): Calculated for $C_{16}H_{13}NO_2$.: C, 76.48; H, 5.21; N: 5.57. Found: C, 76.66; H, 5.20; N: 5.48.

(E)-Ethyl 2-cyano-3-(2-nitrophenyl)acrylate (10f)



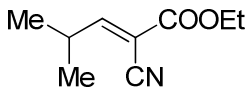
Off-white solid, **m.p.**: 99-100 °C; **R_f** (30% EtOAc/n-hexane): 0.32; **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm) = 8.72 (s, 1H), 8.28 (dd, J = 8.2, 1.1 Hz, 1H), 7.91–7.66 (m, 3H), 4.42 (q, J = 7.1 Hz, 2H), 1.41 (t, J = 7.1 Hz, 3H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm) = 161.1, 153.2, 147.4, 134.5, 132.2, 130.6, 128.2, 125.5, 113.9, 108.7, 63.2, 14.1; **FT-IR ν_{max}** (cm^{-1}): 2999, 1721, 1604, 1570, 1519, 1342, 1259, 1200, 1090, 1007, 930, 886, 857, 798; **Elemental analysis** (%): Calculated for $C_{12}H_{10}N_2O_4$.: C, 58.54; H, 4.09; N: 11.38. Found: C, 58.89; H, 4.17; N: 11.35.

(E)-Ethyl 3-(2-chlorophenyl)-2-cyanoacrylate (10g)



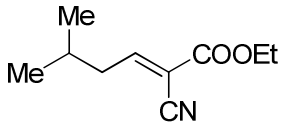
Beige solid, **m.p.**: 52-53 °C; **R_f** (30% EtOAc/n-hexane): 0.57; **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm) = 8.69 (s, 1H), 8.29–8.17 (m, 1H), 7.55–7.35 (m, 3H), 4.40 (q, J = 7.1 Hz, 2H), 1.41 (t, J = 7.1 Hz, 3H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm) = 161.8, 151.2, 136.5, 133.7, 130.4, 129.9, 127.5, 114.9, 106.2, 63.0, 14.2; **FT-IR ν_{max}** (cm^{-1}): 2998, 2224, 1728, 1609, 1468, 1431, 1254, 1199, 1125, 1090, 1019, 893, 758; **Elemental analysis** (%): Calculated for $C_{12}H_{10}ClNO_2$: C, 61.16; H, 4.28; N, 5.94. Found C, 61.37; H, 4,49; N, 5.91; **HRMS**: Calculated for $C_{12}H_{10}ClNO_2$ $(M+H)^+$ 236.0478, found 236.0473 (+APCI GC-MS).

(E)-Ethyl 2-cyano-4-methylpent-2-enoate (10i)



Colourless liquid; **R_f** (20% EtOAc/n-hexane): 0.52; **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm) = 7.45 (d, J = 10.6 Hz, 1H), 4.30 (q, J = 7.1 Hz, 2H), 2.99 (ddt, J = 13.3, 10.6, 6.6 Hz, 1H), 1.34 (t, J = 7.1 Hz, 3H), 1.15 (d, J = 6.6 Hz, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm) = 169.2, 161.5, 113.6, 107.5, 62.5, 31.6, 21.3, 14.1; **FT-IR ν_{max}** (cm^{-1}): 2970, 1729, 1626, 1467, 1369, 1307, 1253, 1157, 1069, 1016, 956, 763; **Elemental analysis** (%): Calculated for $C_9H_{13}NO_2$: C, 64,65; H, 7.84; N, 8.38. Found C, 64.45; H, 7.54; N, 8.21.

(E)-Ethyl 2-cyano-5-methylhex-2-enoate (10j)



Colourless liquid; **R_f** (20% EtOAc/n-hexane): 0.55; **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm) = 7.66 (t, J = 8.0 Hz, 1H), 4.30 (d, J = 7.1 Hz, 2H), 2.45 (dd, J = 8.0, 6.8 Hz, 2H), 1.90 (dp, J = 13.4, 6.7 Hz, 1H), 1.34 (t, J = 7.1 Hz, 3H), 0.98 (d, J = 6.7 Hz, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm) = 162.8, 161.3, 113.8, 110.4, 62.4, 40.7, 28.2, 22.4, 14.1; **FT-IR ν_{max}** (cm^{-1}): 2969, 2876, 2231, 1730, 1627, 1466, 1370, 1281, 1256, 1169, 1072, 1048, 836, 758; **Elemental analysis** (%): Calculated for $C_{10}H_{15}NO_2 \cdot 1/5\ H_2O$: C, 64.98; H, 8.40; N: 7.58. Found: C, 65.21; H, 8.33; N: 7.32.

→ DSC thermograms and TGA curves of the used catalysts, ^{1}H NMR spectra of new products, as well as additional experiments and photographs can be found in the electronic supporting information (ESI) on the enclosed CD.

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[32] Such low reaction conversion was also observed if the CSHB were first stirred in the presence of acetone (30 min) before addition of the aldehyde.

[33] In general, aldol reactions may be catalyzed either by a base or an acid, where the nucleophile is an enolate anion or enol, respectively. In the latter case, catalytic protonation of the carbonyl oxygen increases the electrophilicity of the carbonyl carbon just enough so that it can be attacked by the enol, and promote the dehydration of the aldol product, as water is a good leaving group. Nevertheless, it is well known that aldol reactions proceed more efficiently under basic conditions where dehydration rarely takes place during the reaction since hydroxide is a poor leaving group.

[34] Under the critical assumption that the content of Na^+ ions inside the beads reflect the actual hydroxide catalyst content, atomic absorption spectroscopy (AAS) was used to calculate these values for a series of beads that displayed different pH values of the filtrate after the washing protocol (see Experimental section). Unfortunately, AAS on these materials provided an average value of 9.96 ± 0.16 ppm of Na^+ ions for several batches of beads with pH values ranging from 6.49 to 8.44. Therefore, any attempt to correlate these AAS values with the actual concentration of hydroxide ions inside the beads would not be completely reliable. The same conclusion was reached from ICP measurements of CSHB, from which the theoretical concentration of hydroxide ions (related to the concentration of Na^+ ions) would be well below the minimum catalytic loading necessary for the reaction. Although it was not pursued, a more direct method of analysis could involve the treatment of a Ag(I) solution in the presence of Mn(II) and finely triturated CSHB. The existence of hydroxide ions in the gel matrix could promote the redox formation of MnO_2 and Ag(0), which could be further quantified by AAS.

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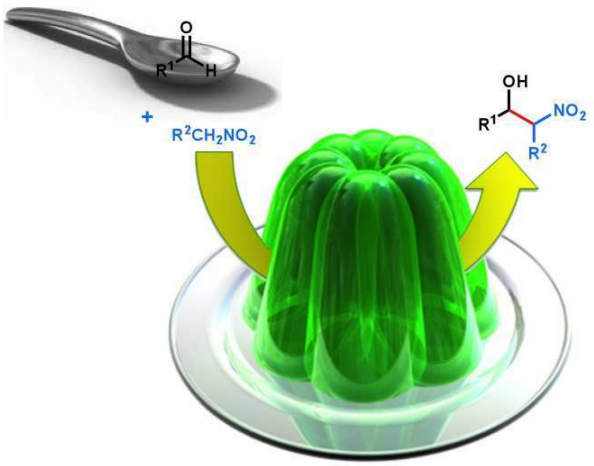
2. Gelatin and Collagen Proteins-mediated Reactions

2.1 C-C Bond Formation Catalyzed by Natural Gelatin and Collagen Proteins[i]



The activity of gelatin and collagen proteins towards C-C bond formation via the Henry (nitroaldol) reaction between aldehydes and nitroalkanes is demonstrated for the first time. Among other variables, protein source, physical state and chemical modification influence product yield and kinetics, affording the nitroaldol products in both aqueous and organic media under mild conditions. Significantly, the scale-up of the process between 4-nitrobenzaldehyde and nitromethane is successfully achieved at 1 g scale and in good yield. A comparative kinetic study with other biocatalysts show an increase of the first-order rate constant in the order chitosan < gelatin < bovine serum albumin (BSA) < collagen. The results of this study indicate that simple edible gelatin can promote C-C bong forming reactions under physiological conditions, which may have important implications from a metabolic perspective.[ii]

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[i] D. Kühbeck, B. B. Dhar, E.-M. Schön, C. Cativiela, V. Gotor-Fernández, D. D. Díaz, *Beilstein J. Org. Chem.* **2013**, *9*, 1111-1118. Copyright by The Authors.

[ii] Calculations and fitting for the kinetic curves were performed by B. B. Dhar. Succinilated and esterificated PSTA gelatin were synthesized and the 2nd run of entries in Table 3 were accomplished by E.-M. Schön. FESEM images were recorded by Prof. Dr. D. D. Díaz. All other experiments were carried out by D. Kühbeck.

2.1.1 Introduction

Gelatin is a mixture of hot-water-soluble proteins of high average molecular weights (50-100 kDa) derived primarily from collagen, which is the main naturally occurring structural protein in animal bones, skin and connective tissue (ca. one-third of the total protein mass in the body). Its low production cost and nontoxic, edible and biodegradable properties have made gelatin a common ingredient in food, pharmaceutical, cosmetic and photographic industries, among others.[1] There are two main types of gelatin: Type-A, with a broad iso-electric point (IEP) range of 6.0-9.0, and type-B, with a rather narrow IEP range of 4.5-5.5. The former results from the acidic hydrolysis of collagen, whereas the latter results from an alkaline treatment that causes a greater degree of deamidation of glutamine and asparagine.

Besides traditional applications in the food industry, gelatin has also gained attention in the scientific community as a reducing ligand and supporting media for the preparation of uniform metal nanoparticle catalysts.[2-4] In addition, the average composition of gelatin in terms of its amino acids content has been reported in several publications (arginine, glutamic acid, alanine, glycine, proline and hydroxyproline are the most abundant amino acids (ca. 10-25%))[1], which makes the protein itself suitable for catalytic studies.

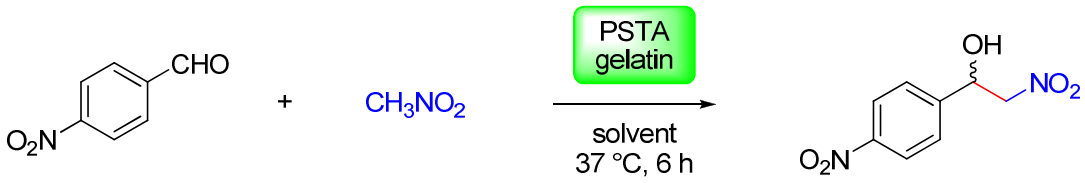
The Henry (nitroaldol) reaction is a versatile and widely used base-catalyzed C-C bond forming reaction between a nitroalkane and an electrophilic carbonyl derivative (aldehyde or ketone) to produce β-nitroalcohols, which can be transformed into valuable synthetic building blocks.[5-10] Some biopolymers such as salmon testes DNA[11] and chitosan[12], as well as various enzymes[13-16], have been reported to catalyze this type of reaction. However, to the best of our knowledge, the role of natural gelatin or collagen proteins as potential biocatalysts for C-C bond formation has not been yet described.[17] A practical importance of this study derives from the fact that gelatin is the protein most commonly associated with food products, in which different aldehydes may be also present. Therefore, the combination of this protein and aldehydes under physiological conditions could generate in vivo the formation of new metabolic products. Herein,

we report for the first time the activity and comparative kinetics of gelatin and collagen proteins in the context of the Henry (nitroaldol) reaction.

2.1.2 Results and Discussion

Reaction between 4-nitrobenzaldehyde (**1a**, 0.1 mmol) and nitromethane (**2a**) in DMSO was considered as the model reaction, observing that 2 mg of gelatin from porcine skin type-A (PSTA) catalyzed the selective formation of the corresponding nitroaldol product **3a** at physiological temperature. Thus, ca. 70% yield of **3a** was attained with 5-fold excess of **2a**, while higher loadings did not significantly improve the results (see Supporting Information File 1). Under these preoptimized conditions, further investigation of the solvent scope revealed DMSO as the most suitable organic solvent to carry out the reaction (Table 1, entries 1 and 2).

Table 1. Solvent screening study for gelatin-mediated Henry reaction.[a]



1a **2a** **3a**

Entry	Solvent	3a, Yield (%)[b]
1	EtOH, DMF, CH_3CN or toluene	0 (0[c])
2	DMSO	70 (0[c])
3	H_2O	14 (8[c])
4	H_2O/TBAB[d]	78 (8[c])

[a] Reaction conditions: **1a** (0.1 mmol), **2a** (0.5 mmol), PSTA gelatin (2 mg), solvent (0.5 mL), 37 °C, 6 h; [b] The ^{1}H NMR yields correspond to the average values of two independent experiments (standard deviation, STDV = ± 2%); [c] Control experiment: Reaction in the absence of gelatin; [d] Tetra-*n*-butylammonium bromide (TBAB, 0.04 mmol). The addition of the phase transfer catalyst did not change the pH of the solution.

Interestingly, the reaction could be also performed in water, although in this case the addition of a phase transfer cocatalyst (e.g., TBAB) was necessary to achieve comparable results (Table 1, entries 3 and 4).[18] In pure water, the yield could be also improved to ca. 60% by a 5-fold increase in the amount of gelatin. The

observed beneficial effect of the phase-transfer catalyst also suggests potential relevance to physiological conditions, where biological membranes can be expected to serve a similar role. The background reaction in DMSO (i.e., control experiment in the absence of protein) was totally inhibited, and in H_2O/TBAB represented only 8% of the product yield obtained in the presence of gelatin (entries 2 and 4), demonstrating the inherent catalytic activity of the protein under both organic and aqueous conditions.

In order to completely suppress the background contribution from the reaction media and concentrate on the pure effect of the protein catalyst, we continued our investigation with DMSO as the model solvent. It is also important to mention that the reaction can be scaled up with modest yields (i.e., **1a** (7.5 mmol), **2a** (37.5 mmol), PSTA gelatin (150 mg), DMSO (37.5 mL), 6 h, 37 °C, ca. 60% yield).

Table 2. Influence of different types of gelatin in the model Henry reaction between **1a** and **2a** in DMSO.[a]

Entry	Gelatin type[b]	3a, Yield (%)[c]
1	PSTA gelatin	70
2	BSTB gelatine	75
3	CWFS gelatine	74
4	Succinylated PSTA gelatin	57[d]
5	Esterificated PSTA gelatin	27[d]
6	Powdered edible gelatin[e]	60[d]
7	Cooked sheet edible gelatin[f]	69[d], 63[g]
8	PSTA gelatin hydrogel	33[h] (33[i], 2[j])

[a] Reaction conditions: **1a** (0.1 mmol), **2a** (0.5 mmol), gelatin (2 mg), DMSO (0.5 mL), 37 °C, 6 h; [b] See Supporting Information File 1 for preparation and experimental details; [c] The ^{1}H NMR yields that correspond to the average values of two independent experiments (unless otherwise indicated, STDV = ± 2%); [d] STDV = ± 5%; [e] Purchased at the supermarket; [f] Purchased at the supermarket and cooked for the experiment; [g] Reaction carried out by using the xerogel material obtained from a cooked sheet of edible gelatin; [h] Experiment performed at rt to preserve the gel phase of the catalyst obtained separately from 6 mg of PSTA gelatin in 0.3 mL of H_2O; [i] Control experiment: Reaction performed in a mixture DMSO (0.5 mL)/H_2O (0.3 mL) and 6 mg of powdered PSTA gelatin (not gel phase); [j] Control experiment: Reaction in DMSO (0.5 mL)/H_2O (0.3 mL) without gelatin.

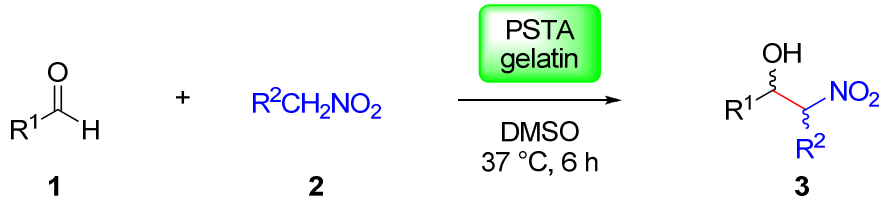
We also studied the effect of different types of gelatin obtained from various natural resources (i.e., PSTA, bovine skin type-B (BSTB) gelatin and cold-water fish skin (CWFS) gelatin). Comparable results were obtained in all cases (Table 2, entries 1-3), illustrating that properties such as IEP, polymer stiffness mass (Bloom number), and the extraction/recovery method used to isolate the protein (i.e., type-A, type-B) have no significant influence on the catalytic activity in DMSO. We also confirmed that there was no effect of possible metal impurities in the gelatin samples on the reaction conversion (see Supporting Information File 1). For further experiments, we used PSTA gelatin based on its lower price, known protein content, and slightly acidic pH value in solution.

Moreover, chemical modification of the side chains of gelatin (i.e., succinylation, esterification)[19,20] suggested the importance of free carboxyl groups on the catalyst activity (e.g., Type A gelatin has ca. 78-80 millimoles of free carboxyl groups per 100 g of protein). The observations are in agreement with the examples reporting individual amino acids (e.g., alanine, proline) as catalysts for similar reactions.[20-27] Very interestingly, even the direct use of edible gelatin obtained from the supermarket (either in powdered or cooked form) promoted the C-C bond formation in good yields (Table 2, entries 6 and 7). On the other hand, although the use of gelatin in hydrogel form did not afford a higher yield than in solution under comparable conditions (Table 2, entry 8), the former provided a suitable way to work in a heterogeneous phase.

We further evaluated the possibility to convert different aldehydes in combination with nitromethane or nitroethane (Table 3). In general, aromatic aldehydes with strong or moderate electron-withdrawing substituents were easily converted into the corresponding nitroaldol products in moderate to very good yields over 6 h at 37 °C (Table 3, entries 1-6). These examples also demonstrated that the *ortho*- or *meta*-substituents did not hinder the reaction at all. In contrast, considerably lower yields were obtained in the cases of aromatic aldehydes bearing weak electron-withdrawing groups or electron-donating groups (Table 3, entries 8–12), while 2-pyridinecarbaldehyde led to 54% yield in the reaction with nitromethane (Table 3, entry 13). Aliphatic aldehydes (Table 3, entries 14 and 15) were poorly converted. However, these yields could be nearly doubled by increasing either the reaction time (e.g., 72 h) and/or the reaction temperature (e.g., 60 °C) (Table 3, entries 7, 9, 11, 12 and 14). Importantly, control

experiments in the absence of gelatin at 60 °C also showed no product formation (Table 3, entries 1 and 7).

Table 3. Substrate scope of the gelatin-catalyzed Henry reaction in DMSO.[a]



1 **2** **3**

Entry	R^1 (1)[b]	R^2 (2)	3, Yield (%)[c]	dr[d]
1	(4-NO_2)-C_6H_4	H	70 (0[e])	NA
2	(4-NO_2)-C_6H_4	CH_3	84 (44[e])	1.1:1
3	(2-NO_2)-C_6H_4	H	77	NA
4	(3-NO_2)-C_6H_4	H	90	NA
5	(4-CN)-C_6H_4	H	78	NA
6	(4-CN)-C_6H_4	CH_3	92	1.1:1
7	C_6H_5	H	5 (11[f], 10[g], 0[h])	NA
8	(4-Br)-C_6H_4	H	24	NA
9	(4-Cl)-C_6H_4	H	13 (22[f])	NA
10	(4-Cl)-C_6H_4	CH_3	39	1.2:1
11	(4-CH_3)-C_6H_4	H	4 (6[f])	NA
12	(4-OH, 3-CH_3O)-C_6H_3	H	6[i] (24[f],[i])	NA
13	Pyrid-2-yl	H	54	NA
14	$(CH_3)_2CHCH_2$	H	8 (13[f])	NA
15	$(CH_3)_2C(CH_2)_2CH(CH_3)CH_2$	H	6[j]	NA

[a] Reaction conditions: **1** (0.1 mmol), **2** (0.5 mmol), PSTA gelatin (2 mg), DMSO (0.5 mL), 37 °C, 6 h; [b] See Supporting Information File 1 for expanded structures; [c] The ^{1}H NMR yields that correspond to the average values of two independent experiments (standard deviation, STDV = ± 2%); [d] Diastereomeric ratio (*anti/syn*) determined by ^{1}H NMR analysis. Relative configurations were assigned by comparison with data in the literature. NA = Not applicable; [e] Control experiment made in the absence of gelatin. Reaction time = 6 h, temperature = 37 °C; [f] Reaction time = 72 h, temperature = 37 °C; [g] Reaction time = 6 h, temperature = 60 °C; [h] Control experiment made in the absence of gelatin. Reaction time = 6 h, temperature = 60 °C; [i] Yield of *β*-nitroalkene; [j] Yield of dinitroalkane. In this case, *β*-nitroalcohol was also identified in trace amounts.

It is important to remark that even vanillin or citronellal, which are also components of many foods, could be partially converted to nitroaldol products by gelatin (Table 3, entries 12 and 15). When nitroethane (pK_a = 8.6) was used as the nucleophile instead of nitromethane (pK_a = 10.2) the yield increased considerably (Table 3, entries 2, 6, 10 *vs.* 1, 5, 9, respectively), albeit without significant diastereoselectivity. Thus, acidity of the nitroalkane plays here a more important role than steric effects.[28] It is worth mentioning that control experiments in the absence of gelatin with nitroethane also provided a much lower conversion than in the presence of the protein (Table 3, entry 2), although it was significant in comparison to the less reactive nitromethane (Table 3, entry 1).

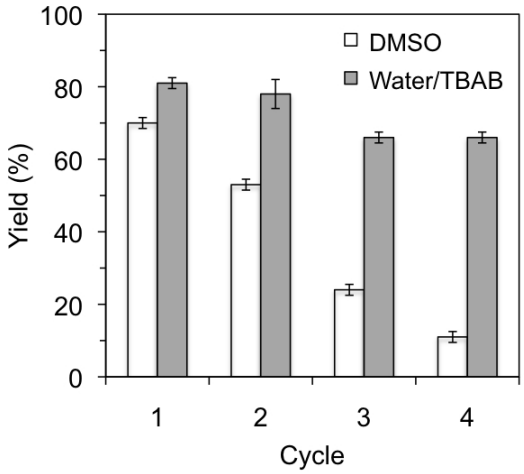


Figure 1. Typical recycling experiments for the gelatine-catalyzed Henry reaction. Reaction conditions: 4-Nitrobenzaldehyde (**1a**, 15.1 mg, 0.1 mmol), nitromethane (**2a**, 27 μL, 0.5 mmol), solvent (0.5 mL), PSTA gelatine (2 mg), 37 °C, 6 h. Yields correspond to ^{1}H NMR values obtained from at least three independent experiments. For the experiments in water/TBAB, additional TBAB was added after each cycle (i.e. 2.6 mg after 1st cycle, 5.2 mg after 2nd cycle, 2.4 mg after 3rd cycle. These quantities correspond to the loss of TBAB after each cycle as determined by ^{1}H NMR analysis).

On the other hand, the gelatin catalyst could be recovered and reused for further cycles, albeit unfortunately with gradual deactivation of the catalyst in both organic and aqueous media (Figure 1). This result may be associated with (1) gradual loss of catalyst loading between cycles and/or (2) possible formation of linear or cyclic aminals.[12] Interestingly, when water/TBAB was used as the solvent system the reduction of the catalytic activity was less dramatic than in the case of DMSO. However, such apparently better performance in water/TBAB was dependent on

the addition of extra TBAB after each cycle in order to ensure a constant concentration of the phase-transfer catalyst during the reaction. The continuing loss of TBAB during the work-up after each cycle was quantified by ^{1}H NMR analysis of the reaction crude.

Very interestingly, we found that the direct use of the precursor collagen as biocatalyst also afforded the desired product in very good yields. In this case, the most common motifs in the amino acid sequence, which could be also associated with catalytic sites, are glycine-proline-X and glycine-X-hydroxyproline, where X is any other amino acid (see Supporting Information File 1). However, despite gelatin and collagen forming triple helices as a chiral secondary structure, HPLC analysis of the reaction mixtures revealed negligible enantioselectivity. This lack of selectivity is in agreement with previous publications dealing with other biocatalysts used in the Henry (nitroaldol) reaction.[11-16]

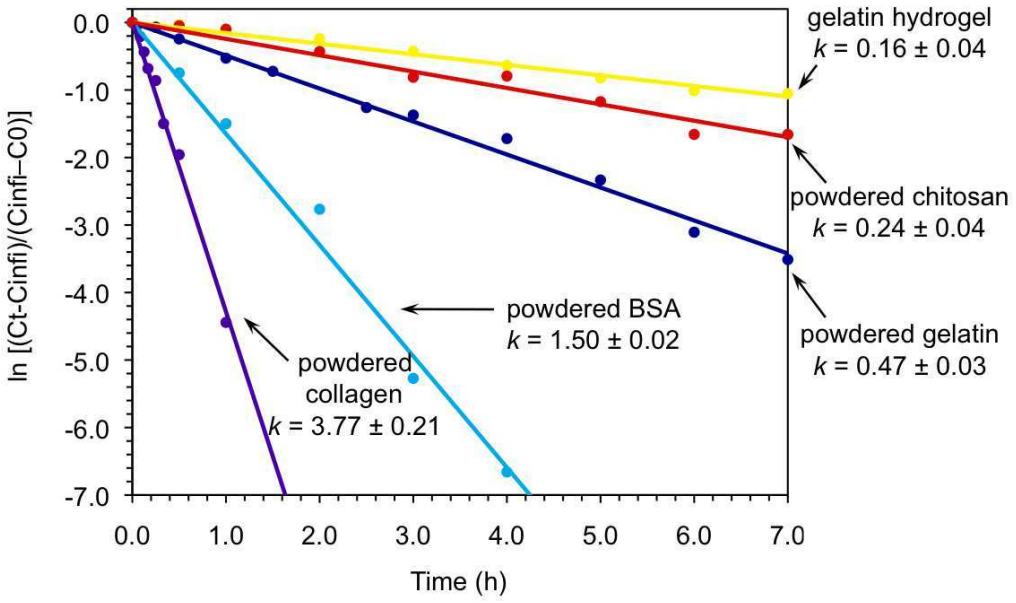


Figure 2. First-order kinetics plots for the model Henry reaction between **1a** and **2a** catalyzed by different systems. Apparent rate constants are in units of h^{-1}. Each data point represents the average of two independent measurements. C_∞ = final concentration at infinite time; C_t = concentration at given time t; C_0 = initial concentration at t = zero time.

At this point, and in order to draw a meaningful comparison with other known biocatalytic systems (i.e., chitosan [29], bovine serum albumin (BSA)[30,31]) we carried out the kinetic analysis of the model reaction for each case.[8] Figure 2

shows the first-order kinetics plots demonstrating the fine-tuning of the reaction rate in response to the biocatalyst used. In the case of biopolymers in powder form, first-order rate constants increased in the order chitosan < gelatin < BSA < collagen, whereas the same concentration of gelatin in hydrogel form showed slower kinetics. These results suggest a detrimental decrease of accessibility to the active groups of the catalyst upon gel formation. Nevertheless, it should also be noted that creating, for example, aerogels of the corresponding powdered materials (e.g., chitosan) would significantly enhance their reactive surfaces and, therefore, their catalytic performance.

Moreover, field-emission scanning electron microscopy (FESEM) images of the biocatalysts associated faster reactions with porous fibrilar morphologies and slower kinetics with thicker and close-grained surfaces (Figure 3). These results suggest that the morphology and/or physical state of the proteins play an important role in the kinetics of the nitroaldol reaction.

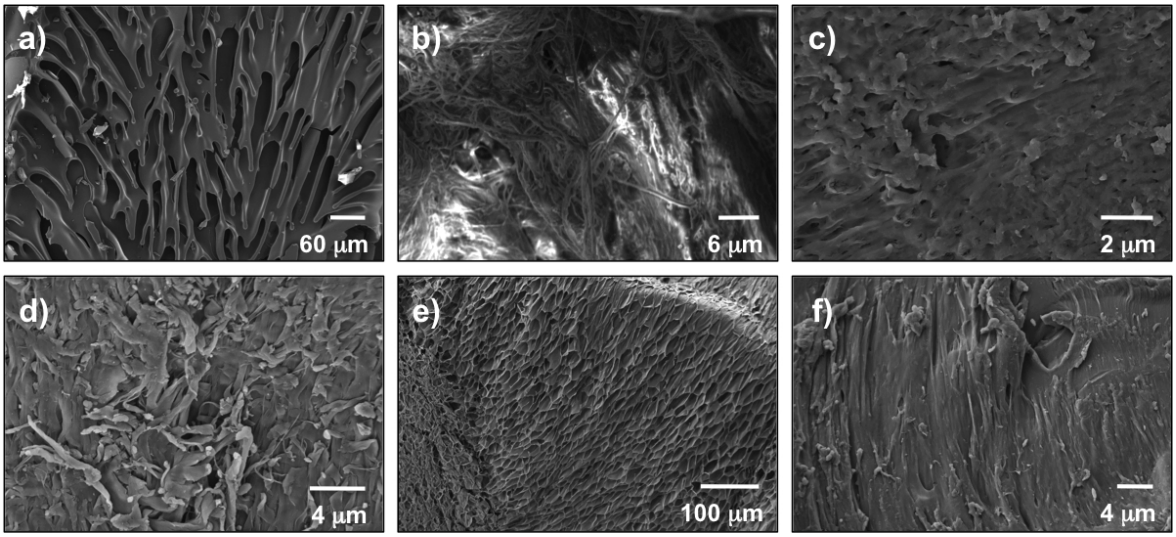


Figure 3. Selected FESEM images of different catalysts used for comparative kinetics: (a) powdered BSA; (b) powdered collagen; (c) powdered PSTA gelatin; (d) powdered chitosan; (e) xerogel prepared by freeze-drying the hydrogel made of PSTA gelatin; (f) commercial powdered edible gelatin.

2.1.3 Conclusion

In conclusion, we have found that natural gelatin and collagen proteins are able to promote C-C bond formation via the Henry (nitroaldol) reaction between various aldehydes and nitroalkanes. Thus, the reaction takes place in both aqueous and organic media under mild conditions, affording the nitroaldol product in variable yields depending on the aldehyde and nitroalkane nature. Moreover, the scale-up of the process between 4-nitrobenzaldehyde and nitromethane could also be achieved on a 1 g scale and in good yield. A comparative kinetics study with other biocatalysts showed an increase of the first-order rate constant as follows: Chitosan < gelatin < BSA < collagen. Remarkably, the morphology and the physical state of the protein play an important role on the kinetics of the nitroaldol reaction. It should be emphasized that although none of the biopolymers are superior to standard base catalysts, such as tetramethylethylenediamine[8], from a synthetic point of view, the former avoided byproduct formation and offered the possibility to work under advantageous ecofriendly, sustainable and heterogeneous conditions. However, it is indeed more important to realize that edible gelatin or collagen have the potential to partially convert aldehydes that are usually present in numerous foods or cosmetics, under physiological conditions, which could modify their metabolic routes.

2.1.4 Experimental Section

Materials and Methods

^{1}H NMR spectra were recorded at 25 °C on a Bruker Avance 300 spectrometer. Chemical shifts are denoted in δ (ppm) relative to tetramethylsilane (TMS $\delta = 0$) as an internal standard or relative to residual solvent peaks. Samples were analyzed by chiral-phase HPLC using a Varian 920-LC Liquid Chromatograph and a column Phenomenex Lux Cellulose-1, 4.6 × 250 mm, 5 μm. TLC was facilitated by the use of the following stains in addition to UV light (254 nm) with fluorescent-indicating plates (aluminium sheets precoated with silica gel 60 F254, Merck): phosphomolybdic acid, vanillin, iodine.

Analytical-grade solvents and commercially available reagents were purchased from TCI Europe or Sigma Aldrich and were used as received. Gelatin Porcine skin type A (PSTA) (Cat. No. G2500-100G; Batch No. 128K0066; CAS 9000-70-8; Type A, derived from acid-cured tissue; ~300 Bloom; 79% protein content by Biuret), gelatin bovine skin type B (Cat. No. G9382-100G; Lot No. 051M0012V; CAS 9000-70-8; Type B, derived from lime-cured tissue; ~225 Bloom; 73% protein content by Biuret) and gelatin from cold water fish skin (Cat. No. G7041-100G; Lot No. 071M0258V; CAS 9000-70-8; ash content 0.3%; heavy metals content 1 ppm; viscosity 8.9 CS, 10% solution, 30 °C) were purchased from Sigma Aldrich and used without further purification. Modification of side chains of gelatin, succinylation and esterification reactions of carboxylic groups of PSTA gelatin were carried out as previously described.

The catalyst samples were observed with a Carl Zeiss Merlin field-emission scanning electron microscope (FESEM, resolution 0.8 nm) equipped with a digital camera and operating at 5 kV (accelerating voltage) and 10 μA (emission current). Xerogel samples of the corresponding hydrogels were prepared by the freeze-drying (FD) method.[32] The resulting material was placed on top of a tin plate and shielded by Pt (40 mA during 30 s for FE-SEM; film thickness ≈ 5 nm).

Typical procedure for gelatin-catalyzed Henry reaction

Nitromethane (27 μL, 0.5 mmol) was added in one portion to a 4 mL screw cap vial containing 4-nitrobenzaldehyde (15.1 mg, 0.1 mmol), PSTA gelatin (2 mg) and DMSO (0.5 mL). The mixture was stirred (250 rpm) for the appropriate time at 37 °C. The reaction was quenched by the addition of EtOAc (1 mL) and EtOH (1 mL) and subsequent filtration of the precipitated catalyst. The filtrate was rinsed three times with EtOAc (1 mL), and the combined organic phases were washed with H_2O (2 × 5 mL) and brine (5 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure to afford the crude product.

Yield was determined by ^{1}H NMR of the crude product in $CDCl_3$ using diphenylmethane (1 mL of a 0.1 M stock solution) as the internal standard. The result was confirmed by a second experiment using directly dimethylacetamide (9.2 μL, added using a Hamilton syringe) as the internal standard. Thus, possible concentration variations of the stock solution of diphenylmethane in $CDCl_3$ could be detected and the values crosschecked. In the case of diphenylmethane, three different methods to introduce the standard were evaluated: (A) Introduction of the standard from the stock solution in $CDCl_3$ after complete work-up of the reaction; (B) internal standard was present in the mixture during the reaction and work-up; (C) internal standard was introduced into the reaction mixture before the work-up. The yields obtained in the above method by using the model reaction between **1a** (0.1 mmol), **2a** (0.5 mmol), DMSO (0.5 mL), and PSTA gelatin (2 mg), at 37 °C, for 6 h, were (A) = 70%; (B) = 2%; (C) 76%. In all further experiments, we used method (A) to quantify the ^{1}H NMR yield.

Typical procedure for gelatin hydrogel-catalyzed Henry reaction

A mixture of PSTA (6 mg) in H_2O (0.3 mL) was gently heated in a sealed screw cap vial (4 mL) until a homogeneous solution was obtained. This solution was stored overnight at rt to promote gel formation, which was confirmed by the complete absence of gravitational flow upon turning the vial upside down. Then, a solution consisting of 4-nitrobenzaldehyde (15.1 mg, 0.1 mmol) and nitromethane

(27 μL, 0.5 mmol,) in DMSO (0.5 mL) was added on top of the gel. The vial was stored without shaking for 24 h at rt to allow diffusion. After this time, EtOAc (1 mL) and EtOH (1 mL) were added to quench the reaction and remove the supernatant organic layer. Next, the gel was gently heated to obtain a solution, which was further diluted with H_2O (2 mL) and EtOAc (2 mL), and finally extracted with EtOAc (2 × 2 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to obtain the crude product. The ^{1}H NMR yield was determined as described above.

NOTES:

a) A control experiment to quantify any possible effect of the hydrogel on the reaction was carried out as follows: PSTA (6 mg), 4-nitrobenzaldehyde (15.1 mg, 0.1 mmol), nitromethane (27 μL, 0.5 mmol,) H_2O (0.3 mL) and DMSO (0.5 mL) were mixed in a screw cap vial (4 mL) and the mixture was stirred for 24 h at rt. After this time, EtOAc (1 mL) and EtOH (1 mL) were added to quench the reaction. The mixture was diluted with H_2O (2 mL) and EtOAc (2 mL), and finally extracted with EtOAc (2 × 2 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to obtain the crude product. The ^{1}H NMR yield was determined as described above.

b) For the reaction with cooked gelatin purchased from the supermarket, 10 g of gelatin sheets were dissolved in 100 mL water by heating it on a heating plate, and the mixture was stored overnight in the fridge. The reactions were carried out with 20 mg of the formed hydrogel.

c) All condensation products are known and the spectroscopic data obtained from the NMR analysis of the reaction mixtures were in agreement with those reported in the literature (see Supporting Information File 1).

Recycling experiments

In general, acetone or ethanol (1 mL per 2 mg of catalyst) could be used to precipitate all of the gelatin catalyst, which could be further separated by centrifugation (10 min, 3800 rpm), washing with EtOAc (2 mL), centrifugation cycles, and finally drying of the residue under vacuum before the next catalytic

cycle. Additionally, direct extraction of aqueous solutions with EtOAc may allow reuse of the aqueous solution of the catalyst in subsequent cycles.

Kinetics studies

Reaction conversions were unambiguously calculated by ^{1}H NMR analysis of the reaction mixtures according to the integration of the characteristic signals of the species in the reaction mixture in the presence of an appropriate internal standard. Each experimental point represents the average of at least two independent experiments. Among various kinetics models, lines presented in the kinetics plots show best-fits of the first-order model for each case (i.e., [NO_2R] ≥ [aldehyde]). Nevertheless, the possibility of more complex kinetics was also suggested in some cases where the fits were not ideal (e.g., TMEDA: presence of a fast introductory phase and subsequent stagnation of the reaction rate: t = 30 s, yield 52%; t = 60 s, yield 60%; t = 90 s, yield 66%; t = 120 s, yield 67%; t = 180 s, yield 63%; t = 240 s, yield 67% (Figure S3)[8]). Due to the fact that not all reactions reached 100% yield, data fitting was made according to the variation of $\ln[(C_t - C_\infty)/(C_\infty - C_0)]$ with time, where C_t is the concentration at a given time t; C_∞ the final concentration (at infinite time) and C_0 the initial concentration (at t = zero time). For reaction conversions close to 100%, plots of $\ln(C_t/C_0)$ versus time provided consistent results (C_∞ = 0). Under these considerations, minor differences were observed between the exponential and linear fits. All errors reported for the rate constants k were calculated by graphical analysis. Solubility of gelatin during the reaction was found to play no role in the product yield.

→ Optimization studies, additional experiments, figures and tables, selected ^{1}H NMR spectra, as well as additional FESEM images can be found in the supporting information (SI) on the enclosed CD.

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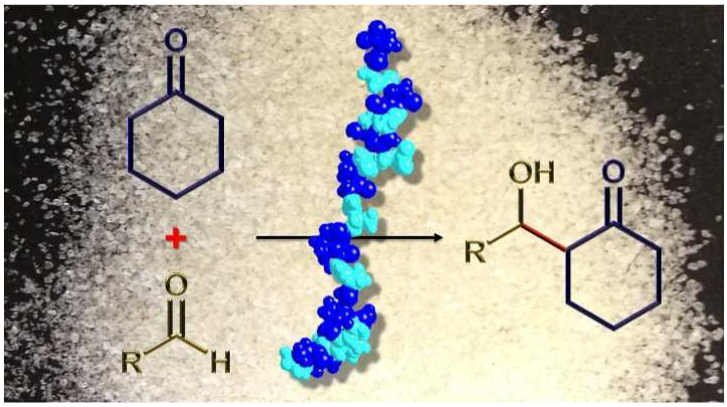
2.2 Gelatin Protein-mediated Direct Aldol Reaction[i]



Gelatin protein was found to catalyze the aldol reaction between cyclohexanone and different aromatic aldehydes under mild reaction conditions. The aldol additions carried out in DMSO at 37 ºC yielded the addition products with moderate diastereoselectivities favoring the *syn* isomers. Appropriate control experiments demonstrated the activity of the protein in the aldol reaction. The kinetic study of the model reaction between 4-nitrobenzaldehyde and cyclohexanone established a first-order rate constant of $k = (7.4 \pm 0.5) \times 10^{-3}$ h^{-1}. Moreover, the scale-up of the process was successfully achieved at 1-g scale in comparable yield to that in small scale.[ii]

[i] Reproduced with permission from: D. Kühbeck, J. Bachl, E.-M. Schön, V. Gotor-Fernández, D. D. Díaz, *Helv. Chim. Acta* **2014**, *97*, 574-580. Copyright 2014 The Verlag Helvetica Chimica Acta AG, Zürich, Switzerland.

[ii] The 2nd run of entries 1-4 in Table 1 and the recycling study were performed by E.-M. Schön. The 2nd run of entries 5-10 in Table 1 were carried out by J. Bachl. FESEM images were recorded by Prof. Dr. D. D. Díaz. All other experiments were carried out by D. Kühbeck.

2.2.1 Introduction

Since the first report in 1872,[1] the aldol reaction between two carbonyl compounds has become one of the most powerful C-C bond-forming methods in organic synthesis. During the last decades, a number of different systems, including small molecules, polymers, enzymes and antibodies, have been reported to serve as efficient catalysts for this important reaction.[2-5]

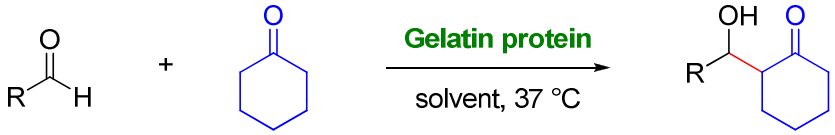
In this context, and motivated by the production of value-added products from sustainable resources, we decided to explore the potential of gelatin as a natural organocatalyst for C-C bond formation. Gelatin is a biodegradable protein (50-100 kD) obtained either by acid (type A) or alkaline (type B) processing of collagen, which is the main component of connective tissues and the most abundant protein in mammals. The average amino acid composition often follows the pattern Gly-Pro-X and Gly-X-Hyp, where X is any other amino acid. In general, commercial samples contain Gly (*ca.* 23%), Pro (*ca.* 12%), and Hyp (*ca.* 11% of the total amino acid content). It should be noted that these values might vary depending on the source of the material and processing method.[6] The fact that some amino acids and peptides have been employed in different catalytic studies[2],[7] prompted us to investigate the potential ability of gelatin to promote some organic reactions. This protein has been traditionally used as a general ingredient in food, cosmetic, pharmaceutical and photographic industries,[8] and only recently as a reducing ligand in the preparation of metal nanoparticles.[9]

As a result of our studies, we have recently reported the addition of nitroalkanes to aldehydes (Henry or nitroaldol reaction) catalyzed by gelatin and collagen proteins in both aqueous and organic media.[10] We have expanded here the potential of the gelatin protein, reporting the results obtained from the direct aldol reaction between cyclohexanone and different aromatic aldehydes in the presence of commercial gelatin under mild reaction conditions.

2.2.2 Results and Discussion

Based on our previous experience and recent reports[10],[11] the reaction between 4-nitrobenzaldehyde (**1a**, 0.1 mmol) and cyclohexanone (**2**) at physiological temperature was chosen as model aldol reaction to study the activity of the gelatin protein (i.e., gelatin from porcine skin type-A (PSTA)). Preliminary screening of the reaction conditions (see Experimental Section) revealed that 10 mg of gelatin and tenfold molar excess of **2** with respect to **1a** were optimal to obtain the corresponding addition product **3a** in reasonable yields.

Table 1. Gelatin protein-catalyzed aldol reaction.[a]



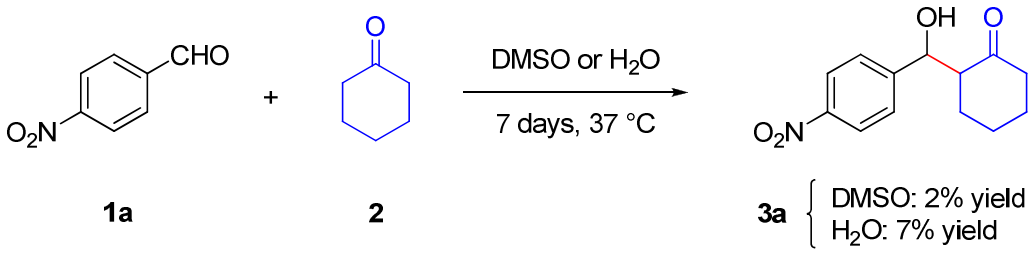
1 **2** **3**

Entry	R	1	Solvent	Yield 3 (%)[b]	d.r.[c] (syn/anti)
1	(4-NO_2)-C_6H_4	**1a**	DMSO	62	3.9/1.0
2	(4-NO_2)-C_6H_4	**1a**	H_2O	14	1.3/1.0
3	(4-NO_2)-C_6H_4	**1a**	H_2O[d]	13	1.5/1.0
4	(3-NO_2)-C_6H_4	**1b**	DMSO	54	4.1/1.0
5	(2-NO_2)-C_6H_4	**1c**	DMSO	15	1.5/1.0
6	(4-NC)-C_6H_4	**1d**	DMSO	52	4.2/1.0
7	Pyrid-2-yl	**1e**	DMSO	24	3.6/1.0
8	C_6H_5	**1f**	DMSO	20	4.2/1.0
9	(4-Br)-C_6H_4	**1g**	DMSO	34	2.8/1.0
10	(4-CH_3)-C_6H_4	**1h**	DMSO	10	2.2/1.0
11	(4-CH_3O)-C_6H_4	**1i**	DMSO	5	2.5/1.0

[a] Reaction conditions: **1** (0.1 mmol), **2** (1.0 mmol), PSTA (10 mg), solvent (0.5 mL), 37 °C, 7 d at 250 rpm; [b] Yields (^{1}H NMR) that correspond to the average values of two independent experiments (standard deviation, ± 2%); [c] Diastereomer ratio *syn/anti* determined by ^{1}H NMR analyses of two independent experiments. Relative configurations were assigned by comparison with the data reported in the literature; [d] Reaction carried out in the presence of TBAB (13 mg, 0.04 mmol).

Higher loadings did not significantly improve the results. For instance, the product yield obtained in the model reaction using 10 equiv. of **2** was ca. 62%, whereas the use of 20 equiv. afforded the product in ca. 64% yield. In contrast, the use of only 5 equiv. of **2** decreased the yield to 38%. Thus, further studies on the substrate scope were carried out using the mentioned pre-optimized conditions (Table 1).

Regarding the solvent, DMSO was found suitable for carrying out a comparative study. For instance, the product yield obtained in DMSO was ca. fourfold higher than in H_2O under the same conditions (entries 1 and 2). In contrast to our previous observations with the Henry (nitroaldol) reaction,[10] the use of a phase transfer co-catalyst such as tetra-*N*-butylammonium bromide (TBAB)[12] did not improve the yield of the gelatin-mediated aldol reaction in H_2O (entries 2 and 3). It is important to highlight that appropriate control experiments confirmed the catalytic activity of the protein. For instance, only a tiny amount of the aldol product **3a** was detected when the model reaction between **1a** and **2** was carried out either in DMSO or in H_2O (pH *ca.* 4-5) for 7 days in the absence of gelatin (Scheme 1).



Scheme 1. Control experiment in the absence of gelatin protein.

In agreement with our previous studies on the Henry reaction,[10] we could also exclude any catalytic effect on the aldol reaction possibly caused by other minor components (*ca.* 10%) such as metal and ash impurities in the gelatin samples.[9a],[13] The aldol product was obtained in only 4% yield when the model reaction of **1a** and **2** was performed only in the presence of metal ions as *Lewis* acids at the concentration reported to be present in gelatin samples (i.e., [metal] $\times$ 10^{-6} mol L^{-1} = 3.92 (Co^{2+}); 5.0 (Cu^{2+}); 9.42 (Fe^{3+}); 2.62 (Pd^{2+}); 9.92 (Ni^{2+}).[13] Most importantly, although the specific mechanism of gelatin catalysis remains unclear,

a simple acid or base catalysis was ruled out by (a) controlling the pH of the reaction medium (pH *ca.* 5-5.5), (b) the poor yields of the blank experiments (*vide supra*), and (c) almost identical results obtained using gelatin samples with different isoelectric points (i.e., PI gelatin type-A ≥ 6; PI gelatin type-B ≤ 5), which was in agreement with our previous observations.[10]

We further studied the gelatin-mediated reaction between different aldehydes **1a**-**1i** and **2** (Table 1). Aromatic aldehydes with strongly or moderately deactivating groups (i.e., electron-withdrawing substituents) were smoothly converted into the corresponding β-hydroxy ketones in modest yields (entries 1, 4, 5 and 6). The results also revealed the influence of the substitution position on the product yield, indicating a significant decrease with the *ortho*-substituted aldehyde **1c** (entries 1, 4 and 5). On the other hand, low yields were obtained with pyridine-2-carbaldehyde **1e** (entry 7), benzaldehyde (**1f**, entry 8), and in the cases of aromatic aldehydes bearing weakly or moderately activating groups (i.e., electron-donating substituents, i.e., **1g-1i**, entries 9-11). As observed with other aldol-like reactions, these yields could be improved by increasing the reaction time and/or the temperature.[10] Unfortunately, aliphatic aldehydes such as isovaleraldehyde were not converted after 7 days under the reported conditions.

Interestingly, a modest *syn* diastereoselectivity was found in all cases (i.e., *ca.* two- to fourfold of *syn vs*. *anti*), which is an uncommon feature in this reaction.[2],[3] The diastereomeric ratio (dr) was reduced either in H_2O, with *ortho*-substituted aromatic aldehydes or with activated aromatic aldehydes. As expected due to the mild reaction conditions, no potential by-products such as dehydrated or self-condensation products were observed. In accordance with our previous studies,[10] the direct use of collagen also afforded the desired aldol product (e.g., **1a** (0.1 mmol), **2** (1.0 mmol), collagen (10 mg), DMSO (0.5 mL), 7 days, 37 ºC: Yield = 46%, *dr* (*syn*/*anti*) = 3.4/1.0). It is also worth mentioning that the reaction can be scaled-up with comparable yields to the small scale (i.e., **1a** (1.13 g, 7.5 mmol), **2** (7.75 mL, 75 mmol), PSTA gelatin (0.75 g), DMSO (38 mL), 7 days, 37 ºC, *ca*. 58% yield). In terms of recycling, gelatin was quantitatively recovered and reused in at least two consecutive runs with maintenance of stereoselectivity. Nevertheless, a major catalyst deactivation was observed in the third run (Figure 1).

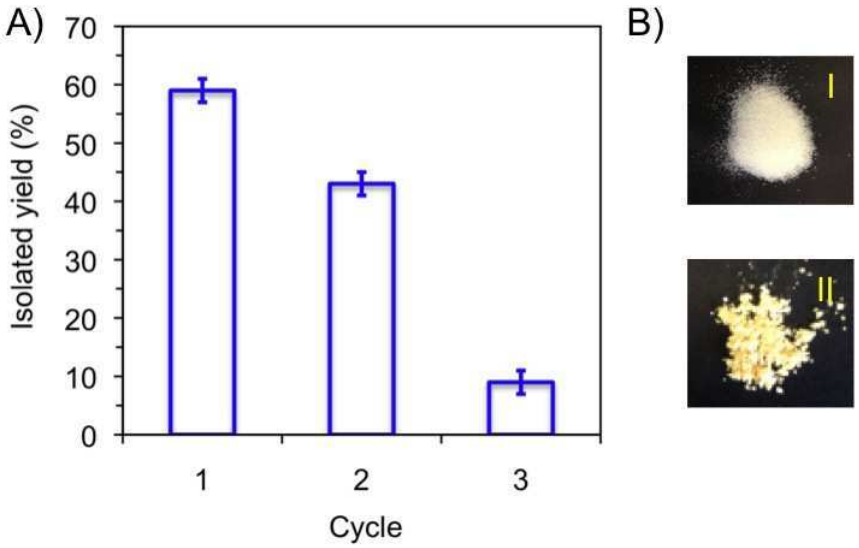


Figure 1. A) Recycling experiment for the model reaction between **1a** (7.5 mmol) and **2** (75 mmol) catalyzede by PSTA gelatin (0.75 g) in DMSO (38 mL) at 37 °C for 7 d. **B)** Gradual coloring and apperent clogging of gelatin. I: Gelatin before 1st run; II: Washed-dried gelatin after 3rd run.

This is presumably due to inefficient molecular desorption from the catalyst, instead of decomposition, as suggested by a visible gradual color change (from light-yellow to intense yellow-orange) and similar FT-IR spectra of the washed-dried catalyst after each run. Either acetone or EtOH could be used to precipitate the protein from DMSO, which was further separated by centrifugation. Alternatively, direct extraction of the aqueous solutions with EtOAc allows the simple isolation and subsequent reuse of the protein-containing aqueous solution in the next cycle.

Despite the chiral secondary structure of gelatin and collagen proteins, chiral high-performance liquid chromatography of the reaction mixtures revealed almost negligible enantioselectivity (i.e., < 5% *ee* for the major diastereomer, *syn*) during the aldol reaction. This lack of enantioselectivity has been also reported with other proteins or biopolymers when used as catalysts in aldol-like reactions.[10],[11],[14] In contrast to the results reported with other biocatalysts,[15] reducing the temperature to 20 ºC did not cause any increase of the enantiomeric selectivity. The first-order kinetics analysis of the model reaction between **1a** and **2** established a slow rate constant k = (7.4 ± 0.5) x 10^{-3} h^{-1} (Figure 2). It is worthwhile to mention that the morphology and/or physical state of a protein

catalyst may also play a significant role in the kinetics of aldol-like reactions under heterogeneous or semi-heterogeneous conditions.[10]

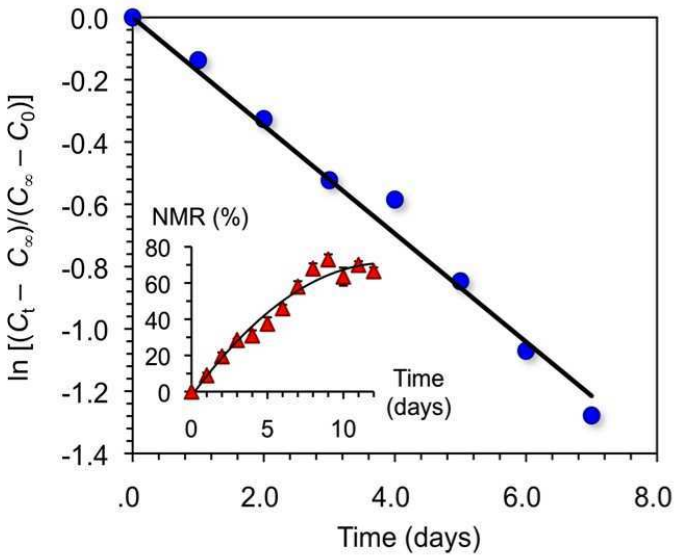


Figure 2. First-order kinetics plot of the model aldol reaction between **1a** and **2** catalyzed by gelatin. C_∞ = final concentration, at infinite time; C_t = concentration at given time t; C_0 = initial concentration, at t = zero time. $R^2 = 0.98$. Inset: Evolution of reaction conversion over time.

2.2.3 Conclusion

In summary, we have demonstrated that C-C bond formation through direct aldol reaction between cyclohexanone and different aromatic aldehydes can be promoted by commercial gelatin in organic medium under mild conditions. The corresponding addition products were obtained in modest yields governed by first-order kinetics. In general, modest *syn*-diastereoselectivity and complete chemoselectivity were also observed, demonstrating the applicability of the catalytic system in a g-scale experiment. Moreover, the protein could be recovered at the end of the reaction and reused in at least two consecutive cycles without major loss of the catalytic activity. Finally, we would like to emphasize that these and our previous investigations indicate that, although edible gelatin is not a competitive catalyst for this type of reactions from a synthetic perspective, the fact that it can mediate to some extent (even under aqueous conditions) the transformation of carbonyl compounds existing in numerous foods or cosmetic formulations could be significant from a metabolic point of view.

2.2.4 Experimental Section

Materials and Methods

Analytical grade solvents and commercially available reagents were purchased from Sigma Aldrich or TCI Europe and used as received. Milli-Q H_2O was used for the experiments in aqueous solutions. Gelatin porcine skin type-A was purchased from Sigma Aldrich (CAS 9000-70-8; Cat. No. G2500-100G; Batch No. 128K0066; Type A, derived from acid-cured tissue; ca. 300 Bloom; 79% protein content by Buiret) and used without further purification. Collagen was purchased from Sigma Aldrich (CAS 9007-34-5; Cat. No. C9879-1G; Batch No. 061M7015V; Type A, derived from bovine achilles tendon). A detailed description of the most important features of both gelatin and collagen can be found in the Supporting Information of our previous work focused on the nitroaldol (Henry) reaction catalyzed by these proteins.[10]

^{1}H NMR spectra were recorded on a Bruker Avance 300 spectrometer at 25 °C. TLC analyses were carried out on fluorescent-indicating plates (aluminium sheets precoated with silica gel 60 F254, Merck) and the products visualized by the use of the phosphomolybdic acid as stain solution and UV light (254 nm). The reaction mixtures were analyzed using a Varian 920-LC Liquid Chromatograph (column Phenomenex Lux Cellulose-2, 4.6 x 250 mm, 5 μm; eluent: *n*-heptane:*i*-PrOH 70:30; flow 1.0 mL/min; λ = 254 nm). FT-IR spectra were recorded using a Diamond ATR (attenuated total reflection) accessory (Golden Gate). Yields were determined by ^{1}H NMR analyses of the crude product in $CDCl_3$ using diphenylmethane (1 mL of a 0.1 M stock solution) as internal standard. The results were confirmed by a second experiment using directly dimethylacetamide (9.2 μL, added using a Hamilton syringe) as internal standard. Thus, possible concentration variations of the stock solution of diphenylmethane in $CDCl_3$ could be detected and the values crosschecked. Relative configurations were assigned by comparison with ^{1}H NMR data reported in the literature.[16] For kinetics calculations, the ^{1}H-NMR analyses of the reaction mixtures were performed in the presence of diphenylmethane (0.1 mmol) as internal standard. Each experimental point was given as the average of at least two independent experiments. Among

various kinetics models, the straight line shown in the kinetics plot show best-fit of the first-order model (i.e., [cyclohexanone] ≥ [aldehyde]). Copies of NMR and IR spectra can be obtained directly on request to the authors.

Preliminary optimization experiments

A) Screening of cyclohexanone (**2**) equivalents: The model reaction between **1a** (15.1 mg, 0.1 mmol) and **2** (5, 10 and 20 equivalents) was carried out in DMSO (0.5 mL) at 37 ºC for 7 days in the presence of PSTA gelatin (10 mg). The results showed that the aldol product **3a** was obtained in 38%, 62% and 64% yield, respectively.

B) Screening of catalyst loading: Table S2 shows the results of selected experiments for the reaction between **1a** (0.1 mmol) and **2** (2 mmol) in DMSO (0.5 mL) under different conditions of solvent temperature, reaction time and catalyst loading.

General Procedure for Gelatin-catalyzed Aldol Reaction

Cyclohexanone (**2**; 104 μL, 1.0 mmol) was added in one portion to a 4 mL screw cap vial containing 4-nitrobenzaldehyde (**1a**; 15 mg, 0.1 mmol), gelatin PSTA (10 mg), and DMSO (0.5 mL). The mixture was stirred (250 rpm) for 7 d at 37 °C. The reaction was quenched by the addition of EtOAc (1 mL) and EtOH (1 mL), and subsequent filtration of the precipitated catalyst. The filtrate was rinsed three times with EtOAc (3 x 1 mL) and the combined organic phases washed with H_2O (2 x 5 mL) and brine (5 mL), dried (Na_2SO_4), filtered and evaporated under reduced pressure to afford the crude product. Yield and diastereomer ratio (*syn/anti*) were determined by ^{1}H NMR analyses of the crude product in $CDCl_3$ (300 MHz) using diphenylmethane (1 mL of a 0.1 M stock solution) as internal standard after complete work-up of the reaction. Relative configurations were assigned by comparison with the spectroscopy data reported in the literature. For instance, in the model reaction between **1a** and **2**, the *anti* diastereomer was clearly identified by a doublet at 4.85 ppm (1H, J = 8.3 Hz), whereas the *syn* diastereomer displayed the doublet at 5.41 ppm (1H, J = 2.4 Hz). Typically, for recycling experiments acetone or EtOH (1 mL per 2 mg of catalyst) was added in order to

precipitate the gelatin from DMSO. The protein was subsequently separated by centrifugation (10 min, 3800 rpm)-washing with EtOAc (2 mL)-centrifugation cycles. The obtained residue was finally dried under vacuum before the next catalytic cycle.

→ Optimization experiments, selected ^{1}H NMR spectra, FT-IR spectra as well as additional FESEM images can be found in the supporting information (SI) on the enclosed CD.

2.2.5 References

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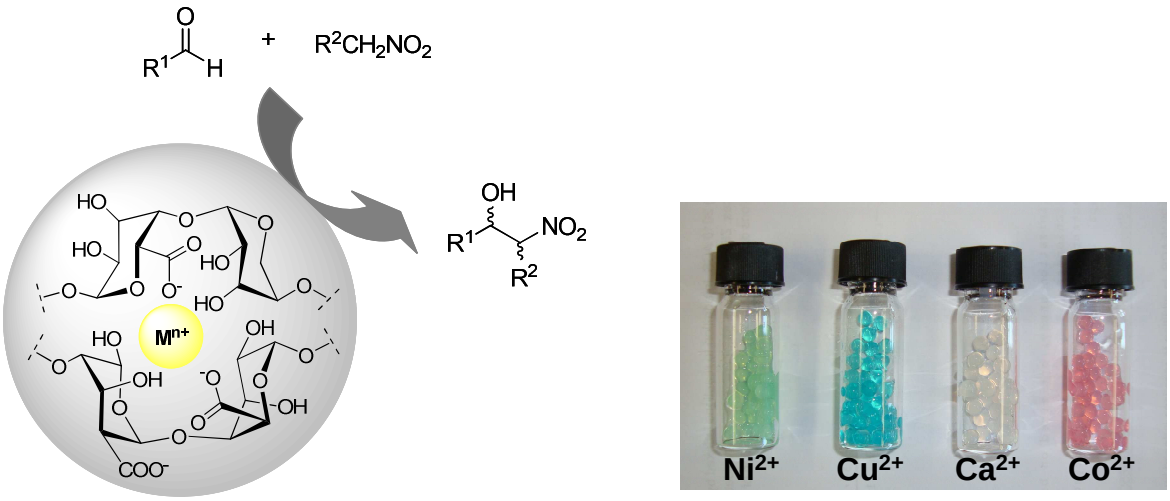
3. Metal Alginate-catalyzed Nitroaldol (Henry) Reaction



In this work the activity of different metal alginate hydrogel beads (i.e. Ca^{2+}-, Cu^{2+}-, Co^{2+}-, Ni^{2+}-, Zn^{2+}- and Fe^{3+}-AHG) in the nitroaldol (Henry) reaction between aldehydes and nitroalkanes under mild conditions was investigated. A comprehensive study showed that Ca^{2+}-AHG in DMSO was the most effective heterogeneous system. The hydrogel generated a slight *syn*-selectivity when nitroethane as nucleophile was established. In addition, it could be applied successfully on a large scale reaction (1 g) between 4-nitrobenzaldehyde and nitromethane in good yields. The recyclability of the catalyst was possible at least three times without significant loss of activity. A comparative kinetic study with calcium 2-ethylhexanoate, an important industrial polymerization catalyst, and our Ca^{2+}-AHG was conducted, whereby the former homogeneous catalyst (TOF = 536.54 h^{-1}) was much faster than the hydrogel (TOF = 0.78 h^{-1}). Furthermore, all materials were characterized by different variables like ICP-OES, IR, TGA and FESEM.[i]

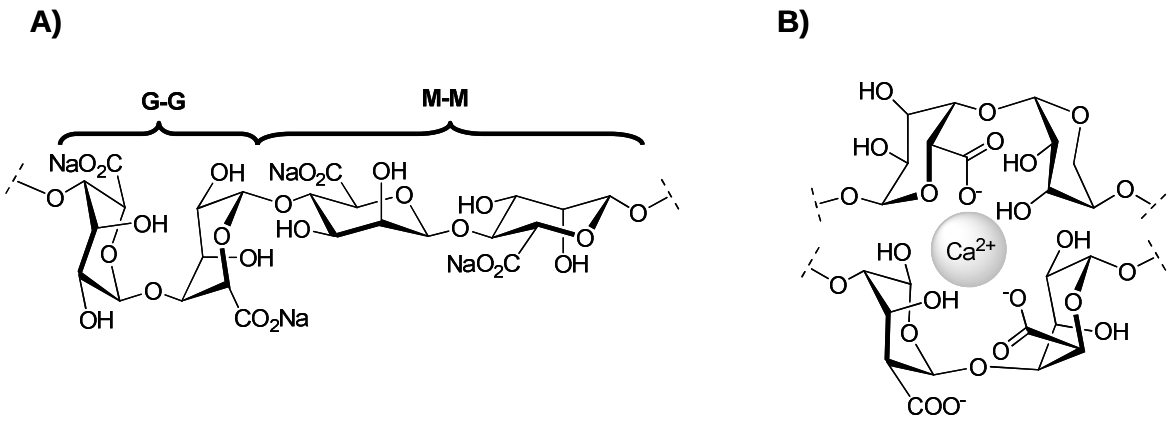
[i] M^{n+}-AHG beads (M^{n+} = Cu^{2+}, Co^{2+}, Ni^{2+} and Fe^{3+}) were prepared by M. Hofmann. ICP-OES measurements were carried out in assistance with J. Rewitzer. FESEM images were recorded by Prof. Dr. D. D. Díaz. All other syntheses and experiments were conducted by D. Kühbeck.

3.1 Introduction

Under consideration of economical aspects in organic synthesis, the investigation of cheap and heterogeneous catalysts has become an important challenge, nowadays. The most typical representatives for this approach are sustainable materials, like the so called biocatalysts (i.e. enzymes) and/or biopolymers (i.e. polysaccharides). Biocatalysis has considerable advantages compared to conventional chemical methods. The catalysts can be applied under mild and heterogeneous conditions, are highly selective and in most cases reusable for several cycles. In addition, the whole process can be named as clean and green due to the common use of water as reaction media.

When especially enzyme catalysis is examined it can be noticed that the stability, heterogeneity and reusability of the enzyme can be considerably enhanced by its immobilization on polysaccharide-based hydrogels, e.g. metal crosslinked alginate gels.[1] Furthermore, this type of combined materials is well investigated in different types of reactions, whereas for the single support only a few contributions as an active catalyst are known.[2]

Alginate is an anionic polymer isolated from brown algae. It has several advantages like biodegradability, non-toxicity and it is wide abundant in nature, thus cheap.



Scheme 1. A) G (α-L-guluronate) and M (β-D-mannurate) blocks in sodium alginate; B) Coordination of Ca^{2+} cation between two adjacent alginate chains (egg-box model).

Furthermore, it is a typical linear unbranched polysaccharide consisting of (1-4)-linked β-D-mannurate (M) and α-L-guluronate (G) monomers (Scheme 1A). Most

importantly stable hydrogels can be made by simple ionotropic gelation with polyvalent metal cations at room temperature. Thereby, in most cases as described by the egg-box model the metal is bound to four G monomers of two adjacent chains of alginate (Scheme 1B).[3]

Based on the chiral backbone and slightly basic carboxylate groups (COO^-) along the polymer, alginate itself owns already promising capabilities for acting as a chiral catalyst. In this context, S. Oudeyer and co-workers were reporting the first catalytic activity of sodium alginate in the cyanomethylation reaction of carbonyl compounds with (trimethylsilyl)acetonitrile.[2c] As the use of catalysts in a powdered form has still limitations in the work-up, we wanted to investigate the use of alginate-based hydrogel beads, which are much easier to separate from the reaction mixture and can be applied without extensive recovery in the next run. For this approach we decided to test this assumption in the nitroaldol (Henry) reaction which is closely related to the cyanomethylation reaction. This type of reaction is the most important synthetic route to create β-nitroalcohols, which are known as useful precursors for compounds, like nitroalkenes, α-nitroketones and 1,2-amino alcohols.[4] The fact that the latter building block can be found in several pharmaceuticals,[5] emphasize the importance of this reaction in organic synthesis. To best of our knowledge we report here the first application of single metal crosslinked alginate hydrogel beads as catalysts in a C-C bond forming reaction.

3.2 Results and Discussion

Several alginate-based hydrogel beads were prepared like previously described.[6] For our library of potential catalysts different metals (i.e. Ca^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Fe^{3+}) were used as gelling agent. In all cases metal chloride salts were applied to avoid any possible catalytic activity by counter ions entrapped in the gel matrix.[7] In Figure 1 the set-up for the beads preparation is shown. Thereby, a simple dropping funnel containing an aqueous 2% (w/v) sodium alginate solution was installed. The viscous polysaccharide solution was dropped with a certain dropping rate and defined gap in an aqueous gelling solution consisting of 0.24 M metal chloride (for further information see Experimental Section). The metal cation

concentration of 0.24 M and the volumetric ratio between the sodium alginate and metal chloride solution (1:2) were kept constant in each case. It is important to mention that the dropping rate and the distance between the tip of the dropping funnel and the surface of the gelling solution was depending on the metal salt used. After maturing the beads over night at room temperature in the gelling solution, they were washed meticulously to remove the excess of metal cations as well as their counter anions.

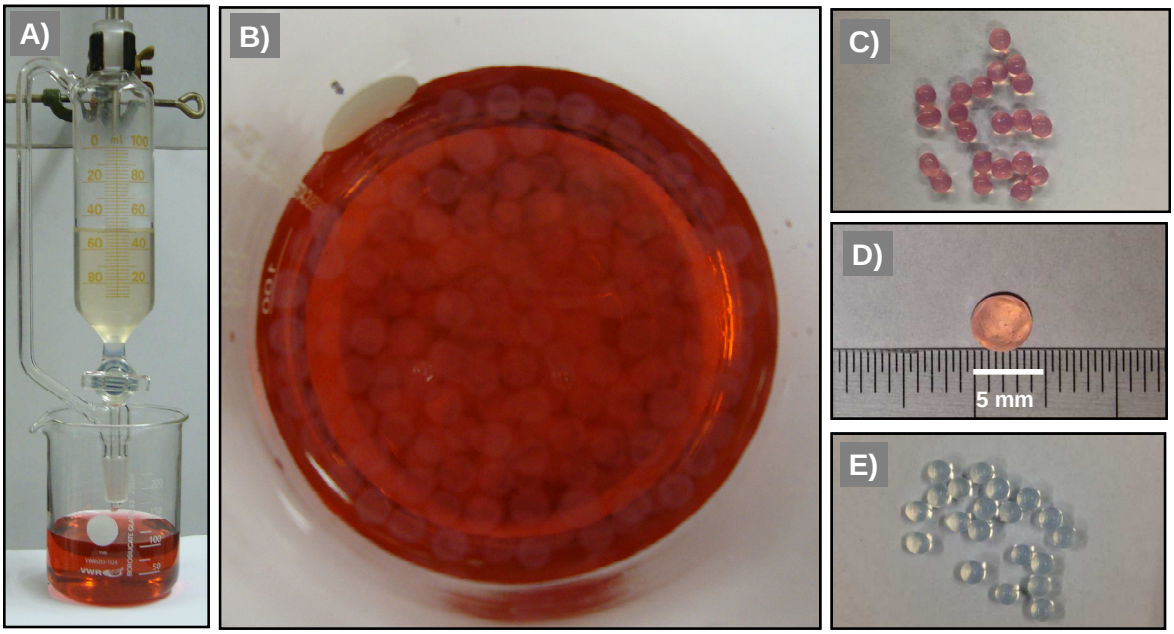


Figure 1. A) Set-up for hydrogel beads preparation: Dropping funnel containing a 2% (w/v) sodium alginate solution and the corresponding jar with an aqueous 0.24 M $CoCl_2 \cdot 6\ H_2O$ solution; B) Co^{2+}-AHG beads matured in the gelling solution; C) Isolated Co^{2+}-AHG beads; D) Amplification of a single Co^{2+}-AHG bead; E) For comparison: Isolated Ca^{2+}-AHG beads.

With this method beads of different diameters were obtained, i.e. in a range from 3.6 mm for Zn^{2+} to 4.9 mm for Fe^{3+}. Thereby, a correlation between the gelling metal and the bead size could be recognized. The metal content of the so prepared hydrogel beads were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Interestingly, a slightly increase of the metal loading with increasing diameter could be monitored (Figure 2). At first glance, this fact should be kind of expected. But when it is considered that in every hydrogel bead the same amount of biopolymer should be found, the attraction/complexation of the metal by alginate plays a more important role than the size. Accordingly, the

highest possible metal-uptake within the alginate gel matrix rises with following order $Ni^{2+} > Cu^{2+} > Zn^{2+} > Co^{2+} > Fe^{3+} > Ca^{2+}$.

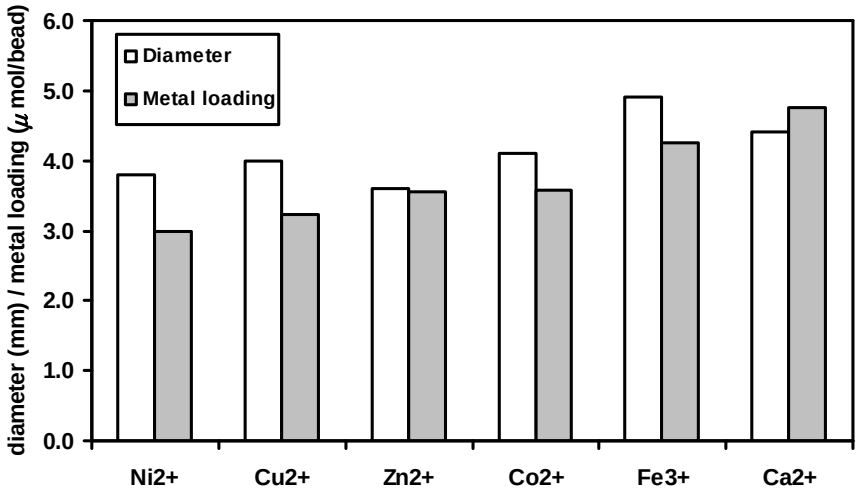


Figure 2. Correlation between M^{n+}-AHG beads diameter (white bars) and the corresponding metal loading (grey bars).

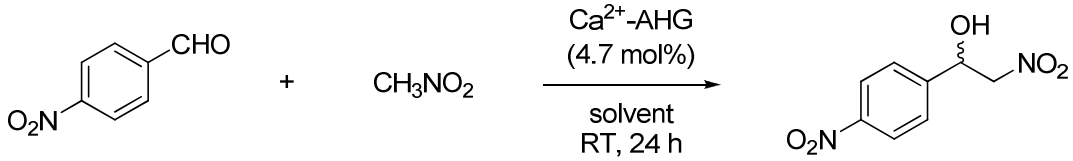
The FT-IR spectra of all alginate-based materials showed all expected bands. In general the used metals had no major impact on the vibration modes. As all samples showed the typical bands at 1597 cm^{-1} for the asymmetric stretching band of the COO^- group (ν_a COO^-) and 1411 cm^{-1} for the symmetric stretching band of the COO^- group (ν_s COO^-), as well as the characteristic bands for polysaccharides between 1176-1028 cm^{-1} (ν C-O, ν C-C and δ C-O-H). The broad band between 3379 and 3076 cm^{-1} corresponds to the –OH absorption (see supporting information for IR spectra, Figure S2 – S4).[8]

Furthermore, the thermal stability for all samples was tested. In the case of all hydrogels the freeze-dried samples were used to obtain better resolutions in the thermograms. The measurements were applied between 25 and 350 °C. In this area all important transitions were noticeable. Generally, the freeze-dried hydrogel samples and the commercial available sodium alginate powder showed a slow weight loss below 100 °C, which could be attributed to the vaporization of moisture and its lactonization. Around 200 °C the polymer decomposition started very rapidly. At this point the weight loss was generated by the degradation of the alginate backbone and the loss of abundant hydroxyl groups in form of water. Thereby, a trend of stability in the area between 200 and 300 °C could be monitored.[9] The stability grew in following order $Fe^{3+} < Cu^{2+} < Na^+ < Ni^{2+} \approx Co^{2+} \approx$

Zn^{2+} ≈ Ca^{2+}. Samples of Ca^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} showed almost the same behaviour in this temperature range, whereas copper and iron doped beads were quite unstable (see supporting information for TGA curves, Figure S5).

As calcium is one of the cheapest commercially available metals, non-toxic and wide abundant on earth,[10] we used the calcium-based hydrogel for further investigations. The results obtained from previous optimization studies showed that 10 equivalents of nitromethane (**2**) in respect to 4-nitrobenzaldehyde (**1a**) and one bead of Ca^{2+}-AHG are necessary to drive the reaction to completion within 24 hours at room temperature in DMSO (see supporting information for optimization table).

Table 1. Solvent screening study for Ca^{2+}-AHG.[a]



1a **2** **3a**

Entry	Solvent	Yield (%)[b]
1	toluene, DCM	0
2	MeCN	< 1
3	THF	2
4	EtOH	9
5	H_2O	16
6	H_2O/TBAB[c]	44 (3[d])
7	DMSO	88 (0[d])

[a] Reaction conditions: **1a** (0.1 mmol), **2** (1.0 mmol), 1 unit of Ca^{2+}-AHG bead, solvent (0.5 mL), room temperature, 24 h; [b] Determined by ^{1}H NMR analysis of the crude product (9.2 μL of DMA was used as internal standard); [c] Tetra-*n*-butylammonium bromide (TBAB, 0.04 mmol); [d] Control experiment: Reaction in the absence of Ca^{2+}-AHG bead.

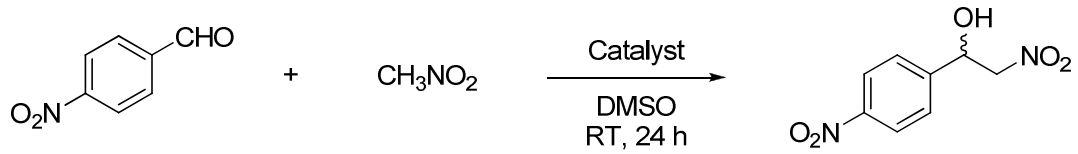
To investigate if the already good yields of 88% obtained in DMSO (Table 1, entry 7) could be increased to excellent yields different solvents in this context were tested and the results are summarized in Table 1. When non-polar solvents like toluene and DCM (Table 1, entry 1) were used, no product formation could be monitored. This could be changed at least to some extent when the solvent had a

polar aprotic nature. In the case of MeCN traces of the product could be obtained (Table 1, entry 2), whereas in THF the yield could be enhanced to 2% (Table 1, entry 3). Much better results could be achieved when the reaction media was shifted towards polar protic solvents. The catalyst showed in EtOH (Table 1, entry 4) almost 5 times higher productivity than in THF. Under consideration of a “green” approach the hydrogel was also tested in pure H_2O which lead to 16% yield of the product (Table 1, entry 5). As this result was not competitive with the yields obtained in DMSO, TBAB was used as phase transfer additive in H_2O. Within this tuned system the yield could be almost tripled up to 44% (Table 1, entry 6). It is worth to mention that the background reaction in DMSO without catalyst under the same conditions is totally inhibited, compared to the background in the H_2O/TBAB system with 3%. Due to these observations the model reaction between 4-nitrobenzaldehyde and nitromethane in DMSO was chosen for further investigations. Additionally, the scale-up of the model reaction from 0.1 to 7.5 mmol (1.13 g) of **1a** leads to a comparable isolated yield of 82%.

We also studied the influence of different metal cations in this reaction, as it is known that the morphology/porosity of the hydrogel network is different for each case.[11] The best result with 88% yield of β-nitroalcohol was obtained with Ca^{2+}-AHG (Table 2, entry 1). Also good yields could be obtained with Co^{2+}-AHG (50%, entry 3) and Ni^{2+}-AHG (59%, entry 4), whereas a modest yield of 36% with Zn^{2+}-AHG (Table 2, entry 5) were monitored. Cu^{2+}-AHG and Fe^{3+}-AHG achieved only poor yields of 4% (Table 2, entry 2) and 5% (Table 2, entry 6), respectively. It has to be mentioned that all hydrogel beads beside Fe^{3+}-AHG were stable under the reaction conditions and were not affected from a macroscopical point of view. Unfortunately, no enantioselectivity in all cases was detected although it could be expected due to the chiral backbone of alginate and a possible transition metal coordination of the substrates. To confirm if the hydrogel is necessary to complete the reaction, other forms of the catalyst were also established. The freeze-dried (FD) form of the Ca^{2+}-AHG afforded 4% yield of the desired product, the air-dried form however only 1% (Table 2, entry 1). With these two drying methods the water content of the hydrogel could be determined to 434 μL per bead. With this in mind another control experiment with the freeze-dried form and the amount of water held in one hydrogel bead were performed. The outcome of 14% yield is still much lower when compared with the yield obtained by Ca^{2+}-AHG itself. This leads to the

conclusion, that the nature of the hydrogel plays an important role in this reaction, which is in contrast to the previously reported result in the cyanomethylation reacton of carbonyl compounds with (trimethylsilyl)acetonitrile.[2c] In addition, the reaction was also performed simply with the commercial available sodium alginate powder (Na^+-AP).[12] In the case of the powder 6% yield could be obtained, whereas the powder and H_2O resulted in 13% yield. The control experiment without powder but with H_2O was completely inhibited. A comparison between the heterogeneous Ca^{2+}-AHG bead and the homogeneous catalyst calcium 2-ethylhexanoate (Ca^{2+}-2EH) showed a significant higher efficiency of the latter (Table 2, entry 8), which confirms the heterogeneous nature of the hydrogel.

Table 2. Influence of different metal alginate-based materials in DMSO.[a]



1a **2** **3a**

Entry	Catalyst	mol (%)[b]	TOF (h^{-1})	Yield (%)[c]
1	Ca^{2+}-AHG	4.76	0.77	88 (4,[d] 1,[e] 14[f])
2	Cu^{2+}-AHG	3.23	0.05	4
3	Co^{2+}-AHG	3.57	0.58	50
4	Ni^{2+}-AHG	2.99	0.82	59
5	Zn^{2+}-AHG	3.56	0.42	36
6	Fe^{3+}-AHG	4.26	0.04	5[g]
7	Na^+-AP	2.08	0.12	6 (13[h], 0[i])
8	Ca^{2+}-2EH	2.08	536.54	93[j]

[a] Reaction conditions: **1a** (0.1 mmol), **2** (1.0 mmol), DMSO (0.5 mL), 1 unit of M^{n+}-AHG bead, room temperature, 24 h; [b] Metal mol% are calculated in respect to the metal/aldehyde ratio; [c] Determined by ^1H NMR analysis of the crude product; [d] Control experiment: freeze-dried form of the hydrogel was used; [e] Control experiment: air-dried form of the hydrogel was used; [f] Control experiment: freeze-dried form of the hydrogel and 434 μL H_2O [g] Fe^{3+}-AHG bead was not stable during reaction; [h] 0.4 mg Na^+-AP and 434 μL H_2O was used; [i] Control experiment: Reaction was performed in absence of Na^+-AP but with 434 μL H_20; [j] Reaction time: 5 min.

With this information in hand we were investigating more control experiments to emphasize the heterogeneous nature of this system. This could be ruled out by a

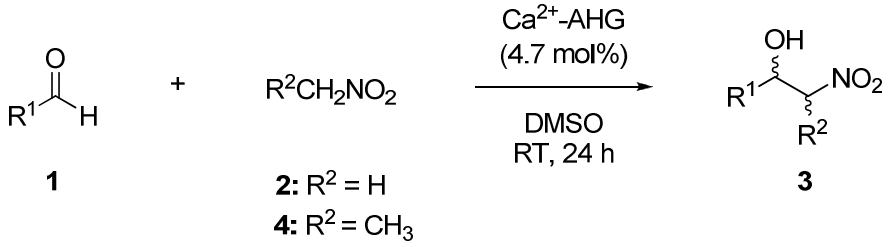
simple experiment, where one bead of the Ca^{2+}-AHG was matured for 24 hours in DMSO without substrates. After this time, the bead was removed and reused in a new reaction mixture including the substrates of the model reaction. With this method almost identical yields could be obtained like in the case without maturing. In addition, a second experiment was run by combining the resulting DMSO after maturing the hydrogel and the substrates. The outcome of 23% can be attributed to a slightly degradation of the hydrogel to small particles, which remained in the matured solution. To exclude the fact that the yield reported above derives from metal leaching, the maximal possible leaching was calculated in the case of Ca^{2+}-AHG and the resulting stock solution (9.52 mM $CaCl_2$) was combined with the substrates of the model reaction and the amount of H_2O held in one HG bead.[13] This reaction was completely inhibited and showed clearly that leaching of the metal has not an impact on the reaction outcome.

We further explored the ability of Ca^{2+}-AHG to convert different aldehydes with either nitromethane or nitroethane. In general, aromatic aldehydes with strong or moderate electron-withdrawing groups were easily converted to the corresponding *β*-nitroalcohols in moderate to very good yields during 24 hours at room temperature (Table 3, entries 1-6). Aldehydes with weak electron-withdrawing groups could be achieved with lower yields (Table 3, entries 8-10), whereas the corresponding *β*-nitroalcohols of benzaldehyde and tolualdehyde could be obtained only in poor yields (Table 3, entries 7 and 11). Interestingly, also heteroaromatic systems like 2-pyridinecarboxaldehyde led to 50% yield (Table 3, entry 12). In the case of aliphatic aldehydes unfortunately no *β*-nitroalcohol was obtained. When the outcome of either nitromethane (pK_a = 10.2) or nitroethane (**4**, pK_a = 8.6) is compared, it can be recognized that almost similar results are monitored in both cases. This leads to the conclusion, that under our conditions neither the sterical aspect nor the acidity of the nucleophile plays a major role for the outcome. The diastereoselectivity in the nitroaldol reaction with nitroethane was not significant, but a small trend towards the *syn* isomer could be noticed, which is in contrast to the control experiment where the *anti* isomer is favoured.

Our Ca^{2+}-AHG catalyst was found to be at least three times reusable without significant loss of activity. But after the fourth cycle a considerably decrease of the

yield to 72% was detectable which was further reduced to 64% after the fifth cycle (Figure 3).

Table 3. Substrate scope of the Ca^{2+}-AHG-catalyzed Henry reaction in DMSO.[a]



1 **2:** $R^2 = H$ **4:** $R^2 = CH_3$ **3**

Entry	R^1 (1)	R^2 (2 or 4)	Yield (%)[b]	dr[c]
1	(4-NO_2)-C_6H_4	H	87 (0[d])	NA
2	(4-NO_2)-C_6H_4	CH_3	91 (47[d])	1:1.3 (1.4:1)
3	(2-NO_2)-C_6H_4	H	92	NA
4	(3-NO_2)-C_6H_4	H	66	NA
5	(4-CN)-C_6H_4	H	91	NA
6	(4-CN)-C_6H_4	CH_3	91	1:1.3
7	C_6H_5	H	7	NA
8	(4-Br)-C_6H_4	H	32	NA
9	(4-Cl)-C_6H_4	H	18	NA
10	(4-Cl)-C_6H_4	CH_3	80	1:1.2
11	(4-CH_3)-C_6H_4	H	3	NA
12	Pyridin-2-yl	H	50	NA
13	$(CH_3)_2CHCH_2$	H	0	NA

[a] Reaction conditions: aldehyde **1** (0.1 mmol), **2** or **4** (1.0 mmol), DMSO (0.5 mL), 1 unit of Ca^{2+}-AHG bead (4.67 mol% in respect to the Ca/aldehyde ratio), room temperature, 24 h; [b] Determined by ^1H NMR analysis of the crude product. The values correspond to the average of two independent experiments (standard deviation, STDV = ± 3%); [c] Diastereomeric ratio (*anti/syn*) determined by ^1H NMR analysis. NA = Not applicable; [d] Control experiment performed in the absence of Ca^{2+}-AHG. Reaction time = 24 h, room temperature.

This could be associated with either leaching of the metal during the recycling or more likely through poisoning of the catalyst by the aldehyde. The latter claim was already noted by C. Verrier and co-workers when they were trying to recycle an alginate-based acetogel in Mukuyma's cyanomethylation reaction.[2c] Beside this

fact further poisoning of sugar backbones are known and were reported in previous works.[14]

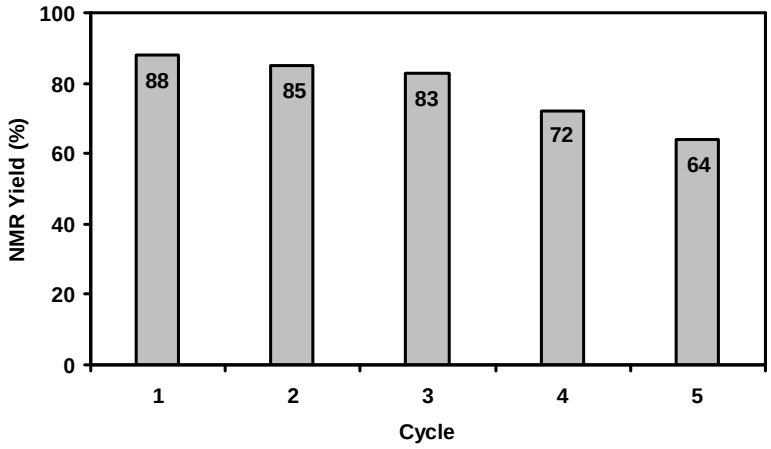


Figure 3. Typical recycling experiment for the Ca^{2+}-AHG-catalyzed Henry reaction. Reaction conditions: **1a** (15.1 mg, 0.1 mmol), **2** (54 μL, 1.0 mmol), DMSO (0.5 mL), Ca^{2+}-AHG (one bead), 24 h, RT.

Since it is unclear if such poisoning and the promoted reaction is taking place only on the surface or in the whole catalyst, further experiments were carried out. For that, a simple comparison was made between one whole bead and one half cutted bead.[7] In both cases the model reaction between **1a** and **2** was used. The NMR yields of the β-nitroalcohols were determined after one, two, three and four hours in each case (Figure 4C). The results obtained showed that our hydrogel is no nanoreactor,[15] but the reaction is taking place on the surface of the bead (Figure 4A, Path I). This can be ruled out by the higher yields obtained by the half cutted beads due to their larger surface area (Figure 4A vs 4B).

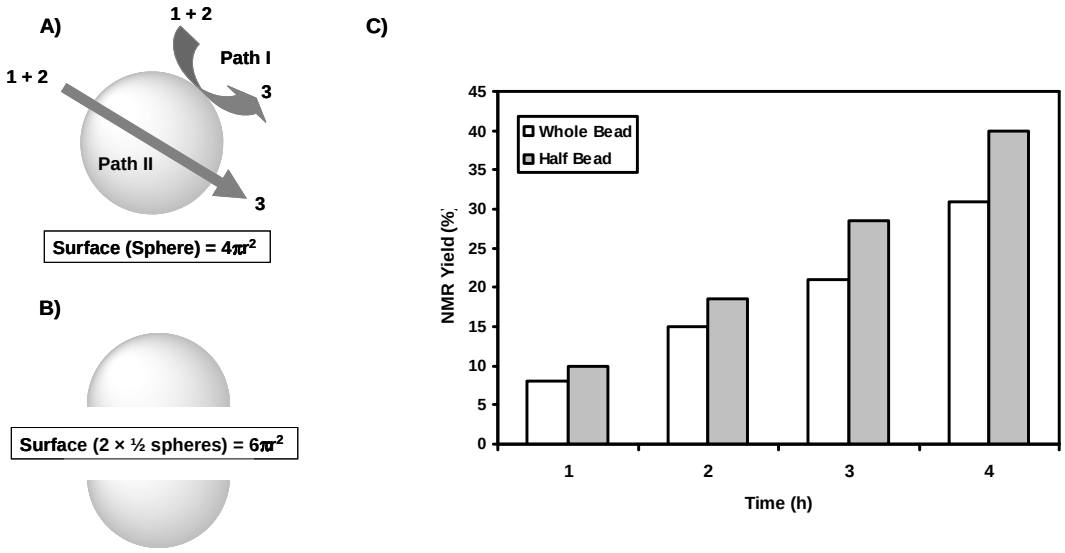


Figure 4. A) Whole Ca^{2+}-AHG bead is used as catalyst. The reaction can take place either on the surface of the catalyst (Path I) or inside the bead based on diffusion of the substrates and subsequent release of the product (Path II); B) Two halves of one Ca^{2+}-AHG bead, resulting in a slightly larger surface than one whole bead; C) Nanoreactor experiment: Henry reaction between **1a** and **2** was catalyzed by either one whole Ca^{2+}-AHG bead or one halved Ca^{2+}-AHG bead. The NMR yield of **3a** is shown in dependency of time.

Moreover, field-emission scanning electron microscopy (FESEM) pictures of all alginate-based materials were monitored to see if the morphology has a major impact on the catalytic performance. In general, catalysts with rough-veined structures showed higher activities than such with closed and agglomerated surfaces.

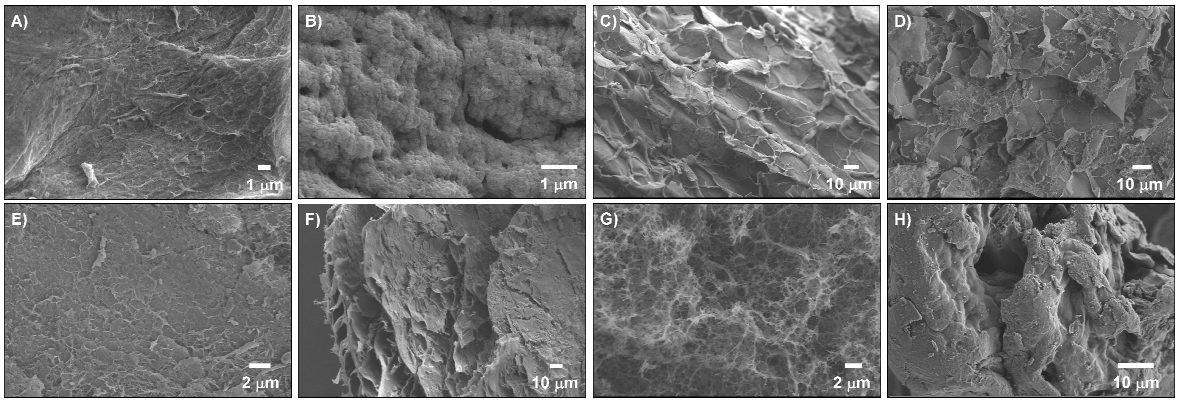


Figure 5. Selected FESEM images of different alginate-based catalysts used in the nitroaldol model reaction: A) freeze-dried Ca^{2+}-AHG, B) air-dried Ca^{2+}-AHG, C) freeze-dried Co^{2+}-AHG, D) freeze-dried Ni^{2+}-AHG, E) freeze-dried Zn^{2+}-AHG, F) freeze-dried Cu^{2+}-AHG, G) freeze-dried Fe^{3+}-AHG, H) Na^{+}-AP.

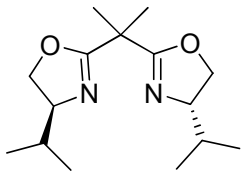
3.3 Conclusion

In conclusion, it has been demonstrated that Ca^{2+}-AHG beads are able to convert aldehydes with nitroalkanes via the nitroaldol (Henry) reaction in organic as well as in aqueous media. Thereby, mild conditions were applied obtaining the corresponding *β*-nitroalcohols in variable yields. In the case of nitroethane the *syn*-isomer was slightly favoured, which shows the important impact of our catalyst in this reaction. Furthermore, the hydrogel was also applicable in a scale-up (1 g) reaction between 4-nitrobenzaldehyde and nitromethane, which resulted in a good yield. Our hydrogel showed lower efficiency in terms of reaction time and turnover frequency (TOF) compared to a well established homogeneous calcium-based catalyst applied in industrial polymerizations (i.e. calcium 2-ethylhexanoate), but was recyclable at least three consecutive runs without significant loss of activity. In addition, further transition metal-based hydrogels (i.e. Co, Ni and Zn) showed remarkable, but lower activities than Ca^{2+}-AHG in this reaction. As the low TOFs and the lack of enantioselectivity are still major drawbacks of these materials, investigations considering immobilization of catalytic active enzymes, asymmetric organocatalysts and metal complexes are ongoing in our laboratory.

3.4 Addendum

As also in our previously reported work about the chitosan-[7] and gelatin-[16] catalyzed Henry reaction, as well as in other cases, i.e. BSA,[17] cyclen,[18] DNA[19] and proline-based gels,[20] no enantioselectivity could be achieved, we wanted to explore more alternatives to induce stereoselectivity in this reaction.

The most promising possibility might be the use of the corresponding metal alginate aerogels (M^{n+}-AAG) instead of the hydrogels. It is well investigated that polysaccharide-based aerogels are porous materials owning large surface areas, which could provide better diffusion properties for the substrates than hydrogels. This fact would also assume that the product formation could take place inside the material, where a highly ordered 3 D network is controlling the transition state in this reaction and supporting therefore stereoselective products. To proof this concept, the most effective hydrogel (i.e. Ca^{2+}-AHG) was transformed to the corresponding aerogel (i.e. Ca^{2+}-AAG) by the reported supercritical CO_2 drying method.[6b], [21] Thereby, the hydrogel was converted to the corresponding alcogel by maturing the beads in different H_2O/EtOH baths (starting from 10%, 30%, 50%, 70%, 90% to 100% EtOH/H_2O) for 15 minutes in each step. After drying the alcogel under supercritical CO_2 conditions, finally the aerogel (Ca^{2+}-AAG) could be obtained. This catalyst was showing a slightly higher yield of **3a** (94%), but could also not induce enantioselectivity in this reaction.



5

Scheme 1. C2-symmetric chiral bisoxazoline (BOX) ligand developed by Evans and co-workers. This ligand shows in combination with $Cu(OAc)_2$ remarkable enantioselection in the nitroaldol (Henry) reaction.[22]

To see whether our used conditions are suitable for stereoselective reactions, we further tested an already known ligand (i.e. 2,2-Bis((4S)-(-)-4-isopropyloxazoline)propane, **5**), which showed in combination with $Cu(OAc)_2 \cdot H_2O$

remarkable enantioselectivities in the nitroaldol reaction under mild conditions (reaction was carried out in either methanol or ethanol at room temperature for 24 hours).[23] As reported, the bisoxazoline complex showed in methanol good enantioselectivity (65% ee), whereas the same complex under our used conditions (DMSO) imparted no asymmetric induction at all and again only the racemate could be isolated. This observation was leading us to the conclusion that DMSO is not a suitable solvent in combination with asymmetric metal complexes. That means it is not supporting highly ordered transition states to induce stereoselectivity in the case of the Henry reaction. In contrast, all other solvent systems were weakly activating our M^{n+}-AHG catalysts. Therefore, the challenge will be in near future to find an agreement between creating a stereoselective catalyst and the right solvent system in which the activity is still acceptable.

3.5 Experimental Section

Materials and Methods

^{1}H NMR spectra were recorded at 25 °C on a Bruker Avance 300 spectrometer. Chemical shifts are denoted in δ (ppm) relative to tetramethylsilane (TMS δ = 0) as an internal standard or relative to residual solvent peaks. Samples were analyzed by chiral-phase HPLC using a Varian 920-LC Liquid Chromatograph and a column Phenomenex Lux Cellulose-1, 4.6 × 250 mm, 5 μm. TLC was facilitated by the use of the following stains in addition to UV light (254 nm) with fluorescent-indicating plates (aluminium sheet precoated with silica gel 60 F254, Merck): phosphomolybdic acid, vanillin, iodine.

Analytical-grade solvents and commercially available reagents were purchased from Merck, TCI Europe or Sigma Aldrich and were used as received. Sodium alginate (Cat. No. A2158-100G; Batch No. 106K0113; CAS 9005-38-3; isolated from brown algae; viscosity of 2% solution at 25 °C: ~ 250 cps) was purchased from Sigma Aldrich and used without further purification.

The hydrogels' metal content was determined via inductively coupled plasma optical emission spectrometry (ICP-OES) with a Spectro Analytical Instruments ICP Modula EOP. IR spectra were recorded using a Bio-Rad FT-IR Excalibur FTS 3000 equipped with a Diamond ATR (attenuated total reflection) accessory (Golden Gate). The catalyst samples were observed with a Carl Zeiss Merlin field-emission scanning electron microscope (FESEM, resolution 0.8 nm) equipped with a digital camera and operating at 5 kV (accelerating voltage) and 10 μA (emission current). Xerogel samples of the corresponding hydrogels were prepared by the freeze-drying (FD) method (Reference). The resulting material was placed on top of a tin plate and shielded by Pt (40 mA during 30 s for FE-SEM; film thickness ≈ 5 nm). Thermal gravimetric analysis (TGA) of the samples was conducted with a Mettler Toledo Thermowaage TG50 apparatus. The samples were combusted under an argon flow (200 mL) during a heating range from 25 to 350 °C with a heating rate of 10 °C/min.

Catalyst preparation

Representative preparation protocol for M^{n+}-AHG beads

50 mL of a 2% (w/v) solution of sodium alginate in water was added dropwise to 100 mL of a 0.24 M aqueous metal chloride solution via a dropping funnel (diameter of the tip = 4.0 mm). After complete addition of the alginate solution, the beads were matured in the metal stock solution over night at room temperature. Finally, the beads were filtered over a Buchner funnel and washed with water until no metal residues could be detected in the washing solution. For qualitative metal detection different compounds for metal complexation were used (see Table 4).

Table 4. Characterization of M^{n+}-AHG beads.

Entry	M^{n+}-AHG	Dropping height (cm)[a]	Dropping rate (drop/s)	Qual. M^{n+} detection	Diameter (mm)[d]	Metal loading (μmol/bead)[e]
1	Ca^{2+}	2.0	1	Na_2CO_3[b]	4.4 ± 0.11	4.76
2	Cu^{2+}	0.5	2	NH_3[c]	4.0 ± 0.13	3.23
3	Co^{2+}	1.0	3	KSCN	4.1 ± 0.17	3.57
4	Ni^{2+}	1.5	1	KCN	3.8 ± 0.16	2.99
5	Zn^{2+}	2.5	1	KFeCN	3.6 ± 0.08	3.56
6	Fe^{3+}	1.5	0.5	KSCN	4.9 ± 0.27	4.26

[a] Dropping height: distance between dropping funnel tip and surface of metal solution; [b] Washing solution was combined with a saturated aqueous Na_2CO_3 solution; [c] Washing solution was combined with a aqueous NH_3 solution (25%); [d] Mean diameter of 20 beads in each case was determined using a vernier calliper; [e] Metal content of each hydrogel was determined by ICP-OES.

Catalysis

Representative procedure for the metal alginate hydrogel-catalyzed Henry reaction

To a mixture of 15.1 mg (0.1 mmol) 4-nitrobenzaldehyde, 54 μL (1 mmol, 10 eq.) nitromethane and 0.5 mL DMSO in a screw cap vial (4 mL), 1 metal alginate

hydrogel bead was added to start the reaction. The resulting reaction mixture was gently stirred for 24 h at room temperature. After completion, 1 mL of EtOAc was added. The diluted supernatant solution was removed and the remaining catalyst washed 3 times with 1 mL of EtOAc for 5 min in each cycle. The combined organic phases were washed 2 times with H_2O (5 mL) and 1 time with brine (5 mL), dried over Na_2SO_4, filtrated and evaporated under reduced pressure to obtain the crude product. To determine the NMR yield, the crude product was dissolved in 1 mL of $CDCl_3$ and 16.7 μL (0.1 mmol) diphenylmethane (or 9.2 μL (0.1 mmol) dimethyl acetamide in the second run) were added.

Typical recycling experiment for the Ca^{2+}-AHG-catalyzed Henry reaction

After the reaction was stopped like previously described, the catalyst was washed with further 0.5 mL of DMSO to remove possible EtOAc residues. Subsequently, the hydrogel bead was added to a new mixture of substrates in DMSO. This procedure was repeated for all further cycles.
The work up and determination of the yield was accomplished like described in the representative procedure for the metal alginate hydrogel-catalyzed Henry reaction.

→ IR spectra and TGA curves of the used catalysts, a selection of ^1H NMR spectra of the crude products, as well as additional experiments can be found in the supporting information on the enclosed CD.

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[12] Calculation of Na-AP amount: Therefore, 20 randomly chosen Ca^{2+}-AHG beads were freeze-dried for 72 hours and weighed afterwards. A mass of 1.55 mg per bead could be determined. Under consideration of the egg-box model, one Ca^{2+}-cation is bound to four alginate units, which lead to $M_{complex}$ = 744.56 g/mol. The mol% was calculated by following equation: mol% = {(1.55 × 10^{-3} g/744.56 g/mol) / 0.1 × 10^{-3} mol} × 100 = 2.08. The amount of Na-AP used in the experiments was calculated by following equation: m = n × $M_{Na\text{-}Alginate}$ = 0.0004 g = 0.4 mg (with: n = 2.08 × 10^{-6}; $M_{Na\text{-}Alginate}$ =198.11 g/mol).

[13] Due to possible insolubility of $CaCl_2$ in DMSO, a second control experiment with a stock solution of $CoCl_2 \cdot 6H_2O$ (7.14 M) was performed yielding no *β*-nitroalcohol in the case of the model reaction.

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Org. Synth. **2006**, *83*, 97-102. All spectroscopic data were in agreement with the reported ones.

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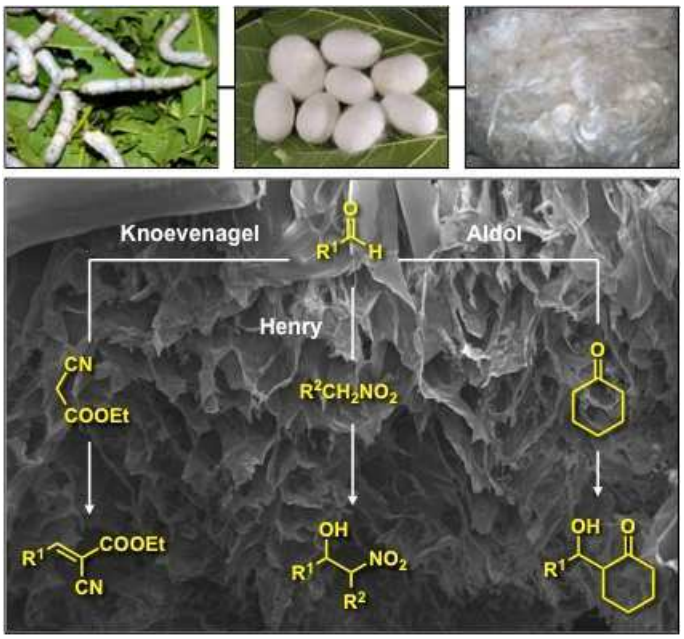
4. Investigation of C-C Bond Formation Mediated by *Bombyx mori* Silk Fibroin Materials[i]



The formation of C-C bonds is a prerequisite for all life on earth. Understanding the role of proteins in mediating the formation of these bonds is important for understanding biological mechanisms in evolution, as well as for designing "green catalysts". In this work, the ability of silk fibroin (SF) proteins to mediate selective C-C bond formation under mild conditions was comprehensively evaluated and compared between different SF-based materials and other proteins. Aqueous SF solution (ASFS), freeze-dried SF (FDSF), mesoporous SF (MPSF) and SF hydrogel (SFHG) materials were prepared and characterized by a variety of techniques including, among others, FE-SEM, ICP-OES, FT-IR, and TGA. The nitroaldol (Henry) reaction, Knoevenagel condensation, and direct aldol reaction were used as models for this study, in which the recovery and reusability of the protein was also evaluated.[ii]

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[i] Reprinted with permission from: D. Kühbeck, M. Ghosh, S. S. Gupta and D. D. Díaz, *ACS Sustainable Chem. Eng.* **2014**, DOI: 10.1021/sc500184r. Copyright 2014 American Chemical Society.

[ii] MPSF, ASFS and FDSF (Batch 2), control experiments with lithium and sericin, optimization reactions in the Henry reaction and ICP-OES measurements were carried out by M. Ghosh. FESEM images were recorded by Prof. Dr. D. D. Díaz. All other syntheses and experiments were conducted by D. Kühbeck.

4.1 Introduction

The formation of C-C bonds is of central importance in modern chemistry and a prerequisite for all life on earth. Understanding the role of natural proteins in promoting the formation or cleavage of C-C bonds may help to find missing links in human evolution and the mechanisms of action of biological systems. On the other hand, the growing environmental concerns coupled with stricter regulations, are driving a gradual shift from the use of petrochemical-based feedstocks to environmentally friendly resources and processes.[1] In this sense, the successful development of protein-based catalysts that are biocompatible and robust and can be mass-produced, which can be very important for designing “green catalysts”.

Motivated by the above combined visions, we have recently found that the morphology and/or physical state of several biopolymers and proteins plays a significant role in the kinetics of some C-C bond forming reactions.[2],[3] Within this context, silk fibroin (SF) constitutes another protein of great interest because it is a water-soluble protein with hydrophobic domains that could solubilize organic substrates. Moreover, we found especially advantageous the possibility of preparing SF materials in different physical forms (e.g., films,[4] porous scaffolds,[5] gels,[6],[7] mats[9]), so the effect of these forms on the chemical reactivity could be studied. SF has a high molecular weight (about 390 kDa) and it is obtained from the cocoons of the larvae of the mulberry silkworm (*Bombyx mori*). In general, SF is composed of three major proteins, a heavy-chain fibroin (H-chain, about 325 kDa), a light-chain fibroin (L-chain, about 25 kDa) and an accessory glycoprotein (P25 protein, about 30 kDa). Glycine (Gly), alanine (Ala), serine (Ser), and tyrosine (Tyr) are the most common amino acids in SF resulting in a total content of about 90 mol%. Repeating units of Gly-Ala-Gly-Ala-Gly-Ser/Tyr forms the core of the protein and the strong hydrophobicity of these amino acids results in van der Waals interactions that drives SF to self-assemble into highly crystalline antiparallel β-sheet structures. The silk polymorphs include silk I, silk II and an air/water interfacial silk III.[9]-[14]

A unique combination of nontoxicity, biocompatibility, tunable biodegradation rates, availability, malleability and excellent mechanical strength, has positioned SF proteins as versatile materials for a number of applications.

Besides the traditional use in textile industry and in biomedicine (e.g., clinical sutures,[15] drug delivery vehicles,[16]-[18] bone,[19]-[21] cartilage,[22],[23] fat,[24] ligament,[25] and vasculature[26]-[28] engineering scaffolds), SF has been also successfully employed as support for a variety of metal-based catalysts. In this regard, metal nanoparticles grafted onto the surface of SF fibers have been used as heterogeneous catalysts for several reactions including hydrogenations,[29]-[31] hydroxylation of phenol,[32] reduction of *p*-nitrophenol[33] and oxidation of methanethiol and hydrogen sulfide.[34]

Herein, we report a comparative study to identify the efficacy of SF in promoting C-C bond forming reactions (i.e., aldol reaction, nitroaldol (Henry) reaction, Knoevenagel condensation). Because SF can be assembled into various physical forms such as mesoporous silk or hydrogels, we also demonstrate the effect of the physical nature of SF on its ability to catalyze these reactions.

4.2 Results and Discussion

Preparation of SF-based materials

The general procedures reported in the literature were used to prepare a variety of silk-based materials (see Experimental Section). Thereby, cocoons of the silkworm *Bombyx mori* were first boiled in aqueous $NaHCO_3$ solution (0.5 wt.%) for 1 h to remove sericin. Silk fibroin fibers (SFF) were isolated after a washing protocol to remove salt residues and subsequent drying under air (Figure 1A). This material was further dissolved under basic conditions and extensively dialyzed against water to obtain a highly pure aqueous silk fibroin solution (ASFS) (Figure 1B) in a range between 2.9 and 5.4 wt%. Neutralization of the slightly basic ASFS with diluted HCl and subsequent freeze-drying afforded the FDSF material as a white solid after 3 days (Figure 1C). Mesoporous silk fibroin (MPSF) was prepared by dissolving FDSF in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to obtain a viscous 17 wt% silk solution, which was then combined with NaCl that acted as porogen. HFIP was allowed to evaporate overnight, and the obtained residue was matured in MeOH to induce the formation of the insoluble β-sheet structure. This obtained material was extensively washed with water to remove possible salt impurities

affording the corresponding MPSF material as a white solid (Figure 1D). Finally, a silk fibroin opaque hydrogel (SFHG) was formed at room temperature from a silk solution (c = 20 g L^{-1}) at pH 5 (Figure 1E). The slightly acidic pH was chosen to form stable and neutral hydrogel material at the given concentration. Characterization of the above materials by FT-IR and TGA was fully consistent with previous reports in the literature (see Experimental Section).

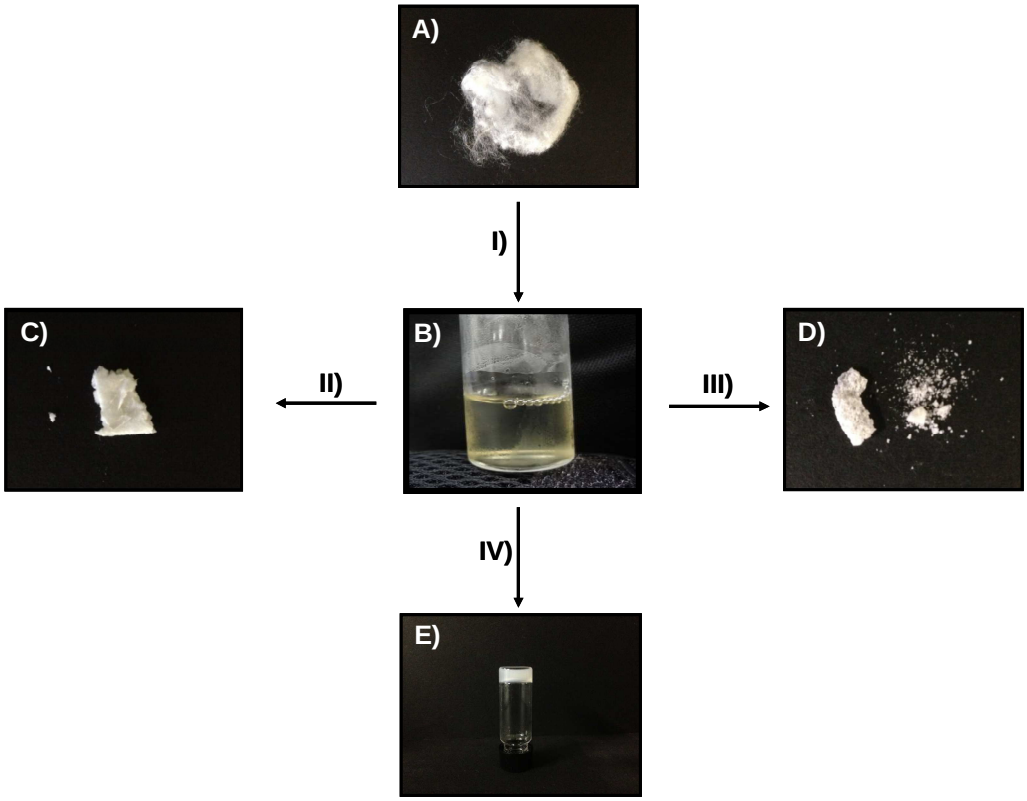


Figure 1. Overview of silk fibroin-based materials used in this work: A) SFF; B) SFAS; C) FDSF; D) MPSF; E) SFHG. Conditions: I) aq. LiBr solution (15.7 M), 24 h, room temperature. II) Freeze-drying of 2.9-5.4 wt% SFAS, pH 7, 3 days. III) i) 17 wt% SFAS, HFIP, ii) NaCl, MeOH. IV) 0.5 mL of a 20 g L^{-1} SFAS, pH 5, 2 days, room temperature.

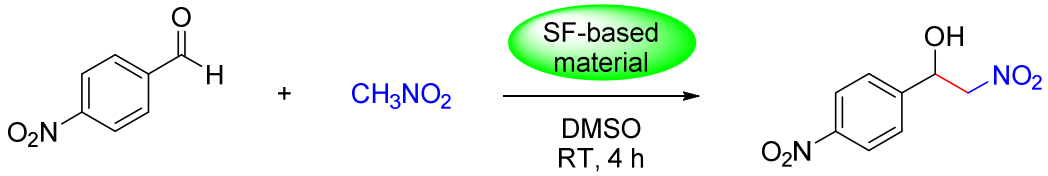
Nitroaldol (Henry) reaction

Nitroaldol (Henry) reaction is a classical and valuable synthetic method for the synthesis of β-nitroalcohols by combination of nitroalkanes with carbonyl compounds (i.e., aldehydes, ketones) in the presence of an ionic or nonionic base-

catalyst.[35],[36] However, one of the main drawbacks of this reaction is the formation of several side products that usually complicate the isolation of the desired compounds. These side products include mainly polymerizable nitroalkenes formed upon dehydration of the *β*-nitroalcohols, self-condensed products especially with sterically hindered substrates (i.e., Cannizaro reaction), epimerized *β*-nitroalcohols and products derived from the Nef reaction.[35] In general, with aromatic aldehydes the selectivity of the reaction is strongly influenced by the electronic nature of the substituents and their ability to favor either the imine or ion-pair mechanism.[35]

In our previous studies with other biopolymers and proteins,[2],[3] we demonstrated that the morphology and/or physical state of a protein catalyst plays a significant role in the kinetics of aldol-like reactions under heterogeneous or semi-heterogeneous conditions. Motivated by this finding, we carried out a comparative evaluation of the effect of the SF-based materials (i.e., ASFS, FDSF, MPSF and SFHG) in the nitroaldol (Henry) model reaction between 4-nitrobenzaldehyde (**1**) and excess of nitromethane (**2**) in DMSO at room temperature over a period of 4 h (Table 1). Preliminary experiments established an optimal use of 10-fold molar excess of nitromethane equivalents for this transformation and 10 mg of catalyst per 0.1 mmol of aldehyde (higher amounts of silk did not improved the yields)**.** The results showed that FDSF catalyzed the formation of the corresponding *β*-nitroalcohol **3a** with an excellent yield of 95% (entry 1). Comparable results were obtained when the reaction was scaled up 10 times. In sharp contrast, MPSF displayed a much slower kinetics as reflected by the very low yield of **3a** (4%) within the same period of time. This yield could be enhanced to 88% upon extension of the reaction time to 48 h (entry 4). The bulk SFHG provided negligible conversions after 4 h when a solution of substrates **1** and **2** in DMSO was layered on top of the gel (entry 6). However, an increase of the surface area by mechanical fragmentation of the gel body into smaller pieces afforded the desired *β*-nitroalcohol **3a** in 83% yield (entry 7). Finally, the incorporation of the substrates in neutral ASFS (*c* = 20 g silk L^{-1}) caused precipitation of the protein and **3a** was obtained in only 22% yield (entry 8). Null conversions in control experiments performed in the absence of SF material (entries 2 and 5) confirmed its catalytic role in the nitroaldol reaction.

Table 1. Screening of different silk-based materials in the nitroaldol (Henry) reaction.[a]



1a **2** **3a**

Entry	SF-based material	Yield 3a (%)[b]
1	FDSF	95
2	FDSF	0 (control)[c]
3	MPSF	4
4	MPSF	88[d]
5	MPSF	0 (control)[e]
6	SFHG	0[f]
7	SFHG	83[g]
8	ASFS	22[h]

[a] Reaction conditions: 4-Nitrobenzaldehyde (**1a**, 0.1 mmol), nitromethane (**2**, 1.0 mmol), SF material (10 mg), DMSO (0.5 mL), 4 h, room temperature; [b] Determined by ^{1}H NMR analysis of the crude product based on aldehyde proton. Reported yields correspond to the average values of two independent experiments (STDV = ± 2); [c] Control experiment performed in the absence of SF material; reaction time = 4 h; [d] Reaction time = 48 h; [e] Control experiment performed in the absence of SF material; reaction time = 48 h; [f] Reaction mixture was layered on top of the hydrogel; [g] Reaction mixture was combined with gel pieces and stirred; [h] Silk protein precipitated during the reaction.

An interesting correlation was found between the reaction outcome (Table 1) and the morphology of the SF materials observed by scanning electron microscopy (Figure 2). The most efficient material, FDSF, displayed a highly ordered, macroporous (ca. 20 μm in diameter) and spongy network structure. Such structure could strongly favor the adsorption of small molecules and ions presented in the medium via electrostatic interactions (e.g., physical adsorption), hydrogen bonding and/or van der Waals forces. However, MPSF showed a closed, amorphous and granular shell that covered a core porous structure with smaller pore diameters (ca. 1.0 - 10 μm) than those observed in FDSF (Figure 2C, D). Interestingly, a highly porous and fibrilar structure was also observed for SFHG (pore diameter ca. 0.2 - 1.0 μm) (Figure 2E, F), in which acceptable conversions

were obtained upon fragmentation of the bulk gel. These results suggest a complex mechanism involving both diffusion-controlled processes and surface reactivity.

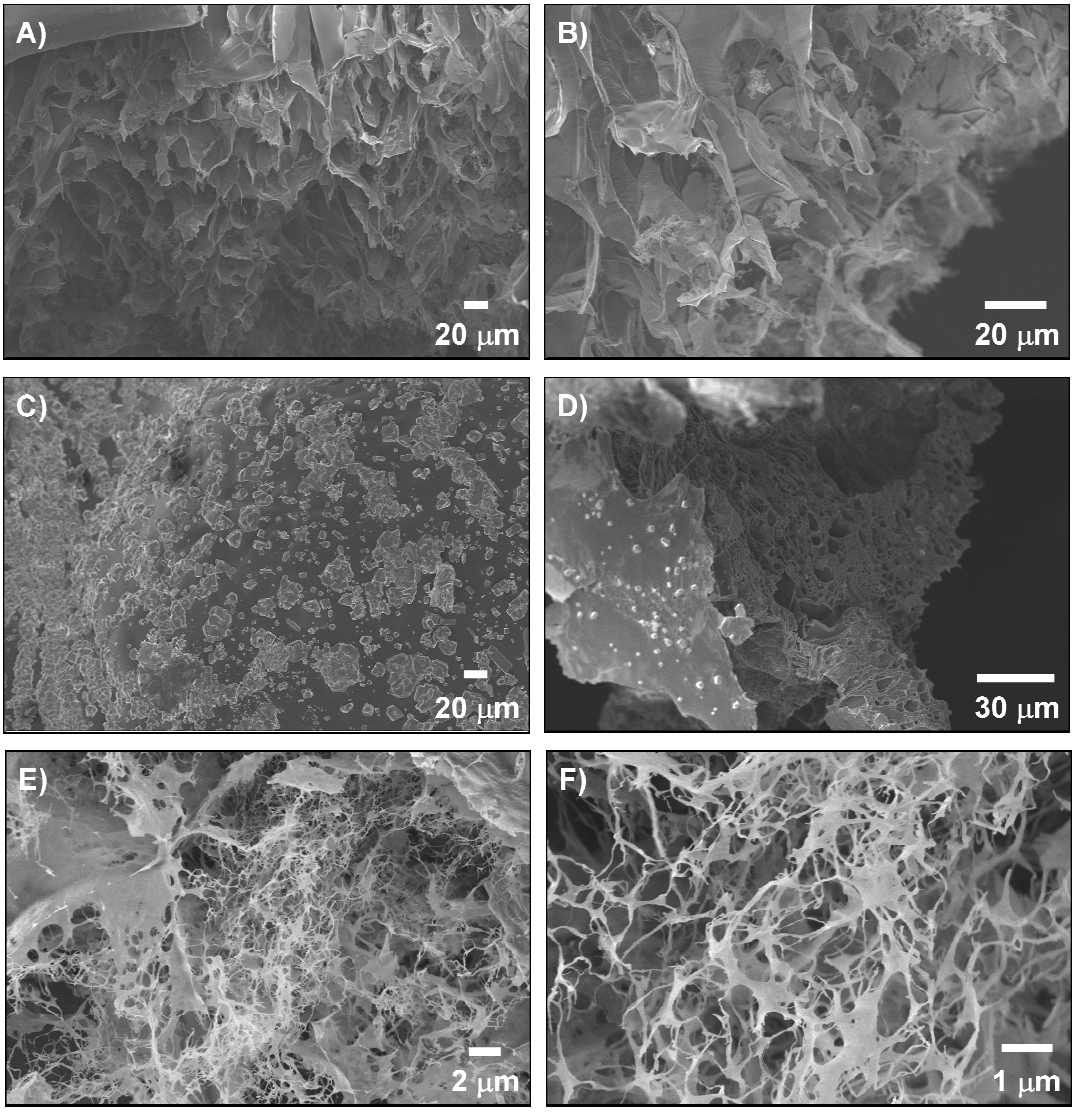
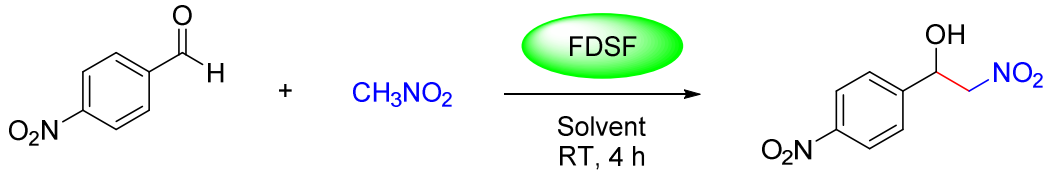


Figure 2. FE-SEM images of different SF-based materials depicted in Table 1: A) FDSF (magnification: 285×); B) FDSF (magnification: 676×); C) MPSF (magnification: 246×); D) MPSF (magnification: 554×); E) xerogel obtained from SFHG (magnification: 3.45K×); F) xerogel obtained from SFHG (magnification: 11.04K×).

The facile preparation of FDSF and its better performance in the catalyst screening made us to use this material for further studies. Table 2 outlines the results of solvent screening for the nitroaldol reaction mediated by FDSF. In agreement with our previous observations the best results were achieved in DMSO (entry 1), whereas modest yields were obtained in DMF (entry 3) and H_2O

(entry 5). In contrast, only traces of the product could be detected when the reaction was carried out in EtOH (entry 7) and no conversion was observed in Et_2O, MeCN, CH_2Cl_2, THF and toluene (entry 8).

Table 2. Solvent screening for FDSF-mediated nitroaldol (Henry) reaction.[a]



1a **2** **3a**

Entry	Solvent	Yield 3a (%)[b]
1	DMSO	95
2	DMSO	0 (control)[c]
3	DMF	36
4	DMF	2 (control)[c]
5	water	31
6	water	0 (control)[c]
7	EtOH	< 2
8	Et_2O, CH_3CN, DCM, THF, toluene	0

[a] Reaction conditions: 4-Nitrobenzaldehyde (**1a**, 0.1 mmol), nitromethane (**2**, 1.0 mmol), FDSF (10 mg), solvent (0.5 mL), 4 h, room temperature; [b] Determined by ^{1}H NMR analysis of the crude product based on aldehyde proton. Reported yields correspond to the average values of two independent experiments (STDV = ± 2); [c] Control experiment performed in the absence of FDSF.

Additional control experiments were also carried out in order to rule out any catalytic effect of possible impurities (e.g., Li^+ ions), which could be leached from the FDSF during the reaction. For these experiments, the FDSF material was matured in DMSO in the absence of the substrates **1** and **2**. After 4 h, the supernatant solution was separated from the solid catalyst. The solution was combined with **1** and **2** (mixture I), and the catalyst was mixed with 0.5 mL of DMSO containing **1** and **2** (mixture II). The molar ratio of the substrates and total concentration in each case were the same than those used in standard experiments. Both reaction mixtures were stirred for additional 4 h. Nitroaldol product **3a** was obtained in only 2% yield using mixture I, whereas mixture II afforded the desired product **3a** in 88% yield. These results confirm the

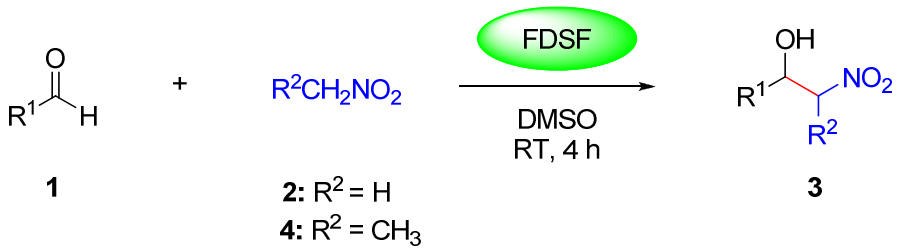
heterogeneous nature of the process and the negligible catalytic effect caused by possible leaching of Li^+ ions. In order to quantify the possible effect of Li^+ ions if they were accessible, the exact amount of Li^+ ions in the FDSF material was estimated by ICP-OES in 0.027 mmol per mg of FDSF. Thus, an equivalent LiBr stock solution was prepared in DMSO and used to carry out the model nitroaldol reaction in homogeneous conditions. In this case, the conversion towards the desired β-nitroalcohol **3a** was only 15%.[37]-[40] In addition, another control experiment was carried out using silk sericin protein instead of FDSF. The silk sericin content lies between 17% and 30% in cocoons of *Bombyx mori*,[41]-[43] which is usually separated from the silk fibroin during the degumming process in $NaHCO_3$ solution. The use of pure silk sericin afforded a considerable lower conversion towards **3a** (20%). All results from the control experiments suggest that mainly the silk fibroin protein is responsible for the catalytic activity in the nitroaldol reaction.

With these results in hand, we further evaluated the substrate scope of FDSF in DMSO (Table 3). In general, aromatic aldehydes with strong electron-withdrawing groups could be converted exclusively to the corresponding β-nitroalcohol in very good yields between 90 and 95% (entries 1, 4, 6-8). No major effects of the position of the substituents in **1** were observed (entries 1, 6, 7). To ensure reproducibility of the process, different batches of FDSF were randomly tested for the reaction between **1a** and **2** resulting in only slight variations in the yield of **3a** within 80% and 95%. It should be noted that the preparation of FDSF at different scales afforded materials with different surface areas, which could explain the variation in reactivity. In general, less spongy and fluffy material was obtained in low scale preparations (entries 1 and 2). Halogenated aromatic aldehydes such as 4-bromobenzaldehyde (entry 11) and 4-chlorobenzaldehyde (entry 12) could be converted into the desired product in good (88%) and moderate yields (59%), respectively. The β-nitroalcohol product derived from benzaldehyde (entry 10) could be also obtained in moderate yield (43%), whereas the use of aromatic aldehydes with electron donating groups such as 4-methylbenzaldehyde (entry 14) gave poor yield (11%). Interestingly, the heteroaromatic aldehyde 2-pyridinecarboxaldehyde (entry 15) could be also transformed in good yield (82%). Very high conversions (ca. 88-100%) were also observed within 14-24 h for other

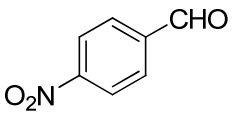
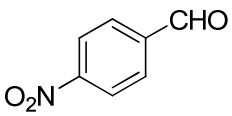
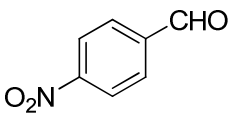
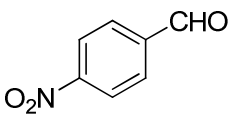
related aldehydes such as 5-bromo salicylaldehyde, 4-bromo benzaldehyde or 4-fluoro benzaldehyde (data not shown). In contrast, aliphatic aldehydes could not converted into the desired product. When nitroethane (**4**) was used as nucleophile in combination with **1a**, no significant differences in comparison to nitromethane were observed (entries 4, 9, 13). Thus, both the size of the nucleophilic carbanion and its pK_a (pK_a values:[44] **2** = 10.2; **4** = 8.6) showed in this case a minor effect on the reaction outcome. In comparison to other standard catalysts, the performance of FDSF was, for example, similar to the catalysis by Et_3N in water (see Supporting Information).

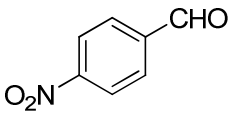
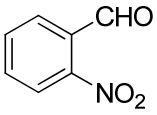
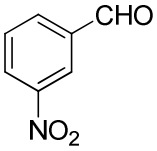
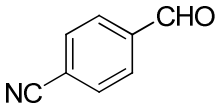
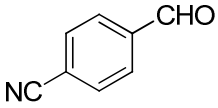
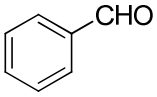
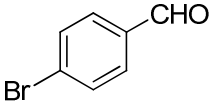
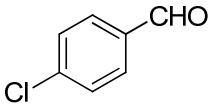
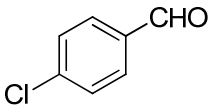
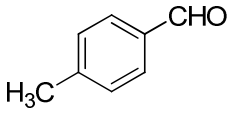
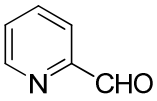
It is worth mentioning that negligible enantioselectivity (ee ≤ 1%)[45]-[47] and low diastereoselectivities (*anti/syn* ratio) were routinely observed. On the basis of the early reports of Evans and co-workers reporting an asymmetric variant of the Henry reaction catalyzed by a chiral copper (II) complex,[48] we also tested Cu(II)-doped FDSF materials.[49] However, this variation also yielded the racemic mixture of the desired nitroaldol product.

Table 3. Substrate scope of the FDSF-mediated nitroaldol reaction in DMSO.[a]



1 **2:** $R^2 = H$; **4:** $R^2 = CH_3$ **3**

Entry	R^1CHO	R^2	Yield 3 (%)[b]	dr[c]
1		H	95[d]	NA
2		H	80	NA
3		H	0 (control)[f]	NA
4		CH_3	94	1:1.1

5		H	8 (control)[f]	1.4:1
6		H	90	NA
7		H	97	NA
8		H	91	NA
9		CH_3	92	1:1.3
10		CH_3	43	NA
11		H	88	NA
12		H	59	NA
13		CH_3	87	1.1:1
14		H	11	NA
15		H	82	NA

[a] Reaction conditions: Aldehyde (**1**, 0.1 mmol), nitroalkane (**2** or **4**, 1.0 mmol), FDSF (batch 1, 10 mg), DMSO (0.5 mL), 4 h, room temperature; [b] Determined by ^{1}H NMR analysis of the crude product based on aldehyde proton. Reported yields correspond to the average values of two independent experiments (STDV = ± 2); [c] Diastereomeric ratio (*anti*/*syn*) determined by ^{1}H NMR analysis (NA = not applicable); [d] FDSF was obtained by freeze-drying ca. 20 mL of ASFS (3.7 wt%) in a 100 mL flask; [e] FDSF was obtained by freeze-drying ca. 2 mL of ASFS (5.4 wt%) in a 5 mL vial; [f] Control experiment performed in the absence of FDSF.

In terms of reusability, FDSF showed a remarkable deactivation after the first almost quantitative cycle (Figure 3). Although the exact deactivation mechanism during the nitroaldol (Henry) reaction remains unclear, blocking of the catalytic sites by chemical poisoning of the surface seems to be crucial. As we have described earlier with other proteins,[2],[3] potential chemical evolution of intermediate imines (e.g., via reductive Cannizaro-like processes or formation of cross-linked aminals) and excess of nitromethane that could undergo partial association with free amine and hydroxyl groups, as well as the slow reaction kinetics, may contribute to form an inactive coat blocking the access to the basic catalytic sites of the protein. In agreement, FE-SEM imaging of FDSF before and after the nitroaldol reaction showed a significant morphological change from a porous and spongy-like structure to a very clotted surface (inset Figure 3).

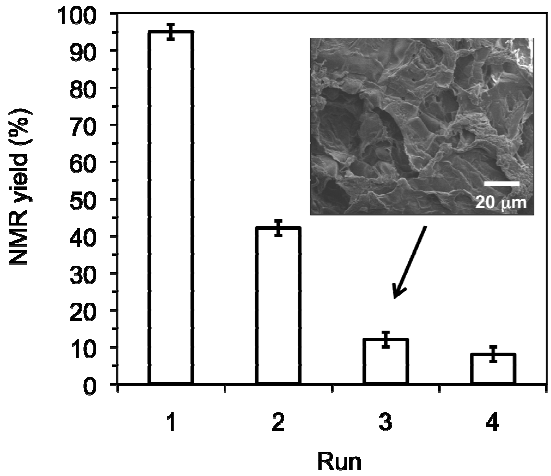


Figure 3. Recycling study of the FDSF-mediated Henry reaction. Inset: FE-SEM image of the clustered surface structure of FDSF after the third run in the nitroaldol reaction (magnification: 634×). Attempts to reactivate FDSF by alternative washing with different solvents after the work-up were fruitless.

Knoevenagel reaction

The potential catalytic effect of FDSF in a model Knoevenagel condensation was also studied. This reaction was carried out between 2-nitrobenzaldehyde (**5**) and ethyl cyanoacetate (**6**) in DMSO at room temperature. As shown in Figure 4, the reaction was completed after 3 h yielding the desired product **7** in 84% (only the *E*-isomer was detected and no self-condensation or Cannizaro products were

observed). The control reaction between **5** and **6** carried out in the absence FDSF showed only traces of the product **7**. Deactivation of the FDSF catalyst after the Knoevenagel condensation was also observed, albeit this process was significantly slower in comparison to that in the nitroaldol reaction (i.e., yield of **7**: 84% (1st run), 60% (2nd run), 34% (3rd run), 20% (4th run)).

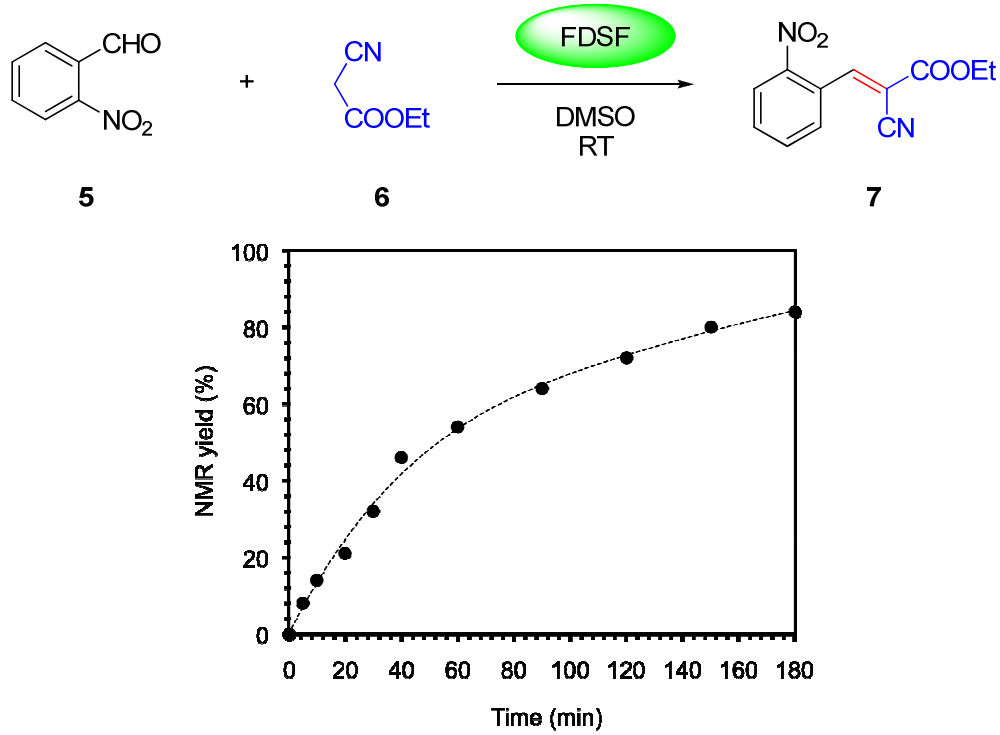


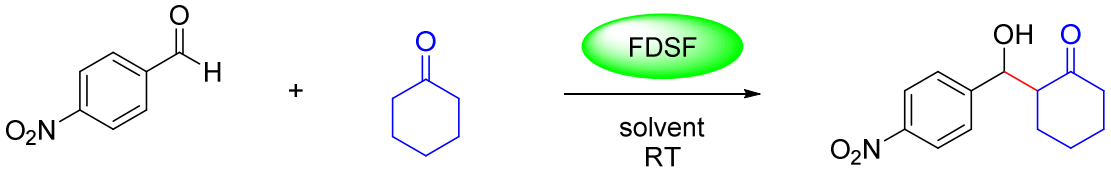
Figure 4. Kinetics of the Knoevenagel model reaction between 2-nitrobenzaldehyde (**5**) and ethyl cyanoacetate (**6**) mediated by FDSF. A comparison with other catalysts is given in the Supporting Information.

Direct aldol reaction

Despite the efficiency of FDSF in both nitroaldol and Knoevenagel reactions, no activity was observed for the direct aldol reaction between acceptor **1** and cyclohexanone (**8**) as donor in different solvents (i.e., DMSO, DMF, water, THF) for 48 h at room temperature (Table 4, entries 1, 5, 9). By increasing the temperature to 37 °C at the aldol product **9** was obtained in 8% yield after 48 h both in DMSO (entry 2). Extension of the reaction time to 7 days provided only a slight increase of the yield (entry 3). The use of water instead of DMSO caused a considerable background reaction in the absence of FDSF (entries 6-8). Only

minor enantioselectivity was observed for the reaction carried out in water (entry 7) (i.e., 6% ee of the major *anti* isomer, 4% ee of the minor *syn* isomer).

Table 4. Solvent scope of the FDSF-mediated aldol reaction.[a]



1a **8** **9**

Entry	Solvent	Temperature (°C)	Time (d)	Yield 9 (%)[b]
1	DMSO	RT	2	0
2	DMSO	37	2	8[c]
3	DMSO	37	7	10[c]
4	DMSO	37	7	< 1 (control)[d]
5	DMF	RT	2	< 1
6	H_2O	RT	2	2
7	H_2O	37	7	13[c]
8	H_2O	37	7	7 (control)[d]
9	THF	RT	2	0

[a] Reaction conditions: 4-Nitrobenzaldehyde (**1a**, 0.1 mmol), cyclohexanone (**8**, 2.0 mmol), FDSF (10 mg), solvent (0.5 mL). RT = room temperature. The molar ratio aldehydes:ketone 1:20 was used to minimize self-condensation of the acceptor and favor cross-condensation; [b] Determined by ^{1}H NMR analysis of the crude product; [c] Approximate diastereomeric ratio anti/syn = 1:1 (for the reaction in DMSO), 1:3 (for the reaction in H_2O); [d] Control experiment performed in the absence of FDSF.

4.3 Conclusion

In conclusion, this study has demonstrated an intrinsic ability of silk fibroin-based materials, derived obtained from the cocoons of the silkworm (*Bombyx mori*), to promote C-C bond formation under mild conditions (i.e., DMSO or H_2O at RT). Among different materials, FDSF was found to be the most active material in the nitroaldol (Henry) and Knoevenagel condensation followed by MPSF and SFHG. In general, aromatic aldehydes with strong electron-withdrawing groups showed the best yields. In sharp contrast, poor activity was observed in the direct aldol reaction, albeit with slight enantioselectivity in some cases. Although the protein catalyst could be reused in further runs, progressive deactivation was observed especially in the nitroaldol reaction, presumably due to the formation of inactive surface coating. Overall, the results confirm that morphology and/or physical state of the silk fibroin plays a significant role in the kinetics when mediating these types of reactions. Studies towards other forms of silk-based materials, including silk-based nanoparticles, their reusability and their potential influence in different chemical processes are currently underway in our laboratories.[50]

4.4 Experimental section

Materials and Methods

^{1}H NMR spectra were recorded at 25 °C on a Bruker Avance 300 spectrometer. Chemical shifts are denoted in δ (ppm) relative to tetramethylsilane (TMS δ = 0) as an internal standard or relative to residual solvent peaks. Reaction products were analyzed by chiral-phase HPLC using a Varian 920-LC liquid chromatograph. The columns Phenomenex Lux Cellulose-1, 4.6 mm × 250 mm, 5 μm, and AS-H, 4.6 mm × 250 mm, 10 μm, were used for the analysis of the nitroaldol and aldol products, respectively. TLC was facilitated by the use of the following stains in addition to UV light (254 nm) with fluorescent-indicating plates (aluminium sheet precoated with silica gel 60 F254, Merck): phosphomolybdic acid, vanillin, iodine. FT-IR spectra were recorded using a Bio-Rad FT-IR Excalibur FTS 3000 equipped with a Diamond ATR (attenuated total reflection) accessory (Golden Gate). The morphology of the materials was observed with a Carl Zeiss Merlin field-emission scanning electron microscope (FESEM, resolution 0.8 nm) equipped with a digital camera and operating at 5 kV (accelerating voltage) and 10 μA (emission current). Xerogel samples of the corresponding hydrogels were prepared by the freeze-drying (FD) method. The resulting material was placed on top of a tin plate and shielded by Pt (40 mA during 30 s; film thickness ≈ 5 nm). Thermal gravimetric analysis (TGA) measurements were carried out under nitrogen with the following program heating rate: (1) equilibration step for 30 min @ 25 °C; (2) heating profile from 25 °C to 800 °C @ 10 °C/min; (3) 15 min @ 800 °C. The lithium content of the samples was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Spectro Analytical Instruments ICP Modula EOP. Analytical-grade solvents and commercially available reagents were purchased from Merck, TCI Europe, or Sigma-Aldrich and were used as received without further purification. Pure H_2O (Milli-Q) was used in all water-included experiments. Cocoons of the *Bombyx mori* (silkworm of the mulberry tree) were provided by the Central Sericulture Training and Research Institute (Mysore, India) and were degummed at the Physical/Materials Chemistry Division, National Chemical Laboratory, Pune, India.

Preparation of Silk Fibroin Materials

The materials for reactivity tests were prepared according to the general procedures reported in the literature with slight modifications.

Preparation of regenerated aqueous silk fibroin solution (ASFS)

Silk cocoons from *Bombyx mori* were boiled for 1 h in a 0.5 wt% aqueous solution of $NaHCO_3$ to remove sericin. The remaining fibroin fibers were washed thoroughly with excess of H_2O to remove $NaHCO_3$. The dried silk fibroin fibers were dissolved during 24 h at room temperature in an aqueous LiBr solution (41 g of LiBr in 30 mL Milli-Q H_2O) stored in a plastic screw cap bottle. The resulting solution was further dialyzed against Milli-Q H_2O for 48 h at 4 °C using a dialysis tube of cellulose acetate membrane with a molecular weight cutoff (MWCO) of 12 kDa. The H_2O was exchanged five times, first after 3 h, then 5 h, and later 12 h each. After the dialyzed solution was centrifuged at 4000 rpm for 40 min at room temperature, the supernatant was collected. This silk fibroin solution had a final concentration between 2.9 and 5.4 wt%, which could be stored in a plastic screw cap bottle at 4 °C for about 1 month. Concentration of the regenerated silk fibroin solution was determined by gravimetric analysis and by measuring the absorption at λ_{max} = 272 nm (ε = 11.8 mol L^{-1} cm^{-1}).

Preparation of freeze-dried silk fibroin (FDSF)

The pH of the obtained ASFS was adjusted to 7. The precipitates formed during neutralization were removed by centrifugation at 4000 rpm for 20 min. The supernatant collected and freeze-dried for 3 days to obtain FDSF. For reactivity studies, bulk FDSF was cut into 10 mg pieces.

Preparation of mesoporous silk fibroin (MPSF)

Thirty millilitres of freshly prepared ASFS was transferred to a slide-a-lyzer dialysis cassette with a MWCO of 3500 (capacity 12-30 mL), and then concentrated by dialysis against an aqueous 25 wt% PEG 20000 solution for about 8 h in total at

room temperature. Additional 10 mL of ASFS were added after 3 h to the dialysis cassette. The concentrated ASFS (CASFS) had a final concentration of 30-40 wt%. CASFS was diluted to a 17 wt% silk solution, prior combination with 5 mL of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). The obtained viscous solution (1 g) was transferred to a Teflon mold, in which NaCl (10 g) was furnished. The mold was covered with a perforated aluminum foil. The setting was left standing for 24 h at room temperature. After this time, the solid film was matured in MeOH for 30 min to obtain the β-sheet structure (MPSF). Finally, the film was washed with Milli-Q H_2O and dried under air. For reactivity studies, bulk MPSF was ground into a fine powder.

Preparation of silk fibroin hydrogel (SFHG)[51],[52]

The pH of freshly prepared ASDS was adjusted to 5. The precipitates formed during neutralization were removed by centrifugation at 4000 rpm for 20 min. The supernatant was diluted to 20 g L^{-1}, and 0.5 mL of this solution (= 10 mg silk fibroin) were transferred to a screw cap vial (4 mL) and left standing for 2 days at room temperature to obtain stable and white hydrogel (SFHG). This gel was found to remain stable for several days in the presence of additional water or organic solvents like MeOH, DMSO or THF.

Reactivity studies

Typical procedure for the FDSF-mediated nitroaldol (Henry) reaction

To a mixture of 4-nitrobenzaldehyde (15.1 mg, 0.1 mmol) and nitromethane (54 μL, 1 mmol) in DMSO (0.5 mL) placed in a screw cap vial (4 mL), FDSF (10 mg) was added in one portion. The resulting reaction mixture was gently stirred for 4 h at room temperature. After this time, 1 mL of EtOAc was added, and the diluted supernatant solution removed by decantation. The remaining catalyst was washed with EtOAc (3 × 1 mL, for 5 min in each cycle). The combined organic phases were washed with H_2O (2 × 5 mL) and brine (5 mL), dried over anhydrous Na_2SO_4, filtrated and evaporated under reduced pressure to obtain the crude product. To determine the NMR yield, the crude product was dissolved in 1 mL of $CDCl_3$ and

diphenylmethane (16.7 μL, 0.1 mmol) or *N,N*-dimethylacetamide (9.2 μL, 0.1 mmol) were added as internal standard. Note that the same procedure and same amount of catalyst (10 mg) was used for testing other silk-based materials. In the case of ASFS, the product was extracted directly from the aqueous phase.

Typical procedure for the FDSF-mediated Knoevenagel condensation reaction

To a mixture of 2-nitrobenzaldehyde (15.1 mg, 0.1 mmol) and ethyl cyanoacetate (11.7 μL, 0.11 mmol) in DMSO (0.5 mL) placed in a screw cap vial (4 mL), FDSF (10 mg) was added in one portion. The resulting reaction mixture was gently stirred for the given time at room temperature. The work-up of the reaction, washing of the catalyst and determination of the NMR yield were carried out as described for the FDSF-mediated nitroaldol reaction.

Typical procedure for the FDSF-mediated direct aldol reaction

To a mixture of 4-nitrobenzaldehyde (15.1 mg, 0.1 mmol) and cyclohexanone (208 μL, 2.0 mmol) in DMSO (0.5 mL) placed in a screw cap vial (4 mL), FDSF (10 mg) was added in one portion. The resulting reaction mixture was gently stirred for the given time at room temperature. The work-up of the reaction, washing of the catalyst and determination of the NMR yield were carried out as described for the FDSF-mediated nitroaldol reaction.

Typical recycling experiment for the FDSF-mediated nitroaldol (Henry) and Knoevenagel condensation reactions

Method A): After quenching the reaction with EtOAc, the catalyst was washed as previously described and the remaining catalyst dried under high vacuum to remove residual solvent. The dried catalyst was then added to the appropriate mixture of substrates in DMSO. This procedure was repeated for all further cycles. The work-up and determination of the yield was accomplished as described for the FDSF-mediated nitroaldol reaction. **Method B)**: The reaction was quenched by

addition of 1 mL of DMSO (instead of EtOAc). The liquid of the reaction mixture was decanted and the remaining catalyst washed DMSO (3 × 1 mL) and used in the next cycle of the reaction. The combined organic a layers were diluted with EtOAc (36 mL) and washed with H_2O (2 × 36 mL) and brine (36 mL), dried over anhydrous Na_2SO_4, filtrated and evaporated under reduced pressure to obtain the crude product. The NMR yield was determined as described in the representative procedure for the FDSF-mediated nitroaldol reaction.

→ Selected IR spectra, TGA curves of the used catalysts, ^1H NMR spectra of the crude products and HPLC chromatograms, as well as additional experiments can be found in the supporting information on the enclosed CD.

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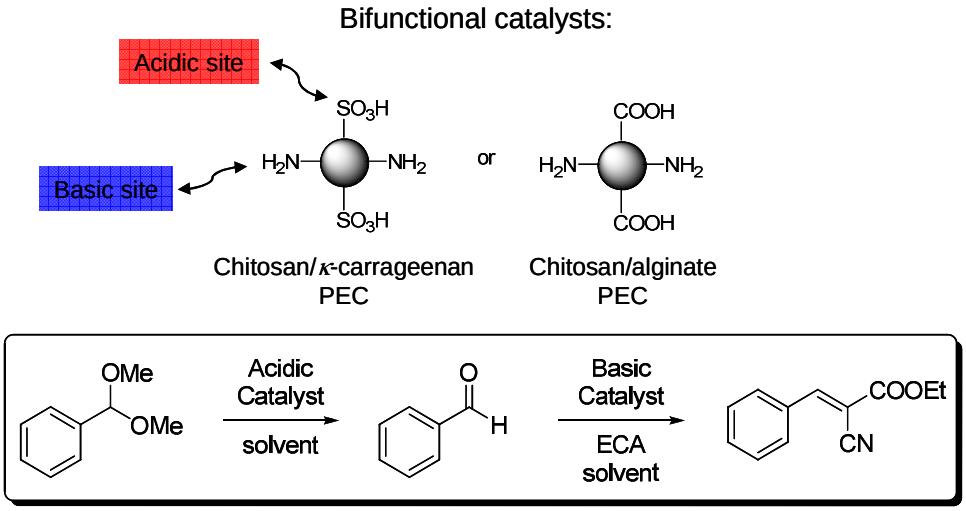
5. Evaluation of Polysaccharide-based Materials in the One-pot Deaceatelyation Knoevenagel Condensation

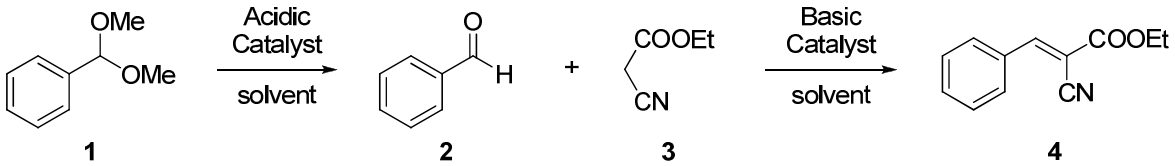


Eigth different types of alginate-, chitosan- and κ-carrageenan-based materials, i.e. the aerogels: P-κ-CGAG, AA-κ-CGAG, HCA-κ-CGAG, CSAG and CS-κ-CGAG; the hydrogels: CS-κ-CGPECHG and CS-AGPECHG; and the powdered material: SFNCS were prepared and comprehensively evaluated as possible multifunctional catalysts in the one-pot deacetylation Knoevenagel condensation. Furthermore, the commercial available powdered forms of chitosan (CSP) and sodium κ-carrageenan (S-κ-CGP) were also considered.[i]

[i] This work was carried out during an exchange program in the laboratories of Dr. F. Quignard at the Institute Charles Gerhardt (ICG) in Montpellier, France between 04.03. and 30.05.2013.

5.1. Introduction

The development of polyfunctional catalysts is a highly important section beyond heterogeneous and industrial applications. In most cases, bifunctional materials consisting of two different reactive centers (e.g. acidic and basic sites) are investigated.[1] The resulting synergistic concept opens new advantageous opportunities for so called tandem or one-pot reactions, which usually show at least two seperate and consecutive chemical transformations within one step/cycle.[2] The savings of high amounts of solvent used during the purification of an intermediate product (e.g. extraction and/or column chromatography) and inserted energy (e.g. heat, light irradiation, etc.) are the most predominant arguments for multistep one-pot reactions. Since, materials based on petrochemical feedstocks are often used for such approaches, the development of natural functional materials should be foregrounded. Suitable compounds offering a variety of functional groups under consideration of green thoughts, are biopolymers, i.e. polysaccharides like chitosan (-NH_2), sodium alginate (-COO^-Na^+), sodium carrageenan (-$SO_3^-Na^+$), etc. In general, polysaccharide composites based on ionic interactions of opposite charges (e.g. chitosan-NH_3^+/$^-$OOC-alginate or chitosan-NH_3^+/$^-OO_2S$-carrageenan), so called polyelectrolyte complexes (PECs), are used as pH-sensitive drug release carriers[3] and support for enzymes[4]. No reports dealing with PEC-mediated chemical transformations based on pure polysaccharides are known. The exact distribution of the charge within these complex materials and the way how the synergistic properties could influence chemical reactions have to be explored more accurate. Therefore, we were interested in this work to prepare already known PECs and design new aerogel PECs based on polysaccharides. The former material is usually limited by substrate diffusion through the hydrogel matrix, whereas the latter could circumvent this limitation by a porous network.[5] For evaluation of the prepared materials the one-pot deacetylation Knoevenagel condensation (Scheme 1) reaction between benzaldehyde dimethylacetal (**1**) and ethylcyanoacetate (**3**) as a model was investigated.



Scheme 1. Model reaction: one-pot deacetylation Knoevenagel condensation reaction between benzaldehyde dimethylacetal (**1**) and ethyl cyanoacetate (**3**).

5.2. Results and Discussion

Preparation of materials

In this work different potential catalysts, so called PECs, i.e. potassium κ-carrageenan aerogel beads (P-κ-CGAG), chitosan aerogel beads (CSAG), two different acid treated κ-carrageenan aerogel beads (HCA-κ-CGAG: hydrochloric acid κ-carrageenan aerogel, AA-κ-CGAG: acetic acid κ-carrageenan aerogel), chitosan-κ-carrageenan composite aerogel beads (CS-κ-CGAG), chitosan-κ-carrageenan polyelectrolyte complex hydrogel (CS-κ-CGPECHG) and chitosan-alginate polyelectrolyte complex hydrogel (CS-AGPECHG) were prepared. As a homogeneous distribution of the functional groups within the PEC is hardly to achieve, attributed to the fast gelation, commercial chitosan was sulfonated creating two functionalities on one biopolymer, yielding sulfonated chitosan (SFNCS) in a powdered form. Moreover, commercial available sodium κ-carrageenan powder (S-κ-CGP) and chitosan powder (CSP) were tested in the model reaction (Scheme 1).

Both, the commercial available powders S-κ-CGP and CSP were used in the reaction without further modifications and purification.[6] P-κ-CGAG and CSAG were prepared like reported in previous publications.[5] In the case of the former a 0.6 M KCl solution was used as gelling media to create stable hydrogels based on the interactions between potassium cations and sulfonic groups of the biopolymer. After drying the gel under supercritical conditions, aerogel beads with an average diameter of 2.10 mm ± 0.26 could be obtained. Compared to the reported preparation method, a syringe without cannula was used to drop the hot polysaccharide solution into the ice-cooled salt solution avoiding partial gelation of

the viscous solution in the cannula. The latter aerogel had an average diameter of 1.65 mm ± 0.23.[7] Based on the ionotropic gelation mechanism through interactions between potassium cations and sulfonic groups in the case of P-κ-CGAG no acidic sites should be expected, which would be essential for the first step (acid-catalyzed deacetylation) in the model reaction (Scheme 1). Therefore, different types of carrageenan-based aerogels creating acidic sites were prepared. For this aim, the hot polymer solution was not dropped in an aqueous KCl solution but in a 0.6 M aqueous acidic solution consisting either of hydrochloric acid (HCA-κ-CGAG) or acetic acid (AA-κ-CGAG). In both cases the ideal concentration of the κ-carrageenan solution was 3% (w/v) obtaining almost uniform beads. Compared to the potassium-based hydrogels, both acidic materials showed considerably lower stability. Consequently, the washing procedure of the acidic hydrogels had to be performed at 5 °C to avoid the complete dissolving of the beads. Hydrogels with a pH between 5 and 6 were directly transferred into the EtOH-H_2O baths for dehydration. In general, after the 70% EtOH-H_2O bath the washing solution had a neutral pH. This indicated that the excess of H^+-cations was successfully removed, which is required for the heterogeneous catalytic approach in the deacetylation step. The first supercritical drying of both materials was not successful. This could be attributed to a possible incomplete EtOH-H_2O exchange at 5 °C. To confirm this claim, beads from the same batch were matured 3 to 6 times in 100% EtOH at room temperature for 15 min in each step followed by the drying under supercritical conditions. The latter drying procedure was leading to a material which is more comparable with an aerogel, but the method has still to be improved. To avoid the problem, the weak gel is creating, a combination of different cations, e.g. K^+ or Ca^{2+} and H^+, should be applied in the preparation to strengthen the hydrogel and to make the washing and the solvent exchange at room temperature possible.

Another design of a possible catalyst is to combine both biopolymers in one bead. This was achieved by dropping the hot κ-carrageenan solution into an ice cooled solution consisting of 0.6 M acetic acid and 1% (w/v) chitosan. The obtained beads were stable due to their enormous rigidity. When a 3% (w/v) carrageenan solution was used the beads were very hard (CS-κ-CGAG3). To facilitate the possible diffusion of the substrates through the catalyst a second

batch with a 2% (w/v) solution was prepared which results in a softer hydrogel (CS-κ-CGAG2). In both cases, the supercritical drying was not successful due to the incomplete EtOH-H_2O exchange at 5 °C. This problem could be circumvented by additional 3 to 6 maturing steps of the beads in 100% EtOH for 15 min in each step at room temperature like previously reported. This second drying could be also improved to obtain aerogel beads.[8]

When carrageenan solutions were mixed with chitosan solutions without any additives like sodium chloride, drugs and plastisizers a gel-like precipitate is formed. This precipitate is described in different references as a polyelectrolyte complex, which is formed due to the electrostatic interactions between the positive charged chitosan (NH_3^+) and the negative charged carrageenan (SO_3^-).[3c],[9] As the precipitate is formed immediately, when no additives are used, no homogeneous composite could be obtained. In literature, there are some examples which describe the preparation of homogeneous materials based on PECs dealing with different biopolymers like chitosan, carrageenan and alginate. In this work two representative polyelectrolyte complex hydrogels were prepared. On the one hand a chitosan-κ-carrageenan (CS-κ-CGPECHG)[10] and on the other a chitosan-alginate polyelectrolyte complex hydrogel (CS-AGPECHG)[3a] was developed. In the case of the former hydrogel, sodium chloride (5.7%) was dissolved in each biopolymer solution before mixing them. The addition of the salt avoided the immediate formation of undefined artefacts, made the mixing of the solutions easier and resulted in a homogeneous rigid gel. In the latter case, a 4% (w/v) chitosan solution in 1% (v/v) aqueous acetic acid was combined at 80 °C with a 4% (w/v) aqueous alginate solution to obtain the corresponding hydrogel.

Moreover, a different strategy was considered. Therefore, chitosan was directly sulfonated with chlorosulfonic acid *via* a nucleophilic substitution to obtain powdered SFNCS.[11]

Catalytic tests

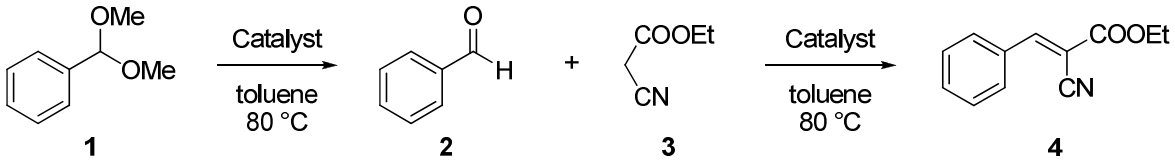
In general all composite materials were tested in the one-pot deacetylation Knoevenagel reaction (Scheme 1). Additionally, pure κ-carrageenan-based

materials were used in the deacetylation reaction (Scheme 2) of benzaldehyde dimethylacetal (**1**).

One-pot deacetalytion Knoevenagel condensation

Before the prepared materials were tested in the one-pot deacetylation Knoevenagel reaction (Scheme 1) between benzaldehyde dimethylacetal (**1**) and ethyl cyanoacetate (**2**), it was investigated if already the commercial available powder of sodium κ-carrageenan (S-κ-CGP) and chitosan (CSP) show catalytic activity in this reaction. Therefore, different experiments were performed in toluene at 80 °C for 30 minutes under slightly modified conditions as reported[2c], i.e. 1 equivalent of **3** instead of 1.2 equivalents was used. The results are summarized in Table 1. When single S-κ-CGP (Table 1, entry 2) was tested in this reaction, as expected, no benzaldehyde (**2**) and subsequent condensation product (**4**) formation occurred due to the missing acidic sites in the salt. To clarify, whether chitosan itself could contribute to the deacetylation reaction also a reaction only with single CSP as catalyst was performed (Table 1, entry 3). In this case also no deacetylated product **2** could be obtained. When both biopolymers were combined (Table 1, entry 4), also negligible formation of **2** and no formation of **4** could be detected. The inefficacy of the commercial powders is probably attributed to the absence of acidic sites in S-κ-CGP and maybe to their low surface area.[5] To overcome the latter limitation, aerogels with high surface areas, of both polymers were prepared and tested. Both materials, P-κ-CGAG as well as CSAG, showed again no activity (Table 1, entry 5) confirming the fact that acidic sites have to be present to promote the deacetylation of **1**. The evaluation of possible catalysts bearing acidic sites is considered in the following tables 2, 5, 6 and 7. Additional, the choice of more polar solvents could facilitate the product formation *via* better interactions between the subtrates and biopolymers. This possible influence of the solvent polarity is also considered in this work and listed in the following tables 2, 6 and 7.

Table 1. One pot deacetylation Knoevenagel reaction catalyzed by different catalysts in toluene.[a]



1 2 3 4

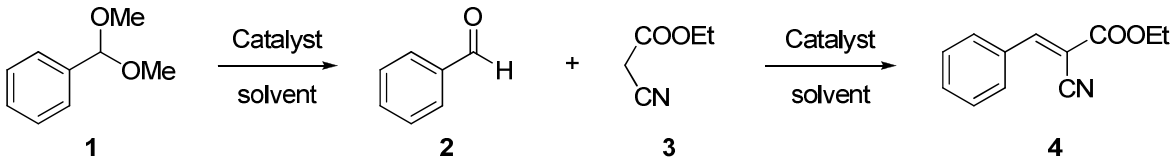
Entry	Catalyst	Yield 2 (%)[b]	Yield 4 (%)[b]
1	-	0	0
2	S-κ-CGP	< 1	0
3	CSP	0	0
4[c]	S-κ-CGP + CSP	< 1	0
5[c],[d]	P-κ-CGAG[e)] + CSAG[f)]	< 1	0

[a] Reaction conditions: 1.0 mmol (150.6 μL) benzaldehyde dimethylacetal, 1.0 mmol (106.4 μL) ethyl cyanoacetate, 0.5 mmol (61.6 μL) p-xylene, 3.0 mL toluene, 20 mg catalyst, 0.5 h, 80 °C; [b] GC yield; [c] 20 mg of each catalyst; [d] Reaction conditions: 0.16 mmol (25 μL) benzaldehye deimthylacetal, 0.16 mmol (18 μL) ethyl cyanoacetate, 0.5 mL toluene, 3.4 mg (4 beads) κ-CGAG, 3.4 mg (15 beads) CSAG, 0.5 h, 80 °C; [e] Aerogel was prepared with a 2% (w/v) κ-carrageenan solution and 0.6 M KCl solution as gelling media; [f] Aerogel was prepared with a 2.5% (w/v) chitosan solution.

A further important parameter is the catalyst design for this reaction. Not only the use of single seperated materials but also the creation and application of combined bifunctional materials consisting of both acidic and basic sites are of interest. These composite materials made of carrageenan and chitosan were applied in the one-pot reaction by varying the reaction time, temperature, solvent and carrageenan content in the composites (Table 2). In general, all tested gels could at least promote the deacetylation of **1** to form **2** in moderate (29%, Table 2, entry 4) up to good yields (80%, Table 2, entry 5). In all cases no or only traces of the condensation product **4** could be detected. In the case of the composite CS-κ-CGAG2 in toluene at 40 °C during 70 h good yields (58%) of **2** could be obtained (Table 2, entry 2). Regarding the corresponding blank reaction (Table 2, etnry 3) the yield with 13% of **2** is already high but still much less than the formation when the catalyst is applied. Changing the solvent from nonpolar to a more polar one (THF) the yield could be enhanced under the same conditions like described in the

latter to 80% (Table 2, entry 5). In addition, the corresponding blank reaction showed only 3% formation of **2** (Table 2, entry 6) in contrast to 13% in toluene. When the commercial available powders of both chitosan and κ-carragenan were used as catalysts in THF (Table 2, entry 7) also no significant yield increase of **2** could be reached, when compared to the reaction in toluene (Table 1, entry 4). Hence, the use of more polar solvents in combination with these catalysts than nonpolar ones should be favoured.

Table 2. Solvent scope of the one pot deacetylation Knoevenagel reaction.[a]



Entry	Catalyst	Solvent	Time (h)	T (°C)	$m_{Catalyst}$ (mg)	Yield 2 (%)[b]	Yield 4 (%)[b]
1	CS-κ-CGAG2	toluene	18	80	4	21[c]	0
2	CS-κ-CGAG2	toluene	70	40	15.7	58	0
3	-	toluene	70	40	-	13	0
4	CS-κ-CGAG2	THF	17	40	4	29	0
5	CS-κ-CGAG2	THF	70	40	16	80	0
6	-	THF	70	40	-	3	0
7	CSP + S-κ-CGP	THF	70	40	8 + 8	3	0

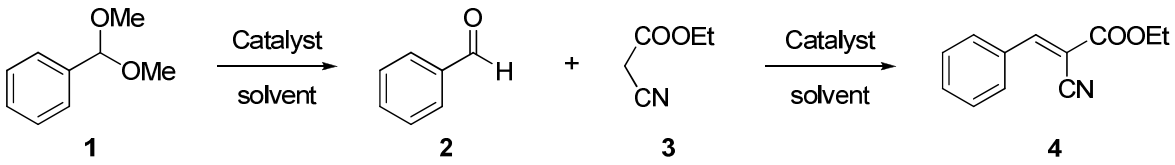
[a] Reaction conditions: 0.16 mmol (25 μL) benzaldehyde dimethylacetal, 0.16 mmol (18 μL) ethyl cyanoacetate, 0.08 mmol (10 μL) p-xylene, 0.5 mL solvent, catalyst; [b] GC yield; [c] Estimated conversion without internal standard.

As the composite aerogels mentioned in Table 2 only produce the intermediate substrate **2** but not the final condensation product **4**, a new strategy for preparing the composite material was investigated. On the one hand the polyelectrolyte complex hydrogel CS-κ-CGPECHG and the corresponding xerogel CS-κ-CGPECXG between chitosan and carrageenan were tested in the one pot reaction

(Table 3). On the other the same hybrid material was obtained between chitosan and sodium alginate (Table 4).

The PEC-based materials between chitosan and carrageenan were tested first under the reported standard conditions, i.e. in toluene at 80 °C, but for longer reaction time (18 h instead of 0.5 h[2c]). As expected the CS-κ-CGPECXG gave poor yields of the intermediate **2** (3%) and no yield of the condensation product **4** (Table 3, entry 1). In contrast, with the CS-κ-CGPECHG, which was used in a ca. 5 fold higher amount than the xerogel, a moderate yield of **2** (27%) (Table 3, entry 2) could be obtained, but no product **4** was formed. When the solvent system was changed to THF, the yields for CS-κ-CGPECXG and CS-κ-CGPECHG were decreasing to 1% (Table 3, entry 4) and 7% (Table 3, entry 5), respectively. It has to be mentioned that in the case of THF the reaction temperature was set to 40 °C due to its' lower boiling point compared to toluene. In general only negligible amounts of the condensation product **4** was formed for all PECs.

Table 3. One pot deacetylation Knoevenagel reaction catalyzed by chitosan-κ-carrageenan polyelectrolyte complex materials in different solvents.[a]



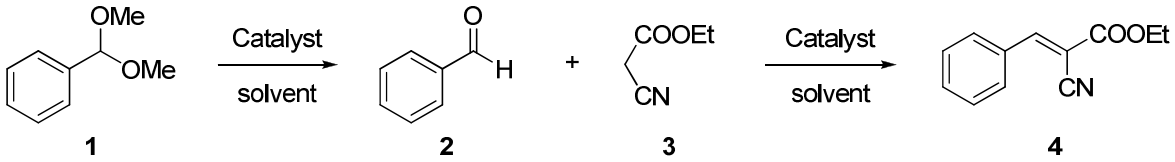
1 **2** **3** **4**

Entry	Catalyst	Solvent	T (°C)	$m_{Catalyst}$ (mg)	Yield 2 (%)[b]	Yield 4 (%)[b]
1	CS-κ-CGPECXG	toluene	80	16.3	3	0
2	CS-κ-CGPECHG	toluene	80	83	27	0
3	-	toluene	80	-	1	0
4	CS-κ-CGPECXG	THF	40	16.8	1	0
5	CS-κ-CGPECHG	THF	40	83	7	0
6	-	THF	40	-	< 1	0

[a] Reaction conditions: 0.16 mmol (25 μL) benzaldehyde dimethylacetal, 0.16 mmol (18 μL) ethyl cyanoacetate, 0.08 mmol (10 μL) p-xylene, 0.5 mL solvent, 18 h, catalyst; [b] GC yield.

The hybrid materials obtained with chitosan and sodium alginate were also first tested under the same conditions like used above. In the case of the xerogel CS-AGPECXG only traces of **2** and no **4** could be obtained (Table 4, entry 1). Interestingly, high yields of **2** (87%, Table 4, entry 2) could be monitored when the hydrogel CS-AGPECHG was applied as catalyst.[12] But also in this case only traces of the condensation product **4** could be detected. Changing the solvent from toluene to THF was resulting in a decrease of the yields of **2** to 20% for the xerogel (Table 4, entry 4) and 13% (Table 4, entry 5) for the hydrogel. The formation of the condensation product **4** for these two materials was taking place to some extent, i.e. only traces could be obtained.

Table 4. One pot deacetylation Knoevenagel reaction catalyzed by chitosan-alginate polyelectrolyte complex materials in different solvents.[a]



1 **2** **3** **4**

Entry	Catalyst	Solvent	T (°C)	$m_{Catalyst}$ (mg)	Yield 2 (%)[b]	Yield 4 (%)[b]
1	CS-AGPECXG	toluene	80	16	1	0
2	CS-AGPECHG	toluene	80	85	87	< 1[c]
3[d]	-	toluene	80	-	1	0
4	CS-AGPECXG	THF	40	16	20	0[e]
5	CS-AGPECHG	THF	40	87	13	0
6[f]	-	THF	40	-	< 1	0

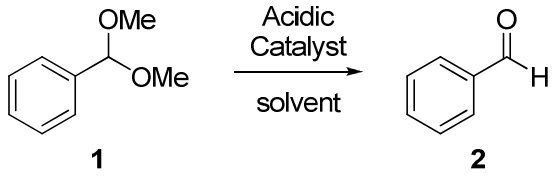
[a] Reaction conditions: 0.16 mmol (25 μL) benzaldehyde dimethylacetal, 0.16 mmol (18 μL) ethyl cyanoacetate, 0.08 mmol (10 μL) p-xylene, 0.5 mL solvent, 20 h, catalyst; [b] GC yield; [c] Traces of the condensation product could be deteceted, exact quantification is missing; [d] Reaction time was 18 h; [e] Different signal (undefined) near to the condensation product retention time is appearing; [f] Reaction time was 18 h.

As only the deacetylation was taking place in the case of all prepared materials, commercial available chitosan powder was modified with chlorosulfonic acid to create a bifunctional material with amine and sulfonic groups (SFNCS). The

material could only be isolated as the corresponding sodium salt. But it should still own acidic sites as the major functionalities as reported by Reddy and co-workers[13], which are necessary for the deacetylation. SFNCS was also tested in the one-pot deacetylation Knoevenagel condensation reaction yielding the condensation product **4** in traces.[14]

Deacetylation reaction

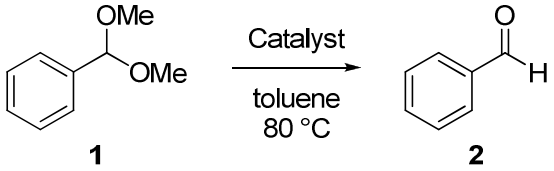
As in the previous investigations of the one-pot deacetylation Knoevenagel condensation reaction in all cases only traces of the condensation product **4** and moderate to high yields of the intermediate **2** could be obtained, a closer look to the deacetylation of benzaldehyde dimethylacetal (**1**) should be of interest (Scheme 2).



Scheme 2. Deacetylation of benzaldehyde dimethylacetal (**1**).

For this reason, first the commercial available powder of the sodium κ-carrageenan (κ-CGP) was tested in the deacetylation reaction (Table 5). As expected no benzaldehyde formation occurred during 0.5 h at 80 °C in toluene (Table 5, entry 1) due to the missing acidic sites in the powder. The same inactivity was also registered, when the catalyst loading was increased (entry 2) and the reaction time extended to 15 h (entry 3). When the acidic aerogel HCA-κ-CGAG is used some catalytic activity could be obtained which could be caused by the higher surface area and mostly by the acidic sites in the material (Table 5, entry 4). Due to the low conversion of 10% towards **2** in toluene two further solvents, i.e. DMSO and THF, were tested. In the former (Table 6, entry 4) the conversion could be enhanced up to 50%, whereas in the latter the conversion dropped to 21% (Table 6, entry 6), but is still higher when compared to toluene. In addition, it has to be emphasized that the aerogel beads were not stable in DMSO reflected by their rapid dissolution under the given conditions.

Table 5. Deacetylation of benzaldehyde dimethylacetal catalyzed by κ-carrageenan-based materials in toluene.[a]



1 **2**

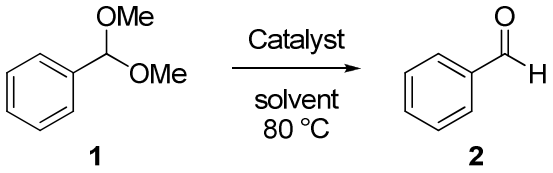
Entry	Catalyst	Time (h)	$m_{Catalyst}$ (mg)	Yield (%)[b]
1	S-κ-CGP	0.5	3.4	0
2	S-κ-CGP	0.5	15	0
3	S-κ-CGP	15	15	0
4	HCA-κ-CGAG	0.5	3.4	10[c]

[a] Reaction conditions: 0.16 mmol (25 μL) benzaldehyde dimethylacetal, 0.08 mmol (10 μL) p-xylene, 0.5 mL toluene, 80 °C, 16 mg catalyst; [b] GC yield; [c] Estimated conversion without internal standard.

For further investigations in THF, the reaction should be in general performed at lower temperatures than 80 °C due to its' low boiling point of 65 °C to avoid too much pressure in the closed reaction vessels.

As shown in Table 6, DMSO and THF are more suitable for the deacetylation reaction catalyzed by HCA-κ-CGAG than toluene. But as the conversions are still low, longer reaction times were applied. The results are summarized in Table 7. When only the potassium carrageenan-based aerogel P-κ-CGAG was used as single catalyst, interestingly **2** was obtained in 16% yield, although in the material no acidic active sites are expected.[15] In the case of the acidic aerogel AA-κ-CGAG the yield of **2** could be increased remarkably to 81%.

Table 6. Deacetylation of benzaldehyde dimethylacetal catalyzed by κ-carragenan-based materials in different solvents.[a]

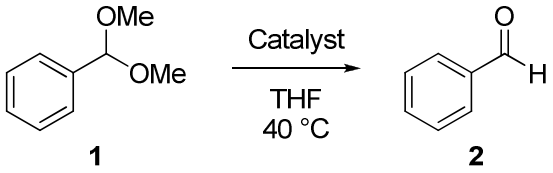


1 **2**

Entry	Catalyst	Solvent	Yield (%)[b]
1	-	toluene	0
2	HCA-κ-CGAG	toluene	10[e]
3[c]	-	DMSO	0
4[c]	HCA-κ-CGAG	DMSO	50[e]
5	-	THF	0
6[d]	HCA-κ-CGAG	THF	21[e]

[a] Reaction conditions: 0.16 mmol (25 μL) benzaldehyde dimethylacetal, 0.5 mL solvent, 0.5 h, 80 °C, catalyst; [b] GC yield; [c] No p-xylene was used due to immiscibility with DMSO; [d] 0.08 mmol (12.5 mL) benzaldehyde dimethylacetal, 0.25 mL THF, 0.5 h, 80 °C, 1.7 mg catalyst; [e] Estimated conversion without internal standard.

Table 7. Deacetylation of benzaldehyde dimethylacetal catalyzed by κ-carragenan-based materials in different solvents.[a]



1 **2**

Entry	Catalyst	Solvent	Time (h)	Yield (%)[b]
1	P-κ-CGAG	THF	70	16
2	AA-κ-CGAG	THF	70	81

[a] Reaction conditions: 0.16 mmol (25 μL) benzaldehyde dimethylacetal, 0.08 mmol (10 μL) p-xylene, 0.5 mL solvent, 70 h, 40 °C, 16 mg catalyst; [b] GC yield.

5.3 Conclusion

In summary, we discussed the preparation of different polysaccharide-based materials (i.e. aerogel (AG), hydrogel (HG) and powder (P). Thereby, polymers like sodium alginate, chitosan and sodium κ-carrageenan were considered and tested in the one-pot deacetylation Knoevenagel condensation reaction. Bifunctional PECs like CS-κ-CGAG, CS-κ-CGPECHG, CS-AGPECHG and modified SFNCS powder showed no condensation product formation of **4** in the model reaction under different tested conditions, but at least activities in the deacetylation of **1**. Thereby, both PECs CS-κ-CGAG2 and CS-AGPECHG showed good GC yields with 80% and 87% of the deacetylated product (**2**), respectively. Acidic κ-carrageenan-based aerogels, i.e. AA-κ-CGAG and HCA-κ-CGAG, were directly tested in the deacetylation of **1**, whereby the former showed high effectivity with a yield of 81% of the corresponding deprotected compound **2**, whereas the latter was less active.

To enhance the stability and to facilitate the H_2O-EtOH exchange of some κ-carrageenan-based materials, incorporation of metals (e.g. K, Ca, Cu, Co, etc.) should be considered. With this strategy the catalytic performance could also be increased attributed to new created lewis acidic sites in the multifunctional material. In addition, acid treated alginate gels could be a good alternative to κ-carrageenan-based materials. Moreover, screening of different reaction conditions including other substrates with lower pK_a values or aromatic compounds with electron withdrawing groups are ongoing efforts in our laboratory.

5.4 Experimental Section

Materials and Methods

^{1}H NMR spectra were recorded at 25 °C on a Bruker Avance 300 spectrometer. Chemical shifts are denoted in δ (ppm) relative to tetramethylsilane (TMS $\delta = 0$) as an internal standard or relative to residual solvent peaks. Aerogels were obtained under supercritical CO_2 conditions (85 bar, 37 °C) in a Polaron 3100 apparatus. GC yields were determined by a GC Varian 3900 with a capillary column (HP-5: 30 m × 0.32 mm × 0.25 μm) and FID. GC program: Injector temperature: 280 °C, column flow: 1.0 mL/min, column oven: heating from 50 °C to 290 °C with a rate of 15 °C/min and a hold time of 5 min. Retention times: t_R (**1**): ca. 5.7 min, t_R (**2**): ca. 4.4 min, t_R (**3**): 4.2, t_R (**4**): 10.1, t_R (p-xylene): 3.8.

Analytical-grade solvents and commercially available reagents were purchased from Merck, TCI Europe or Sigma Aldrich and were used as received. Chitosan (Batch No. 04809DH, low molecular weight (LMW) 69000 g/mol, degree of acetylation (DA) 8%) was purchased from Sigma Aldrich and purified analogue to reference [16] before use. Sodium κ-carrageenan was provided from the ICG. Alginic acid sodium salt (Batch No. 056K0005; CAS 9005-38-3; isolated from brown algae) was purchased from Sigma Aldrich and used without further purification.

Preparation of materials

Preparation of chitosan aerogel beads (CSAG)

A 2.5% (w/v) aqueous chitosan solution was obtained by dissolving 1.0 g purified chitosan in 40 mL H_2O including 208 μL acetic acid overnight at 25 °C. The solution was then dropped *via* a syringe pump into 100 mL of a 4 M aqueous NaOH solution with a flow of 1.0 mL/min. The distance between the syringe cannula tip and the surface of the gelling solution was set to 10 cm to ensure the formation of almost uniform beads. The so obtained spheres were matured for 2 h

in the gelling solution without stirring at room temperature and were washed with distilled H_2O (26 times, 250 mL portions) until a pH of 6.68 could be obtained (Ø (hydrogel beads) = 2.35 mm ± 0.28). The hydrogel beads were dehydrated by immersion in a series of successive EtOH-H_2O baths with increasing EtOH concentration (10, 30, 50, 70, 90 and 100%) for 15 min in each step (Ø (alcogel beads) = 1.93 mm ± 0.18). Finally, the alcogel beads were dried under supercritical CO_2 conditions to obtain the corresponding aerogel (Ø (aerogel beads) = 1.65 mm ± 0.23).

Preparation of κ-carrageenan aerogel beads (P-κ-CGAG)

A 2.0% (w/v) aqueous κ-carrageenan solution was obtained by dissolving 0.32 g κ-carrageenan in 16 mL H_2O by heating the suspension for 30 min at 80 °C. The hot solution was dropped via a syringe (without cannula) into an ice-cooled 0.6 M KCl solution. The distance between the syringe tip and the gelling solution was set to 7 cm. The beads were matured in this gelling media at 5 °C for 20 h without stirring. Then they were washed 20 times with 250 mL portions of H_2O until a pH of 6.40 could be obtained. The alcogel was obtained by storing the beads in different H_2O-Ethanol baths for 15 min at 5 °C with increasing EtOH content (10, 30, 50, 70, 90 and 100%). Finally the alcogel beads were dried under supercritical CO_2 conditions.

Preparation of κ-carrageenan aerogel beads (HCA-κ-CGAG)

A 3% (w/v) κ-carrageenan aqueous solution was obtained by dissolving 0.48 g κ-carrageenan in 16 mL H_2O by heating the suspension for 30 min at 80 °C. The hot solution was directly dropped via syringe (without cannula) into an ice cooled 0.6 M HCl aqueous solution. The distance between the syringe tip and the gelling solution was set to 5 cm. The so obtained beads were matured without stirring at 5 °C for 17 h. Then the beads were washed with 4.5 L deionized H_2O until neutral pH. The alcogel was obtained by storing the beads in different EtOH-H_2O baths for 15 min at 5 °C with increasing EtOH content (10, 30, 50, 70, 90 and 100%). Finally, the alcogel beads were dried under supercritical CO_2 conditions.

Preparation of κ-carrageenan aerogel beads (AA-κ-CGAG)

The beads were prepared analogue to the method described for HCA-κ-CGAG by dissolving 1.5 g κ-carrageenan in 50 mL H_2O and dropping the hot solution into an ice-cooled 0.6 M acetic acid aqueous solution (120 mL). The distance between the syringe tip and gelling solution was set to 6 cm. The beads were matured at 5 °C for 19 h. Afterwards, the hydrogel was washed by keeping it in ice-cooled deionized water for 5 min in each step (150 mL portions H_2O, 10 washing steps) to reach a pH of ca. 5. Then H_2O was exchanged by storing the beads in EtOH-H_2O baths with increasing EtOH content (10, 30, 50, 70, 90 and 100%) at 5 °C. Finally, the beads were dried under supercritical conditions.

Preparation of chitosan-κ-carrageenan composites aerogel beads (CS-κ-CGAG3)

0.96 g κ-carrageenan were dissolved in 32 mL deionized H_2O by heating the suspension at 80 °C for 30 min (3% (w/v)). The hot solution was dropped *via* a syringe (without cannula) into 80 mL of an ice-cooled aqueous solution consisting of 0.80 g chitosan and 0.6 M acetic acid. The distance between the syringe tip and gelling solution was set between 10 and 12 cm. After maturing the beads without stirring at 5 °C for 19 h, they were washed with deionized H_2O at 5 °C by storing them 8 times in 50 mL portions for 5 min to reach a slightly acidic pH (between 5 and 6). The washing was continued by exchanging H_2O by EtOH by keeping the beads in EtOH-H_2O baths with increasing content of EtOH (10, 30, 50, 70, 90 and 100%) for 15 min at 5 °C. Finally, the beads were dried under supercritical conditions.

Preparation of chitosan/κ-carrageenan polyelectrolyte complex hydrogel (CS-κ-CGPECHG)

The polyelectolyte complex gel was prepared like described in the reference with slight modifications. 0.4 g chitosan were dissolved in 9.6 g of a 1% aqueous acetic acid solution. 0.4 g κ-carrageenan was dissolved in 9.6 g deionized H_2O by

heating the suspension at 80 °C for 30 min. To each solution 0.57 g NaCl was added. Then both hot solutions (80 °C) were combined and further stirred for 15 min at 100 °C. After the mixture was cooled down to room temperature the complex was stored over night (24 h) at 5 °C. The obtained gel was then washed by keeping it in deionized H_2O. The H_2O was exchanged after 18 h and the gel was matured for further 18 h in deionized H_2O.

Preparation of chitosan-alginate polyelectrolyte complex hydrogel (CS-AGPECHG)

The polyelectrolyte complex gel was prepared like described in the reference with slight modifications. 0.4 g chitosan was dissolved in 10 mL of a 1% aqueous acetic acid solution. 0.4 g sodium alginate was dissolved in 10 mL deionized H_2O. Both solutions were heated for 30 min at 80 °C and combined under stirring. The mixture was cooled down to room temperature during 3 h without stirring. The obtained polyelectrolyte gel complex was washed 4 times with 30 mL H_2O portions by storing the gel for 10 min in H_2O baths. After the last washing step, the gel was kept in H_2O at 5 °C over night. Then the H_2O-gel mixture was centrifuged for 30 min at 10000 rpm, stored again at 5 °C for 48 h and finally centrifuged again to remove the excess of H_2O.

Synthesis of sulfonated chitosan (SFNCS)

Chitosan was activated like described in the reference [11] with slight modifications. 2.0 g chitosan were dissolved over night in 200 mL of a 2% aqueous acetic acid solution at 25 °C. The almost clear solution was centrifuged for 20 min at 5000 rpm to remove some solid particles. The decanted solution was neturalized with a 2.5 N aqueous NaOH solution (pH between 7 and 8). The resulting suspension was stored over night at 5 °C and then centrifuged for 25 min at 2500 rpm. The resulting residue was washed 4 times with H_2O by centrifugation for 25 min at 2500 rpm in each step. The gel-like residue was kept over night at 5 °C before being washed by centrifugation 2 times with EtOH and 1 time with Et_2O. The obtained residue was air-dried over night. To dry and ice-cooled pyridine (12 mL) chlorosulfonic acid (2 mL) was added very slowly *via* a dropping funnel.

Afterwards, a suspension of dried chitosan in 8 mL dry pyridine was added to this mixture and heated on a boiling water bath for 1 h. After the mixture had reached room temperature it was poured into 40 mL of water and mixed subsequently with 15 mL of 2.5 M NaOH. The sodium salt of sulfated chitosan was precipitated with 100 mL EtOH. This salt was again redissolved with 40 mL water and dialyzed against deionized water for 3 days by changing the water 3 times. The purified solution of sulfated chitosan was concentrated under reduced pressure to ca. 20 mL and combined with 2 mL of saturated NaCl solution. The pure sodium salt of sulfated chitosan was obtained by precipitation with 30 mL EtOH, followed by filtration and excessive washing with EtOH.

Catalysis

General procedure for the one-pot deacetylation Knoevenagel condensation reaction

To a mixture of 0.16 mmol (25 μL) benzaldehyde dimethylacetal, 0.16 mmol (18 μL) ethyl cyanoacetate and 0.08 mmol (10 μL) p-xylene in 0.5 mL toluene, 3.4 mg of κ-carrageenan (KCl) and 3.4 mg of chitosan aerogel beads were added. The reaction was heated at 80 °C for 0.5 h. Then the reaction mixture was allowed to cool down to room temperature, filtrated over silica, which was further washed with 2 mL toluene. The diluted sample was then filtrated over a syringe filter (0.2 μm, PP) before it was injected for GC analysis.

General procedure for the deacetylation reaction of benzaldehyde dimethylacetal

To a solution of 0.16 mmol (25 μL) benzaldehyde dimethylacetal and 0.08 mmol (10 μL) p-xylene in toluene, 3.4 mg of the catalyst were added. The reaction mixture was heated at 80 °C for 0.5 h. The sample for GC analysis was prepared like described above.

5.5 References

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[6] Catalytic tests with purified chitosan could be of interest to crosscheck some results.

[7] For the preparation of CSAG purified chitosan was used.

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[12] It should be considered that a possible influence in the catalytic activity could be attributed to NaCl which was used when the material was prepared. Therefore, the sodium content in the gel should be determined and a corresponding control experiment should be applied to exclude any salt effect in the catalyzed reaction.

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[14] Reaction conditions: toluene, 4 d, 80 °C or THF, 4 d, 40 °C.

[15] Due to time limitations the blank experiment could not be performed. But in general 0% yield could be expected because the blank experiment in the one-pot deacetylation Knoevenagel reaction under the same conditions (Table 3, entry 6) resulted **2** only in traces.

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E List of Abbreviations

1D	1-dimensional
3D	3-dimensional
AAC	alkyne-azide cycloaddition
AAS	atomic absorption spectroscopy
Ac	acetyl
APCI	atmospheric pressure chemical ionization
Ar	aryl
BSA	bovin serum albumin
C-C	carbon carbon
CO_2	carbon dioxide
d	day(s), dublett
DCM	dichloromethane
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNP	2,4-Dinitrophenol
dr	diastereomeric ratio
DSC	differential scanning calorimetry
ECA	ethyl cyanoacetate
*Ec*GGT	γ-glutamyltraspeptidase from *Escherichia coli*
ee	enantiomeric excess
Et	ethyl
EtOAc	ethyl acetate
EtOH	ethanol
eq.	equivalent
EWG	electron withdrawing
FESEM	field emission scanning electron microscopy
FID	flame ionization detector
FT	fourier transformation
GC	gas chromatography
h	hour
HCl	hydrochloric acid
HEPES	2-(4-(2-hydroxyethyl)-piperazin-1-yl)ethane-sulfonic acid
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectroscopy
ICP-OES	inductively coupled plasma optical emission spectrometry
IEP	iso-electric point
IR	infrared spectroscopy
LMW	low molecular weight
LP	lipase
PECs	polyelectrolyte complexes
m	meter
M	metal
MBA	*N,N'*-Methylenebis-Acrylamide

ME	*Manihot esculenta*
Me	methyl
MeCN	acetonitrile
MeOH	methanol
min	minute
MS	mass spectroscopy
NA	not applicable
n.d.	not determined
NIPA	*N*-isopropylacrylamide
NMR	nuclear magnetic resonance
NP	nanoparticle
p	para
PDI	polydisperity index
pH	potential of hydrogen
Ph	phenyl
PMA	potassium methacrylate
ppm	parts per million
rpm	revolutions per minute
RT	room temperature
s	second(s), singulett
SA	surface area
SEM	scanning electron microscopy
T	temperature
t	triplett
TBAB	tetrabutylammonium bromide
TGA	thermal gravimetric analysis
THF	tetrahydrofuran
TLC	thin layer chromatography
TMEDA	*N,N,N',N'*-tetramethylethylenediamine
TMS	trimethyl silane
TOF	turn over frequency
TON	turn over number
t_R	retention time
UV	ultra violet
V	volume
wt%	weight percentage

F Curriculum Vitae

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- EuCheMS 2012, Prague, Czech Republic: “Critical Assessment of the Efficiency of Chitosan-based Materials as Potential Organocatalysts for C-C Bond Formation”

Oral Communications

- Christmas colloquim, December 2013, University of Regensburg: “Helpful Gels Beyond The Baby Diaper Secret”

- Christmas colloquium, December 2011, University of Regensburg: “Functional Supramolecular Gel-based Materials: Synthesis and Applications”

List of Publications

- “Investigation of C-C Bond Formation Mediated by *Bombyx Mori* Silk Fibroin Materials”
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Declaration

Herewith I declare that this present dissertation is a presentation of my original work prepared single-handed. Wherever contributions from others are involved, all of them are marked clearly, with reference to the literature, licence, and acknowledgement of collaborative research.

Regensburg, 17 March 2014

Dennis Kühbeck